HLA-G 3′ Untranslated Region Polymorphisms Are Associated with Systemic Lupus Erythematosus in 2 Brazilian Populations

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ABSTRACT. ***Objective.*** HLA-G has well recognized tolerogenic properties in physiological and nonphysiological conditions. The 3' untranslated region (3'UTR) of the *HLA-G* gene has at least 3 polymorphic sites (14-bpINS/DEL, +3142C/G, and +3196C/G) described as associated with posttranscriptional influence on messenger RNA production; however, only the 14-bpINS/DEL and +3142C/G sites have been studied in systemic lupus erythematosus (SLE).

Methods. We investigated the *HLA-G* 3'UTR polymorphic sites (14-bpINS/DEL, +3003C/T, +3010C/G, +3027A/C, +3035C/T, +3142C/G, +3187A/G, and +3196C/G) in 190 Brazilian patients with SLE and 282 healthy individuals in allele, genotype, and haplotype analyses. A multiple logistic regression model was used to assess the association of the disease features with the *HLA-G* 3'UTR haplotypes.

Results. Increased frequencies were observed of the 14-bpINS ($p = 0.053$), +3010C ($p = 0.008$), +3142G ($p = 0.006$), and +3187A ($p = 0.013$) alleles, and increased frequencies of the 14-bpINS-INS ($p = 0.094$), +3010 C-C ($p = 0.033$), +3142 G-G ($p = 0.021$), and +3187 A-A ($p = 0.035$) genotypes. After Bonferroni correction, only the +3142G ($p = 0.05$) and +3010C ($p = 0.06$) alleles were overrepresented in SLE patients. The UTR-1 haplotype (14-bpDEL/+3003T/+3010G/+3027C/+3035C/+3142C/+3187G/+3196C) was underrepresented in SLE ($p_{corr} = 0.035$).

Conclusion. These results indicate that *HLA-G* 3'UTR polymorphic sites, particularly +3142G and +3010C alleles, were associated with SLE susceptibility, whereas UTR-1 was associated with protection against development of SLE. (J Rheumatol First Release May 15 2013; doi:10.3899/jrheum.120814)

Key Indexing Terms:
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Systemic lupus erythematosus (SLE) is characterized by hyperreactivity of several B and T lymphocyte populations against a myriad of self-antigens, leading to deposition of immune complexes and tissue damage[1]. Familial clustering of patients with SLE and disease association with many genes of the immune response confirm the involvement of genetic factors in SLE susceptibility[2,3], emphasizing the role of MHC class I and II genes[4,5]. Important MHC class II gene associations are related to the presence of the HLA-DRB1*03 allele group in whites, the DRB1*08 allele in African Americans, and the DRB1* allele 15 in Hispanics and Egyptians[6,7], and major class I associations include several allele groups such as HLA-A*01, A*03, A*11, A*23, A*26, and A*69[8].

Human leukocyte antigen G (HLA-G) is a nonclassical MHC class Ib molecule that has a restricted tissue distribution in nonpathological conditions. Four membrane-bound (HLA-G1 to 4) and 3 soluble HLA-G (HLA-G5 to 7) isoforms have been described, and play a pivotal role in the modulation of the maternal immune response during pregnancy, thus facilitating the tolerance of a semiallogenic fetus by the immune system cells of the mother[9]. The tolerogenic properties of HLA-G are primarily mediated by the interaction of the molecule with several leukocyte receptors (ILT-2, ILT-4, and KIR2DL4, among others), which may (1) inhibit cytolysis mediated by CD8+ T cells and natural killer cells[10], (2) impair transendothelial migration of lymphocytes[11], (3) shift the balance of cytokines to a predominant Th2 immune response[12], (4) suppress T CD4+ lymphocyte proliferation[13,14], and (5) induce apoptosis of activated T CD8+ cells by soluble HLA-G isoforms[15,16], among others. Thus, the ectopic expression of HLA-G has been suggested as a possible mechanism of tissue protection against autoimmune inflammatory responses during the process of immune surveillance[17].

