Human Immunology 77 (2016) 358–364

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

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

Rafael S. de Albuquerque [a,b], Celso Teixeira Mendes-Junior [c,*], Norma Lucena-Silva [d], Camila Leal Lopes da Silva [c], Diane Meire Rassi [e], Luciana C. Veiga-Castelli [e], Maria Cristina Foss-Freitas [e], Milton César Foss [e], Neifi Hassan Saloum Deghaide [e], Philippe Moreau [f], Silvia Gregori [b], Erick C. Castelli [g], Eduardo Antônio Donadi [e]

[a] Faculty of Medicine of Ribeirão Preto, University of São Paulo, Departament of Genetics, Ribeirão Preto, SP, Brazil
[b] Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), San Raffaele Scientific Institute, Milan, Italy
[c] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[d] Aggeu Magalhães Research Center, Department of Immunology, Oswaldo Cruz Fundation (Fiocruz), Recife, PE, Brazil
[e] Faculty of Medicine of Ribeirão Preto, University of São Paulo, Department of Medicine, Ribeirão Preto, SP, Brazil
[f] UMR Commissariat à l'Energie Atomique et aux Energies Alternatives/Université Paris Diderot-Paris 7, Sorbonne Paris-Cité/iMETI/Service de Recherches en Hémato-Immunologie, Hôpital Saint-Louis, IUH, Hôpital Saint-Louis, 1, avenue Claude Vellefaux, 75475 Paris cedex 10, France
[g] Departamento de Patologia, Faculdade de Medicina de Botucatu, Universidade Estadual Paulista Júlio de Mesquita Filho, Botucatu, SP, Brazil

ARTICLE INFO

Article history:
Received 11 April 2015
Revised 19 November 2015
Accepted 2 February 2016
Available online 13 February 2016

Keywords:
Type 1 diabetes mellitus
HLA-G
3′ untranslated region
Gene regulation
Brazilians

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.

* Corresponding author at: Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, 14040-901, Ribeirão Preto, SP, Brazil.
E-mail address: ctmendes@ffclrp.usp.br (C.T. Mendes-Junior).

http://dx.doi.org/10.1016/j.humimm.2016.02.001
0198-8859/

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

Table 1
Allele and genotype frequencies of the nine polymorphic sites (14bp DEL/INS, +3001C/T, +3003C/T, +3010C/G, +3027A/C, +3035C/T, +3142C/G, +3187A/G and +3196C/G) observed at the 3′ untranslated region (UTR) of the HLA-G gene in patients with type 1 diabetes mellitus and healthy controls. The Fisher exact test *P*-values, odds ratio (OR), 95% confidence interval (95%C.I.), probability of adherence to Hardy–Weinberg expectations (PHWE), and preventive (PF) and etiologic (EF) fractions are also shown. Significant *P*-values ($P < 0.05$) are represented in boldface and indicate statistically significant associations.