The *HLA-G* coding region has a low level of polymorphism compared to the classical HLA genes; however, the regulatory gene regions (promoter and 3'UTR) have relevant genetic variations. In the 3'UTR of the *HLA-G* gene there are several polymorphic sites that define at least 15 different known haplotypes in Brazil[18,19] and a number of rare haplotypes found in other populations[20,21]. Three of these polymorphic sites have been previously associated with the magnitude of *HLA-G* messenger RNA (mRNA) expression: (1) the insertion of 14 bp at position +2961 has been associated with a more stable mRNA[22]; (2) the +3142C/G polymorphic site has been described as influencing the binding of microRNA, particularly the +3142G allele, increasing the affinity of some microRNA for the *HLA-G* mRNA, thus decreasing mRNA availability[23]; and (3) the +3187A allele that has been associated with a less stable mRNA because of its vicinity to an adenine and uridine-rich motif[24]. Although the other 5 described polymorphic sites have not been studied in relation to their post-transcriptional influence on *HLA-G* mRNA production, they may influence the binding of several microRNA[25].

There are only a few, controversial studies regarding the role of *HLA-G* 3'UTR in patients with SLE, evaluating only the 14-bp insertion/deletion (14-bpINS/DEL)[26,27,28] and the +3142 C/G polymorphisms[29]. Because the polymorphic sites at *HLA-G* 3'UTR are in linkage disequilibrium (LD)[18], 3'UTR haplotypes may differentially influence HLA-G expression, and because HLA-G has immunomodulatory properties, we evaluated the *HLA-G* 3'UTR polymorphic sites in patients with SLE from 2 Brazilian populations for allele, genotype, and haplotype frequencies.

MATERIALS AND METHODS

Population. We designed a case-control study encompassing 190 patients with SLE (180 women) aged 17–66 years (median 36 yrs) from 2 distinct geographical regions of Brazil, i.e., 50 patients followed at the Rheumatology Outpatient Clinic of the University Hospital of the Federal University of Pernambuco (northeastern Brazil), and 140 patients followed at the University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo (southeastern Brazil). All patients met at least 4 of the 11 criteria established by the American College of Rheumatology, revised in 1997, for the diagnosis of SLE[30]. Demographic, clinical, and laboratory data were obtained from medical records or from interviews (Table 1). In the northeastern population, original skin color classification of the patients (white and nonwhite followed an adaptation of the Fitzpatrick classification) and controls (white, mulatto, and black) did not use the same categorization pattern. Because of this, for the analysis of this variable all subjects were reclassified as whites or nonwhites to allow comparison. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was determined in 107 patients with SLE[31].

A total of 283 healthy unrelated blood donors from the same geographical regions as the patients were also studied, i.e., 128 individuals from the Pernambuco Northeastern Hematology Institute and 155 from the Ribeirão Preto Blood Center, for whom data for *HLA-G* 3'UTR have been published[18,19]. The haplotype diversity between these 2 populations has been described[19].

The study protocol was approved by the Ethics Committee of the Agamenon Magalhães Hospital in Pernambuco (CAAE 0091.0.236.000-11) and by the Ethics Committee of the University Hospital of Ribeirão Preto (HCRP 2234/2007), and written informed consent was obtained from all participants.

Amplification and sequencing of 3'UTR of the HLA-G gene. Genomic DNA was extracted from peripheral blood mononuclear cells using the DNAzol reagent (Invitrogen), according to the manufacturer's instructions. The *HLA-G* 3'UTR was evaluated by nucleotide sequence variations between positions +2945 and +3259, as described[18]. Amplification was performed in a final volume of 25 μl containing 1× PCR buffer (20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 0.3 mM of each dNTP, 25 pmol of each primer (HG08F and HG08R[18]), 1.0 unit *Taq* DNA polymerase (BioTools), and 50 ng DNA. The initial denaturation cycle was carried out at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 60°C for 60 s, 72°C for 30 s, and a final extension step at 72°C for 7 min. The amplification product was evaluated using 2% agarose gel. PCR products containing the amplified fragment of about 350 bp were directly sequenced. Individuals homozygous for the presence or absence of the 14-bp fragment were sequenced in both directions using the Big Dye Terminator kit and the ABI3100 Genetic Analyzer (Applied Biosystems). Individuals who were 14-bp heterozygous were sequenced only with the reverse primer. All polymorphic sites found in *HLA-G* 3'UTR were individually annotated.