Polymorphic sites	Patients		Controls		*P*	OR	95%C.I.
	Absolute frequency	Relative frequency	Absolute frequency	Relative frequency			
14bp	*n* = 120		*n* = 120				
Ins	101	0.4208	107	0.4458	0.6452	0.9032	0.6293–1.2960
Del	139	0.5792	133	0.5542	0.6452	1.1070	0.7715–1.5890
Del–Del	35	0.2916	39	0.3250	0.7024	0.8780	0.5324–1.4480
Ins–Del	69	0.5750	55	0.4583	0.0929	1.5990	0.9602–2.6630
Ins–Ins	16	0.1333	26	0.2166	0.1256	0.5562	0.2811–1.1010
*P*HWE	0.0624		0.4606				
+3001	*n* = 120		*n* = 120				
C	234	0.9750	240	1.0000	**0.0303**[a]	0.0750	0.0041–1.3400
T	6	0.0250	0	0.0000	**0.0303**[b]	13.3330	0.7464–238.1700
C–C	114	0.9500	120	1.0000	**0.0293**[c]	0.0730	0.0040–1.3130
C–T	6	0.0500	0	0.0000	**0.0293**[d]	13.6810	0.7615–245.8000
T–T	0	0.0000	0	0.0000	–	–	–
*P*HWE	1.0000		–				
+3003	*n* = 119		*n* = 120				
C	26	0.1092	33	0.1375	0.4045	0.7693	0.4445–1.3320
T	212	0.8908	207	0.8625	0.4045	1.3000	0.7510–2.2500
C–C	1	0.0084	0	0.0000	0.4979	3.0510	0.1229–75.7000
C–T	24	0.2016	33	0.2750	0.2248	0.6660	0.3651–1.2150
T–T	94	0.7899	87	0.7250	0.2912	1.4260	0.7858–2.5880
*P*HWE	1.0000		0.1233				
+3010	*n* = 119		*n* = 120				
C	116	0.4874	136	0.5667	0.0990	0.7271	0.5071–1.0430
G	122	0.5126	104	0.4333	0.0990	1.3750	0.9592–1.9720
C–C	24	0.2016	39	0.3250	**0.0395**[e]	0.5247	0.2912–0.9453
C–G	68	0.5714	58	0.4833	0.1958	1.4250	0.8558–2.3740
G–G	27	0.2268	23	0.1916	0.5282	1.2380	0.6624–2.3130
*P*HWE	0.1445		0.8539				
+3027	*n* = 120		*n* = 120				
A	12	0.0500	15	0.0625	0.6927	0.7885	0.36141.7240
C	228	0.9500	225	0.9375	0.6927	1.2670	0.3614–1.7240
A–A	0	0.0000	1	0.0083	1.0000	0.3306	0.0133–8.2030
A–C	12	0.1000	13	0.1083	1.0000	0.9145	0.3991–2.0950
C–C	108	0.9000	106	0.8833	0.8359	1.1890	0.5254–2.6890
*P*HWE	1.0000		0.3725				
+3035	*n* = 118		*n* = 120				
C	198	0.8390	201	0.8375	1.0000	1.0110	0.6206–1.6470
T	38	0.1610	39	0.1625	1.0000	0.9891	0.6071–1.6110
C–C	83	0.7033	85	0.7083	1.0000	0.9765	0.5590–1.7060
C–T	32	0.2711	31	0.2583	0.8835	1.0680	0.6004–1.9010
T–T	3	0.0254	4	0.0333	1.0000	0.7565	0.1656–3.4570
*P*HWE	1.0000		0.5114				
+3142	*n* = 118		*n* = 120				
C	120	0.5085	101	0.4208	0.0659	1.4240	0.9915–2.0440
G	116	0.4915	139	0.5792	0.0659	0.7024	0.4892–1.0090
C–C	28	0.2372	20	0.1666	0.1980	1.5560	0.8196–2.9520
C–G	64	0.5423	61	0.5083	0.6064	1.1460	0.6889–1.9080
G–G	26	0.2203	39	0.3250	0.0814	0.5870	0.3289–1.0480
*P*HWE	0.4609		0.7104				
+3187	*n* = 100		*n* = 120				
A	145	0.7250	183	0.7625	0.3811	0.8212	0.5342–1.2620
G	55	0.2750	57	0.2375	0.3811	1.2180	0.7923–1.8720
A–A	51	0.5100	67	0.5583	0.4994	0.8233	0.4834–1.4020
A–G	43	0.4300	49	0.4083	0.7846	1.0930	0.6383–1.8720
G–G	6	0.0600	4	0.0333	0.5179	1.8510	0.5073–6.7540
*P*HWE	0.6154		0.2125				
+3196	*n* = 69		*n* = 109				
C	100	0.7246	155	0.7110	0.8102	1.0700	0.6653–1.7109
G	38	0.2754	63	0.2890	0.8102	0.9349	0.5816–1.5030
C–C	36	0.5217	57	0.5229	1.0000	0.9952	0.5441–1.8200
C–G	28	0.4057	41	0.3761	0.7529	1.1330	0.6109–2.1000
G–G	5	0.0724	11	0.1009	0.5993	0.6960	0.2309–2.0980
*P*HWE	1.0000		0.3588				

[a] PF = 0.9232.
[b] EF = 0.0231.
[c] PF = 0.9234.
[d] EF = 0.0463.
[e] PF = 0.1545.

these polymorphic was observed in the Brazilian [17,19] and in several worldwide populations [32], the Bonferroni correction for multiple testing was not taken into account to adjust the significance levels in the case-control comparisons. The etiologic fraction, which indicates how much the allele, genotype or haplotype contributes to susceptibility to disease development at the population level, and the preventive fraction, which indicates how much the allele, genotype or haplotype contributes to protection against disease development at the population level, were also estimated [33].

3. Results

Nine polymorphic sites were analyzed at the *HLA-G* 3′UTR segment, including the 14bp DEL/INS (rs1704), +3001C/T (rs116414017), +3003C/T (rs1707), +3010C/G (rs1710), +3027A/C (rs17179101), +3035C/T (rs17179108), +3142C/G (rs1063320), +3187A/G (rs9380142) and +3196C/G (rs1610696), previously described in the Brazilian and in other populations [19,32,34]. *HLA-G* 3′UTR allele and genotype frequencies of these polymorphic sites are shown in Table 1. The genotypes of all polymorphic sites detected fit Hardy–Weinberg expectations.

We found that the + 3001 T allele ($P = 0.0303$; OR = 13.3330, 95%C.I.: 0.75–238.17) and the + 3001 CT genotype ($P = 0.0293$; OR = 13.6810, 95%C.I.: 0.76–245.80) were present only in T1D patients. Likewise, the frequencies of +3001 C allele ($P = 0.0303$; OR = 0.0750, 95%C.I.: 0.004–1.34) and of the +3001 CC genotype ($P = 0.0293$; OR = 0.0730; 95%C.I.: 0.004–1.31) were overrepresented in healthy subjects. The frequency of the +3010 CC genotype was also underrepresented in T1D patients as compared to healthy subjects ($P = 0.0395$; OR = 0.5247, 95%C.I.: 0.29–0.95). The genotypic and allelic frequencies of the other polymorphic sites showed no significant differences between T1D patients and healthy subjects, except for the slightly decreased frequency of the +3142 G allele ($P = 0.0659$; OR = 0.7024, 95%C.I.: 0.49–1.01) and of the +3142 GG genotype ($P = 0.0814$; OR = 0.5870, 95%C.I.: 0.32–1.05) in T1D patients when compared to controls. If the Bonferroni correction was taken into account, no significant *HLA-G* 3′UTR associations would be observed; however, as addressed in the Patients and Methods section, the 3′UTR represents a very small gene segment in strong LD.

The linkage disequilibrium (LD) analyses revealed significant LD ($P < 0.05$) for 23 (82.1%) out of the 28 possible pairs of *HLA-G* 3′UTR polymorphic sites in T1D patients and for 24 (66.7%) out of the 36 possible pairs in controls (Table 2). The *HLA-G* 3′UTR haplotype inferences, encompassing the nine (eight currently described and including the +3001C/T) polymorphic sites in T1D patients, showed a high rate of concordance (98.3%) using the PHASE and EM algorithms, with each haplotype pair being inferred with average probabilities of 0.94 (PHASE) and 0.95 (EM). The two individuals (1.7%) who presented different inferences by the two methods were excluded from all haplotype analyses. Haplotype frequencies for the control sample were previously inferred and we classified the *HLA-G* 3′UTR haplotypes according to a previously reported study [19].

A low frequent haplotype, designated as UTR-17 (14bpINS, +3001T, +3003T, +3010C, +3027C, +3035T, +3142G, +3187A and +3196C) [34], which differed from the previously reported UTR-5 by the presence of the +3001 T allele, was found only in T1D patients ($P = 0.0148$; OR = 13.4470, 95%I.C.: 0.75–240.30) (Table 3). We also observed a significantly decreased frequency of UTR-3 (14bpDEL, +3001C, +3003T, +3010C, +3027C, +3035C, +3142G, +3187A and +3196C) haplotype ($P = 0.0078$; OR = 0.4063, 95%C.I.: 0.21–0.78), and a marginally increased frequency of the UTR-6 (14bpDEL, +3001C, +3003T, +3010G, +3027C, +3035C, +3142C, +3187A and +3196C) haplotype ($P = 0.069$; OR = 2.02, 95%C.I.: 0.95–4.28) in T1D patients as compared to healthy subjects.

To evaluate the influence of 3′UTR haplotypes inferred with probabilities lower than 0.90 in the previous analyses, we excluded these haplotypes and verified that the association of UTR-3 with T1D still remained ($P = 0.0412$, OR = 0.4946; 95%C.I.: 0.26–0.96), as well as the marginally increased frequency of UTR-6 in T1D patients ($P = 0.0623$; OR = 2.1030, 95%C.I.: 0.98–4.50).