Statistical analysis. Allelic and genotypic frequencies were estimated with

Table 1. Demographic, clinical, and laboratory features of the Brazilian study populations.

Population Features	Northeastern Region				Southeastern Region				Total	
	Patients,		Control,		Patients,		Control,		Patients	
	n	%	n	%	n	%	n	%	n	%
Sex										
Female	46	92	18	14	134	96	39	26	149	94
Male	4	8	110	86	6	4	108	74	8	6
Age, yrs										
Median	39		30		39		32		36	
Minimum	23		18		17		19		17	
Maximum	58		60		66		52		66	
Skin color										
White	25	50	32	25	109	78	116	79	113	72
Nonwhite	25	50	—	—	—	—	—	—	44	28
Mulatto	—	—	88	69	19	14	17	12	—	—
Black	—	—	8	6	12	9	13	9	—	—
Asian ancestry	—	—	—	—	—	—	1	< 1	—	—
Clinical and laboratory features										
Malar rash									67	43
Discoid rash									57	36
Photosensitivity									76	48
Oral/nasopharyngeal ulcers									19	12
Nonerosive arthritis									89	57
Pericarditis/pleurisy									33	21
Renal involvement[1]									71	45
Convulsion/psychosis									27	17
Hematological alterations[2]									112	71
Immunological alterations[3]									102	65
Positive ANA, in the absence of drug inductors									147	94
Total[4]	50	100	128	100	140	100	147	100	157	100

[1] Proteinuria > 0.5 g or cell cylinders; [2] hemolytic anemia with reticulocytosis and/or leukopenia < 4000 and/or lymphopenia < 1500 and/or thrombocytopenia < 100,000 twice or more; [3] anti-DNA and/or anti-Sm and/or antiphospholipid antibodies (anticardiolipin, lupus anticoagulant, or false Venereal Disease Research Laboratory serology); [4] information was not available for 8 southeastern controls and 33 patients with SLE. ANA: antinuclear antibody; SLE: systemic lupus erytheamtosus.

the GENEPOP software version 4.0.10[32], using Fisher's exact test with Levene's correction to calculate the number of expected homozygotes or heterozygotes. Adherences of genotypic proportions to Hardy-Weinberg equilibrium (HWE) expectations were estimated by the exact test of Guo and Thompson, also using the GENEPOP software[33]. The exact test of population differentiation, based on genotypic frequencies, was carried out with the GENEPOP program. LD between each pair of loci was evaluated for each group as well as for the entire population using the Arlequin program, version 3.5.1.2[34].

Given the positive LD between alleles from the studied polymorphisms, but unknown gametic phase, the most likely haplotype pair for each sample was determined by 2 independent computational methods, without taking into account any prior information. The PHASE method[35] was applied using the PHASE program version 2.1. The expectation-maximization (EM) algorithm[36] was also used to estimate haplotype frequencies using the Arlequin program.

To compare the allele frequencies between patients and controls we constructed 2 × 2 contingency tables, and p values were calculated using the 2-tailed Fisher's exact test. The relative risk was estimated by calculating the OR with 95% CI. For genotype frequency analysis, tables were constructed by fixing 1 genotype and comparing it against the others. A similar strategy was applied to haplotype association studies. Analyses of contingency tables were performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software).

The association of disease features with the *HLA-G* 3'UTR haplotypes was assessed by a multiple logistic regression model. Covariants that showed the likelihood ratio statistic with a probability up to 0.25 in univariate analysis were included in the model using the Forward method[37] and the Software R, version 2.10.1 (R Foundation for Statistical Computing).

For all analyses, p values were considered to be significant at < 0.05. For the analyses encompassing allele, genotype, and haplotype comparisons between patients and controls, p values obtained before and after Bonferroni correction (p_c) were also calculated.

RESULTS

Influence of demographic variables. Patient and control groups showed differences in sex distribution, with a high frequency of females in the patient group and males among blood donors. The median age of healthy donors was slightly lower than that of patients. Ancestry information, evaluated by the perception of skin color, showed a similar distribution between white and nonwhite individuals (p = 0.886; OR 0.94, 95% CI 0.54–1.65) in the southeastern population. In the northeastern population, the proportions of whites and nonwhites differed between patients and controls; however, the multivariate analyses stratified according to skin color showed no significant differences.

Frequencies of HLA-G 3'UTR polymorphic sites differed

between SLE and healthy controls. Because the 3'UTR of *HLA-G* gene was sequenced, all described polymorphic sites were observed, including the 14-bp INS/DEL (GenBank ID rs1704), +3003C/T (GenBank ID rs1707), +3010C/G (GenBank ID rs1710), +3027A/C (GenBank ID rs17179101), +3035C/T (GenBank ID rs17179108), +3142C/G (GenBank ID rs1063320), +3187A/G (GenBank ID rs9380142), and +3196C/G (GenBank ID rs1610696).

Considering the Brazilian population as a whole, controlled for place of origin, sex, age, and skin color, the SLE group presented differences in the allele frequencies of +3010 C ($p = 0.008$; OR 1.69, 95% CI 1.15-2.56), +3142 G ($p = 0.006$; OR 1.74, 95% CI 1.17-2.61), and +3187 A ($p = 0.013$; OR 1.75, 95% CI 1.12-2.78), and a borderline value for the 14-bp INS allele ($p = 0.053$; OR 1.49, 95% CI 1.00-2.23) in relation to controls. Some comparisons continued to be significant after Bonferroni correction; i.e., $p_c = 0.048$ for +3142 G and a borderline value for +3010 C ($p_c = 0.064$). After stratification according to place of origin, but still controlling for confounding variables (sex, age, and skin color), we observed important differences in 3'UTR HLA-G allele frequencies between patients with SLE and controls in each population. In the northeastern population, most allele frequencies differed significantly between the SLE group and controls, but the +3187 A allele was the only one that continued to show a significant difference after Bonferroni correction ($p = 0.0003$ and $p_c = 0.002$). In contrast, in the southeastern population there were no significant differences in allele frequencies between SLE and controls after control for confounders (Table 2).

Patients from different regions showed different genotype distribution. HWE, LD, and differences in genotype distribution were determined, considering patients and controls as whole groups. None of the studied loci showed significant departures from HWE expectations ($p > 0.05$) in the control population; however, the 14-bp polymorphism did not fit HWE in the patient group, exhibiting a deficit of heterozygosis ($p = 0.010$). Overall, most polymorphic sites in the *HLA-G* 3'UTR presented high LD, allowing the use of probabilistic models to infer haplotypes. Exceptions are made for the +3003 T/C and +3027 A/C pair in patients and controls, and for the +3027 A/C and +3196 C/G pair in the patient group. The exact test of population differentiation based on genotype frequencies revealed different genotype distribution for the 14-bp ($p = 0.037$), +3010 ($p = 0.001$), +3142 ($p = 0.007$), and +3187 ($p = 0.043$) loci between patients with SLE and controls, considering the northeastern and southeastern populations as a whole. Differences in genotypic distribution between the southeastern and northeastern control groups were significant only for the +3003 locus ($p = 0.020$); and the exact test of population differentiation based on genotype frequencies revealed a similar result for the control groups of both populations ($p = 0.449$). Considering the whole population controlled for place of origin, sex, age, and skin color, we found an overrepresentation of +3010 C-C ($p = 0.033$; OR 1.96, 95% CI 1.06-3.70), +3142 G-G ($p = 0.021$; OR 2.04, 95% CI 1.14-3.85), and +3187 A-A ($p = 0.035$; OR 1.85, 95% CI 1.05-3.23) and concomitant underrepresentation of the +3010 G-G ($p = 0.027$; OR 0.44, 95% CI 0.21-0.92) and +3142 C-C ($p = 0.034$; OR 0.46, 95% CI 0.22-0.94) genotype frequencies in the SLE group. However, none of the genotype frequency differences remained significant after Bonferroni correction. Interestingly, all individuals from the patient and control groups exhibiting the 14-bp INS-INS genotype showed homozygosis for the +3010 C-C, +3142 G-G, and +3187 A-A loci as well, as detected by Castelli, *et al*[18].