No association was observed regarding the stratification of T1D patients according to clinical (body mass index, disease duration, macro and microvascular complications) and laboratory findings (HbA1c levels, microalbuminuria and dyslipidemia). It should be emphasized that these clinical and laboratory parameters were retrieved from a small group of patients ($n = 68$), and the stratification resulted in very small subgroups which compromise the statistical power of the test.

4. Discussion

The analysis of *HLA-G* 3′UTR in a cohort of 120 Brazilian T1D patients revealed: (i) the presence of the +3001 T allele only in patients, (ii) the exclusive presence of the UTR-17 haplotype, which contains the +3001 T allele, (iii) the decreased frequency of the +3001 CC and +3010 CC genotypes, and (iv) the decreased frequency of the UTR-3 haplotype.

Considering the immunomodulatory properties of HLA-G, the expression of HLA-G in tissues of patients with autoimmune diseases would have a desirable effect, since HLA-G may cause inhibition of auto-reactive cells. There are few studies evaluating this protective effect in autoimmune diseases. One of them reported that the expression of HLA-G in the skin specimens of patients with systemic sclerosis was associated with a more benign course of the disease as compared to patients who did not express HLA-G in skin biopsies. Although associated with a milder disease, the increased expression of HLA-G in skin specimens of these patients did not prevent the development of skin thickening in systemic sclerosis patients [13]. In the case of T1D, there are no studies evaluating the expression of HLA-G in pancreatic tissues obtained from biopsies or autopsies. The pancreas is one of the few adult tissues that

Table 2
Probabilities of exact test of linkage disequilibrium encompassing the nine polymorphic sites observed at *HLA-G* 3′ untranslated region regarding patients with type 1 diabetes (under the diagonal) and controls (above the diagonal). Significant *P*-values ($P < 0.05$) are represented in boldface and indicate statistically significant pairwise LD.

Polymorphisms	14bp	+3001	+3003	+3010	+3027	+3035	+3142	+3187	+3196
14bp Del–Ins	–	–	**0.00000**	**0.00000**	**0.00083**	**0.00000**	**0.00000**	**0.00000**	**0.00000**
+3001 C–T	0.11655	–	–	–	–	–	–	–	–
+3003 C–T	**0.00017**	0.37229	–	**0.00000**	0.91692	0.05112	**0.00000**	0.05028	**0.00300**
+3010 C–G	**0.00000**	0.33833	**0.00000**	–	**0.01665**	**0.00000**	**0.00000**	**0.00000**	**0.00000**
+3027 A–C	**0.00266**	1.00000	0.14868	**0.00866**	–	**0.00000**	**0.02065**	0.11339	0.05828
+3035 C–T	**0.00000**	**0.00000**	0.06327	**0.00000**	**0.00000**	–	**0.00000**	**0.00266**	**0.00283**
+3142 C–G	**0.00000**	0.35997	**0.00000**	**0.00000**	**0.00833**	**0.00000**	–	**0.00000**	**0.00000**
+3187 A–G	**0.00000**	0.66101	**0.00583**	**0.00000**	0.42341	0.21445	**0.00000**	–	**0.00033**
+3196 C–G	**0.00000**	0.28671	0.25824	**0.00000**	**0.04645**	0.00100	**0.00000**	**0.00000**	–

Table 3
Frequency of the *HLA-G* 3′UTR haplotypes encompassing the nine polymorphic sites observed in patients with type 1 diabetes mellitus and healthy individuals. The Fisher exact test *P*-values, odds ratio (OR) and 95% confidence interval (95%C.I.) for comparisons between patients and controls are also shown. Significant *P*-values ($P < 0.05$) are represented in boldface and indicate statistically significant associations.