HLA-G 3'UTR haplotypes were associated with SLE susceptibility. Given the positive LD between alleles of the 3'UTR *HLA-G* polymorphisms, but unknown gametic phase, the expectation-maximization algorithm and the PHASE method were applied to define haplotypes from SLE patient samples, as described[35,36]. Haplotypes from the control southeastern[18] and northeastern[19] samples were defined in previous studies. In our current study, haplotypes from 2 patients with SLE were not concordant by either method and were not considered for further analysis. The remaining samples did present the same haplotype inferred by both methods, with a mean probability of 0.998 and 0.964 for the EM and PHASE algorithms, respectively. The haplotyping methods used did reveal the most frequent haplotypes, named according to our previous studies[18,19].

The UTR-1 haplotype (14-bpDEL, +3003T, +3010G, +3027C, +3035C, +3142C, +3187G, +3196C) was associated with protection against development of SLE ($p < 0.005$; OR 0.52, 95% CI 0.33-0.82, $p_c = 0.035$), whereas UTR-5 (14-bpINS, +3003T, +3010C, +3027C, +3035T, +3142G, +3187A, +3196C) was associated with susceptibility to the development of SLE only before Bonferroni correction ($p = 0.031$; OR 2.10, 95% CI 1.09-4.08, $p_c = 0.215$; Table 3). Haplotype frequency distributions for both control populations were different when all haplotypes were considered simultaneously ($p = 0.003$). The haplotypes that were associated with protection from (UTR-1) and susceptibility to (UTR-5) SLE did not differ significantly between northeastern and southeastern Brazilians[19].

To further explore these associations, we grouped haplotypes exhibiting shared susceptibility or protective alleles. The frequency of the occurrence of both haplotypes bearing the SLE susceptibility sequence (14-bpINS, +3010C, +3142G, +3187A) was overrepresented in patients compared to controls ($p = 0.029$; OR 1.67, 95% CI 1.07-2.61). Considering that UTR-1 is the only haplotype that lacks the entire sequence for susceptibility, we examined the existence of a protective consensus sequence by grouping haplotypes that lacked at least 3 of the 4 susceptibility alleles. With this strategy, we found that haplotypes

Table 2. Comparisons of the 3'UTR *HLA-G* allele and genotype frequencies between patients with systemic lupus erythematosus (SLE) and healthy blood donors from 2 Brazilian populations: southeastern (SE) from Ribeirão Preto and northeastern (NE) from Recife.

Variables	SE Brazil*					NE Brazil*					Total**					
	Case %	Cont. %	OR	95% CI	p	Case %	Cont. %	OR	95% CI	p	Case %	Cont. %	OR	95% CI	p^a	p_c
Alleles																
'14bp INDEL																
14-bp DEL	52.50	59.18	1.00			55.00	62.20	1.00			53.16	60.58	1.00			
14-bp INS	47.50	40.82	1.30	0.81–2.10	0.283	45.00	37.80	2.02	0.94–4.48	0.075	46.84	39.42	1.49	1.00–2.23	0.053	0.424
'+3003 C/T																
'+3003 C	8.57	13.27	1.00			12.00	6.69	1.00			9.47	10.22	1.00			
'+3003 T	91.43	86.73	1.68	0.80–3.45	0.165	88.00	93.31	0.35	0.10–1.19	0.096	90.53	89.78	1.13	0.60–2.10	0.705	
'+3010 C/G																
'+3010 C	61.79	52.40	1.00			68.00	54.33	1.00			63.42	53.30	1.00			
'+3010 G	38.21	47.60	0.71	0.44–1.14	0.151	32.00	45.67	0.40	0.18–0.86	0.021	36.58	46.70	0.59	0.39–0.87	0.008	0.064
'+3027 A/C																
'+3027 A	3.57	5.78	1.00			6.00	3.54	1.00			4.21	4.74	1.00			
'+3027 C	96.43	94.22	2.27	0.75–6.48	0.131	94.00	96.46	0.17	0.03–1.09	0.054	95.79	95.26	1.25	0.46–3.19	0.654	
'+3035 C/T																
'+3035 C	83.57	85.37	1.00			80.00	87.01	1.00			82.63	86.13	1.00			
'+3035 T	16.43	14.63	1.15	0.60–2.25	0.681	20.00	12.99	4.33	1.47–3.85	0.010	17.37	13.87	1.66	0.95–2.95	0.080	
'+3142 C/G																
'+3142 C	38.21	46.60	1.00			31.00	44.88	1.00			36.32	45.80	1.00			
'+3142 G	61.79	53.40	1.41	0.88–2.28	0.153	69.00	55.12	2.74	1.26–6.13	0.012	63.68	54.20	1.74	1.17–2.61	0.006	0.048
'+3187 A/G																
'+3187 A	75.36	74.15	1.00			86.96	70.08	1.00			78.23	72.26	1.00			
'+3187 G	24.64	25.85	0.86	0.51–1.48	0.585	13.04	29.92	0.15	0.05–0.40	0.000	21.77	27.74	0.57	0.36–0.89	0.013	0.104
'+3196 C/G																
'+3196 C	68.21	74.14	1.00			76.09	76.38	1.00			70.16	75.18	1.00			
'+3196 G	31.79	25.86	1.28	0.76–2.20	0.352	23.91	23.62	0.91	0.37–2.21	0.833	29.84	24.82	1.20	0.77–1.88	0.428	
Genotypes***																
'14-bp DEL/																
14-bp DEL	30.71	38.78	0.71	0.36–1.45	0.344	38.00	40.16	0.63	0.21–1.85	0.403	32.63	39.42	0.67	0.38–1.19	0.169	
'14-bp DEL/																
14-bp INS	43.57	40.82	1.14	0.58–2.27	0.697	34.00	44.09	0.62	0.20–1.85	0.391	41.05	42.34	0.98	0.56–1.78	0.942	
'14-bp INS/																
14-bp INS	25.71	20.41	1.32	0.60–3.03	0.503	28.00	15.75	1.32	0.60–3.03	0.503	26.32	18.25	1.82	0.92–3.70	0.094	
'+3003 C/+3003 C						2.00	0.00			0.992	0.53	0.00			0.987	
'+3003 C/+3003 T	17.14	26.53	0.55	0.25–1.22	0.138	20.00	13.39	2.94	0.71–12.5	0.140	17.89	20.44	0.80	0.40–1.59	0.516	
'+3003 T/+3003 T	82.86	73.47	1.82	0.82–4.00	0.138	78.00	86.61	0.31	0.07–1.22	0.100	81.58	79.56	1.19	0.60–2.33	0.605	
'+3010 C/+3010 C	36.43	28.77	1.67	0.81–3.57	0.177	46.00	29.13	2.44	0.81–8.33	0.116	38.95	28.94	1.96	1.06–3.70	0.033	0.792
'+3010 C/+3010 G	50.71	47.26	0.83	0.42–1.61	0.590	44.00	50.39	1.12	0.39–3.33	0.834	48.95	48.72	0.90	0.52–1.56	0.717	
'+3010 G/+3010 G	12.86	23.97	0.64	0.27–1.56	0.316	10.00	20.47	0.20	0.04–0.83	0.032	12.11	22.34	0.44	0.21–0.92	0.027	0.648
'+3027 A/+3027 A	0.00	0.68			0.986	0.00	0.79			0.994	0.00	0.73			0.983	
'+3027 A/+3027 C	7.14	10.20	0.68	0.21–2.38	0.532	12.00	5.51	8.33	1.08–100.0	0.044	8.42	8.03	1.28	0.45–3.85	0.647	
'+3027 C/+3027 C	92.86	89.12	1.00	0.35–2.70	0.998	88.00	93.70	0.13	0.02–0.98	0.049	91.58	91.24	1.01	0.35–2.70	0.992	
'+3035 C/+3035 C	68.57	72.79	0.78	0.36–1.61	0.497	66.00	76.38	0.78	0.36–0.63	0.493	67.89	74.45	0.53	0.27–1.00	0.055	
'+3035 C/+3035 T	30.00	25.17	1.43	0.62–3.13	0.362	28.00	21.26	4.55	1.16–25.0	0.042	29.47	23.36	1.92	1.00–3.85	0.055	
'+3035 T/+3035 T	1.43	2.04	0.37	5.26–9.18	0.414	6.00	2.36	5.88	0.36–100.0	0.255	2.63	2.19	1.16	0.20–8.33	0.873	
'+3142 C/+3142 C	13.57	21.77	0.68	0.29–1.69	0.395	10.00	20.47	0.20	0.04–0.83	0.032	12.63	21.17	0.46	0.22–0.94	0.034	0.816
'+3142 C/+3142 G	49.29	49.66	0.78	0.39–1.52	0.454	42.00	48.82	0.95	0.33–2.78	0.927	47.37	49.27	0.83	0.48–1.45	0.525	
'+3142 G/+3142 G	37.14	28.57	1.72	0.84–3.70	0.147	48.00	30.71	2.86	0.95–9.09	0.066	40.00	29.56	2.04	1.14–3.85	0.021	0.504
'+3187 A/+3187 A	58.57	53.06	1.22	0.63–2.38	0.559	76.09	49.61	5.88	1.82–25.0	0.005	62.90	51.46	1.85	1.05–3.23	0.035	0.840
'+3187 A/+3187 G	33.57	42.18	0.83	0.42–1.67	0.611	21.74	40.94	0.46	0.13–1.52	0.206	30.65	41.61	0.70	0.39–1.25	0.230	
'+3187 G/+3187 G	7.86	4.76	0.89	0.27–3.57	0.857	2.17	9.45	0.03	0.00–0.29	0.008	6.45	6.93	0.43	0.16–1.14	0.080	
'+3196 C/+3196 C	46.43	55.17	0.68	0.34–1.32	0.253	63.04	60.63	1.35	0.45–4.17	0.585	50.54	57.72	0.80	0.45–1.41	0.442	
'+3196 C/+3196 G	43.57	37.93	1.47	0.74–2.94	0.281	26.09	31.50	1.47	0.74–2.94	0.281	39.25	34.93	1.16	0.65–2.08	0.607	
'+3196 G/+3196 G	10.00	6.90	1.10	0.35–4.00	0.883	10.87	7.87	1.67	0.24–1.25	0.613	10.22	7.35	1.28	0.48–3.70	0.632	