Haplotype	*HLA-G* 3′UTR haplotypes									Patients		Controls		*P*	OR	95%C.I.
	14bp	+3001	+3003	+3010	+3027	+3035	+3142	+3187	+3196	Absolute frequency	Relative frequency	Absolute frequency	Relative frequency			
UTR-1	Del	C	T	G	C	C	C	G	C	72	0.3050	57	0.2375	0.1224	1.3930	0.9277–2.0900
UTR-2	Ins	C	T	C	C	C	G	A	G	64	0.2721	65	0.2708	0.6044	1.1190	0.7444–1.6820
UTR-3	Del	C	T	C	C	C	G	A	C	14	0.0593	32	0.1333	**0.0078**	0.4063	0.2108–0.7828
UTR-4	Del	C	C	G	C	C	C	A	C	25	0.1059	33	0.1375	0.3271	0.7362	0.4231–1.2810
UTR-5	Ins	C	T	C	C	T	G	A	C	17	0.0720	24	0.1000	0.3273	0.6923	0.3617–1.3250
UTR-6	Del	C	T	G	C	C	C	A	C	21	0.0889	11	0.0458	0.0693	2.0150	0.9489–4.2780
UTR-7	Ins	C	T	C	A	T	G	A	C	12	0.0508	15	0.0625	0.6927	0.7965	0.3646–1.7400
UTR-8	Ins	C	T	G	C	C	G	A	G	1	0.0042	3	0.0125	0.6234	0.3333	0.0344–32.2290
UTR-10	Del	C	T	C	C	C	G	A	G	1	0.0042	–	–	0.4979	3.0380	0.1230–75.0070
UTR-13	Del	C	T	C	C	T	G	A	C	2	0.0084	–	–	0.2474	5.0850	0.2426–106.5500
UTR-14	Del	C	T	G	C	T	C	A	C	1	0.0042	–	–	0.4979	3.0380	1.1230–75.0070
UTR-17	Ins	T	T	C	C	T	G	A	C	6	0.0254	–	–	**0.0148**	13.4470	0.7528–240.2300
Total										236	1.0004	240	0.9999			

constitutively express HLA-G [12]; however, the role of HLA-G expression in this endocrine organ has not been clarified. Since the regulatory regions of the *HLA-G* gene are involved in the magnitude of production of the molecule, the search for additional genetic markers in this MHC region is pertinent.

Our group described the structure of 3′UTR of the *HLA-G* gene in bone marrow donors from the Northeastern region of the State of São Paulo, Brazil, reporting the frequencies of the three polymorphic sites that have been associated with the regulation of *HLA-G* mRNA production (14bpDEL/INS, +3142C/G and +3187A/G), as well as of the five SNPs (+3003C/T, +3010C/G, +3027A/C, +3035C/T and +3196C/G) that have not yet been studied in relation to their influence on post-transcriptional gene control. This set of polymorphic sites has produced at least eight combinations (haplotypes) with polymorphic frequencies, i.e., greater than 1% (UTR-1 to UTR-8), and other low-frequency ones (UTR-9 to UTR-11). In addition, we found strong linkage disequilibrium between these polymorphic sites [21], particularly the combination of the 14bp INS, +3142 G and +3187 A alleles, each of them individually associated with low *HLA-G* mRNA levels.

Although at low frequency, in the present study we found the presence of the +3001 T allele in T1D patients. The +3001 T/C polymorphic site was previously reported in populations from Portugal and Guinea Bissau [34], and in the Northeastern Brazilian population [35], but it was not observed in our control population. The +3001 CC and the +3010 CC genotypes were significantly decreased in T1D patients, conferring preventive fractions of 0.9234 and 0.1545, respectively, which are considerable when compared to that conferred by the *HLA-DQB1**06:02/*06:03 alleles (preventive fraction = 0.20) in the Brazilian population [36]. To understand the possible influence of 3′UTR polymorphic sites in *HLA-G* mRNA production, we have previously performed an *in silico* study to evaluate microRNAs that can target this region, disclosing at least six microRNAs (hsa-mir-1262, hsa-mir-92nd-1, hsa-mir-92nd-2, hsa-mir-661, hsa-mir-1266, hsa-mir-1293) that can potentially target the +3010C/G segment in *HLA-G* 3′UTR [23]. However, no reports evaluating the profile of microRNAs in pancreatic tissue of T1D patients have been reported. On the other hand, the +3010 CC genotype was associated with a reduced level of soluble HLA-G in Brazilian and French individuals [20]. Considering that HLA-G is constitutively expressed in pancreas, where it is supposed to protect the organ against the attack of cytotoxic cells, the decreased expression at the pancreas level should be detrimental in individuals genetically prone to produce less HLA-G.

The fact that +3001T allele and +3001CT genotype are only present in 6 T1D patients and absent in controls resulted in a weak association with large 95% confidence intervals. Moreover, the analysis of clinical and laboratory data from these patients revealed no particular clinical or epidemiological features in comparison to other diabetic patients without the +3001T allele. The structure of *HLA-G* 3′UTR that includes this allele is exactly the same of UTR-5 (associated with low HLA-G soluble levels) [20], except for the C to T mutation at the +3001 position. The other polymorphic sites found together with the +3001 T allele are 14bpINS/+30142G/+3187A, all of them associated with low production of *HLA-G* mRNA [19,34], corroborating the idea of low HLA-G expression at the pancreas level in T1D, an issue that deserves further attention.

The UTR-3 haplotype was underrepresented among T1D patients, and this haplotype carries one allele associated with high production of HLA-G (14bp DEL) and two alleles (+3142 G and +3187 A) associated with decreased production of *HLA-G* mRNA [21,22]. In addition, the UTR-3 haplotype is associated with moderate expression of soluble HLA-G [20].

Unlike many tissues where the HLA-G has no detectable basal expression, the pancreas constitutively expresses HLA-G. Thus,

given that functionally relevant *HLA-G* 3′UTR haplotypes are associated with susceptibility/protection to T1D, one may argue that the constitutive expression of HLA-G in pancreatic cells may protect these cells against the action of immune system lymphocytes. According to this, the expression of HLA-G could be reduced in patients with T1D compared to control, rendering patients' pancreas more susceptible to immune mediated destruction when for some reason, such as upon viral infection, cytotoxic lymphocytes reach the pancreas. Some evidence supports these ideas: (i) development of T1D after infection with mumps and other viruses [37], which may be accompanied by a large influx of cytotoxic lymphocytes, (ii) some viruses may decrease classical and non-classical HLA molecule expression to escape the attack of cytotoxic cells [38].

In conclusion, in this study we reported polymorphic sites at the *HLA-G* 3′UTR that are associated with T1D development in Brazilian population. Since some of these polymorphic sites, including +3010 CC and UTR-3, have been previously associated with decreased and moderate soluble *HLA-G* production, and a decreased expression of HLA-G at the pancreas level could be detrimental to this endocrine organ, the present results suggest that the *HLA-G* gene may play a role in T1D susceptibility, pathogenesis or both. The evaluation of other *HLA-G* gene segments such as the promoter and coding regions may help the definition of *HLA-G* extended haplotypes, and the study of the expression of HLA-G in the pancreas at various stages of T1D development may unveil the role of HLA-G on the surface of pancreatic cells.

Author contributions

Conceived and designed the experiments: RSDA, CTMJ, PM, SG, ECC, EAD. Performed the experiments: RSDA, NLS, CLLS, NHSD.

Analyzed the data: RSDA, CTMJ, LCVC, ECC.

Contributed reagents/materials/analysis tools: NLS, DMR, MCFF, MCF.

Wrote the paper: RSDA, CTMJ, NLS, PM, SG, ECC, EAD.

Acknowledgements

We thank Viviane Carvalho for invaluable technical assistance and the Program for Technological Development in Tools for Health (PDTISFIOCRUZ). This study was supported by Conselho Nacional de Pesquisa e Desenvolvimento (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), CAPES/COFECUB (Project # 653/09) and Fundação de Apoio ao Ensino Pesquisa e Assistência (FAEPA). C.T.M.J. (309572/2014-2), E.A.D. (304931/2014-4) and E.C.C. (304471/2013-5) are supported by Research fellowships from CNPq/Brazil. P.M. is supported by Programa Ciência sem Fronteiras "Pesquisador Visitante Especial" (grant 406594/2013-9) from CNPq/Brazil.

References

[1] J. Klein, A. Sato, The HLA system. First of two parts, N. Engl. J. Med. 343 (2000) 702–709.
[2] J. Klein, A. Sato, The HLA system. First of two parts, N. Engl. J. Med. 343 (2000) 782–786.
[3] U. Kanga, B. Vaidyanathan, R. Jaini, P.S. Menon, N.K. Mehra, HLA haplotypes associated with type 1 diabetes mellitus in North Indian children, Hum. Immunol. 65 (2004) 47–53.
[4] C. Gorodezky, C. Alaez, A. Murguía, A. Rodríguez, S. Balladares, et al., HLA and autoimmune diseases: Type 1 diabetes (T1D) as an example, Autoimmun. Rev. 5 (2006) 187–194.
[5] M.C. Eike, T. Becker, K. Humphreys, M. Olsson, B.A. Lie, Conditional analyses on the T1DGC MHC dataset: novel associations with type 1 diabetes around HLA-G and confirmation of HLA-B, Genes Immun. 10 (2009) 56–67.
[6] A.1 Solini, E. Muscelli, M. Stignani, L. Melchiorri, E. Santini, C. Rossi, B.D. Astiarraga, R. Rizzo, O.R. Baricordi, Soluble human leukocyte antigen-g expression and glucose tolerance in subjects with different degrees of adiposity, J. Clin. Endocrinol. Metab. 95 (2010) 3342–3346.
[7] E.D. Carosella, P. Moreau, J. Le Maoult, M. Le Discorde, J. Dausset, Rouas-Freiss, HLA-G molecules: from maternal-fetal tolerance to tissue acceptance, Adv. Immunol. 81 (2003) 199–252.
[8] E.D. Carosella, B. Favier, N. Rouas-Freiss, P. Moreau, J. Lemaoult, Beyond the increasing complexity of the immunomodulatory HLA-G molecule, Blood 111 (2008) 4862–4870.
[9] L.C. Sullivan, H.L. Hoare, J. McCluskey, J. Rossjohn, A.G. Brooks, A structural perspective on MHC class Ib molecules in adaptive immunity, Trends Immnunol 27 (2006) 413–420.
[10] A. King, S.E. Hiby, L. Gardner, S. Joseph, J.M. Bowen, S. Verma, et al., Recognition of trophoblasts HLA class I molecules by decidual NK cell receptors-a review, Placenta 21 (2006) 81–85.
[11] V. Cirulli, J. Zalatan, M. MCmaster, R. Prinsen, D.R. Salomon, et al., The class I HLA repertoire of pancreatic islets comprises the nonclassical class Ib antigen HLA-G, Diabetes 55 (2006) 1214–1222.
[12] M. Shiroishi, K. Tsumoto, K. Amano, Y. Shirakihara, M. Colonna, et al., Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 8856–8861.
[13] I.J. Wastowski, P.D. Sampaio-Barros, E.M. Amstalden, G.M. Palomino, J.F. Marques-Neto, et al., HLA-G expression in the skin of patients with systemic sclerosis, J. Rheumatol. 36 (2009) 1230–1234.
[14] J.E. Boyson, R. Erskine, M.C. Whitman, M. Chiu, J.M. Lau, et al., Disulfide bond-mediated dimerization of HLA-G on the cell surface, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 16180–16185.
[15] E.A. Donadi, E.C. Castelli, A. Arnaiz-Villena, M. Roger, D. Rey, P. Moreau, Implications of the polymorphism of HLA-G on its function, regulation, evolution and disease association, Cell. Mol. Life Sci. 68 (2011) 369–395.
[16] C. Ober, C. Billstrand, S. Kuldanek, Z. Tan, The miscarriage associated HLA-G -725G allele influences transcription rates in JEG-3 cells, Hum. Reprod. 21 (2006) 1743–1748.
[17] E.C. Castelli, C.T. Mendes-Junior, L.C. Veiga-Castelli, M. Roger, et al., A comprehensive study of polymorphic sites along the HLA-G gene: implication for gene regulation and evolution, Mol. Biol. Evol. 28 (2011) 3069–3086.
[18] P. Rousseau, M. Le Discorde, G. Mouillot, C. Marcou, E.D. Carosella, P. Moreau, The 14 bp deletion-insertion polymorphism in the 3′ UT region of the HLA-G gene influences HLA-G mRNA stability, Hum. Immunol. 64 (2003) 1005–1010.
[19] E.C. Castelli, C.T. Mendes-Junior, N.H. Deghaide, R.S. de Albuquerque, Y.C. Muniz, et al., The genetic structure of 3′untranslated region of the HLA-G gene: polymorphisms and haplotypes, Genes Immun. 11 (2010) 134–141.
[20] G. Martelli-Palomino, J.A. Pancotto, Y.C. Muniz, C.T. Mendes-Junior, E.C. Castelli, et al., Polymorphic sites at the 3′ untranslated region of the HLA-G gene are associated with differential hla-g soluble levels in the Brazilian and French population, PLoS ONE 8 (2013) e71742.
[21] Z. Tan, G. Randall, J. Fan, B. Camoretti-Mercado, R. Brockman-Schneider, L. Pan, et al., Allele-specific targeting of microRNAs to HLA-G and risk of asthma, Am. J. Hum. Genet. 81 (2007) 829–834.
[22] S.M. Yie, L.H. Li, R. Xiao, C.L. Librach, A single base-pair mutation in the 3′-untranslated region of HLA-G mRNA is associated with pre-eclampsia, Mol. Hum. Reprod. 14 (2008) 649–653.
[23] E.C. Castelli, P. Moreau, A. Oya e Chiromatzo, C.T. Mendes-Junior, L.C. Veiga-Castelli, et al., In silico analysis of microRNAS targeting the HLA-G 3′ untranslated region alleles and haplotypes, Hum. Immunol. 70 (2009) 1020–1025.
[24] S.A. Miller, D.D. Dykes, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acids Res. 16 (1988) 1215.
[25] L. Excoffier, M. Slatkin, Incorporating genotypes of relatives into a test of linkage disequilibrium, Am. J. Hum. Genet. 62 (1998) 171–180.
[26] L. Excoffier, H. Lischer, Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows, Mol. Ecol. Resour. 10 (2010) 564–567.
[27] F. Rousset, GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux, Mol. Ecol. Resour. 8 (2008) 103–106.
[28] S.W. Guo, E.A. Thompson, Performing the exact test of Hardy–Weinberg proportion for multiple alleles, Biometrics 48 (1992) 361–372.
[29] L. Excoffier, M. Slatkin, Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population, Mol. Biol. Evol. 12 (1995) 921–927.
[30] M. Stephens, N.J. Smith, P. Donnelly, New statistical method for haplotype reconstruction from population data, Am. J. Hum. Genet. 68 (2001) 978–989.
[31] Z.S. Qin, T. Niu, J.S. Liu, Partition-ligation-expectation-maximization algorithm for haplotype inference with single-nucleotide polymorphisms, Am. J. Hum. Genet. 71 (2002) 1242–1247.
[32] A. Sabbagh, P. Luisi, E.C. Castelli, L. Gineau, D. Courtin, et al., Worldwide genetic variation at the 3′ untranslated region of the HLA-G gene: balancing selection influencing genetic diversity, Genes Immun. 15 (2014) 95–106.
[33] A. Svejgaard, B.K. Jakobsen, P. Platz, L.P. Ryder, J. Nerup, et al., HLA associations in insulin-dependent diabetes: search for heterogeneity in different groups of patients from a homogeneous population, Tissue Antigens 28 (1986) 237–244.
[34] M. Alvarez, J. Piedade, S. Balseiro, G. Ribas, F. Regateiro, HLA-G 3′-UTR SNP and 14-bp deletion polymorphisms in Portuguese and Guinea-Bissau populations, Int. J. Immunogenet. 36 (2009) 361–366.
[35] N. Lucena-Silva, A.R. Monteiro, R.S. de Albuquerque, R.G. Gomes, C.T. Mendes-Junior, et al., Haplotype frequencies based on eight polymorphic sites at the 3′

untranslated region of the HLA-G gene in individuals from two different geographical regions of Brazil, Tissue Antigens 79 (2012) 272–278.
[36] A.P. Fernandes, P. Louzada-Junior, M.C. Foss, E.A. Donadl, HLA-DRB1, DQB1, and DQA1 allele profile in Brazilian patients with type 1 diabetes mellitus, Ann. N. Y. Acad. Sci. 958 (2002) 305–308.
[37] N. Van Der Werf, F.G. Kroese, J. Rozing, J.L. Hillebrands, Viral infections as potential triggers of type 1 diabetes, Diabetes Metab. Res. Rev. 23 (2007) 169–183.
[38] M. Grommé, J. Neefjes, Antigen degradation or presentation by MHC class I molecules via classical and non-classical pathways, Mol. Immunol. 39 (2002) 181–202.