[a] Some comparisons did not remain statistically significant at the 0.05 significance level for the alleles ($alpha_c = 0.0063$) and genotype ($alpha_c = 0.0021$) distribution, after the Bonferroni correction. * Adjusted by sex, skin color, and age. ** Adjusted by sex, skin color, age, and place of origin. *** Genotype frequencies were calculated by comparing each genotype against the others.

Table 3. *HLA-G* 3' untranslated region (UTR) haplotype frequencies observed for systemic lupus erythematosus (SLE) patients and healthy controls from the 2 Brazilian populations: southeastern (SE) from Ribeirão Preto and northeastern (NE) from Recife.

Haplotypes	SE Brazil*					NE Brazil*					Total**				
	Pt. %	Cont. %	Or	95% CI	p	Pt. %	Cont. %	OR	95% CI	p	Pt. %	Cont. %	OR	95% CI	p
UTR1 (DTGCCCGC)															
Others	76.79	74.15	1.00			88.00	70.87	1.00			79.74	72.63	1.00		
UTR 1	23.21	25.85	0.81	0.48–1.40	0.451	12.00	29.13	0.13	0.05–0.36	0.000[a]	20.26	27.37	0.52	0.33–0.82	0.005[b]
UTR2 (ITCCCGAG)															
Others	69.29	74.83	1.00			74.00	77.17	1.00			70.53	75.91	1.00		
UTR 2	30.71	25.17	1.26	0.75–2.16	0.395	26.00	22.83	1.01	0.42–2.41	0.985	29.47	24.09	1.21	0.78–1.90	0.408
UTR3 (DTCCCGAC)															
Others	86.07	87.41	1.00			78.00	84.25	1.00			83.95	85.95	1.00		
UTR 3	13.93	12.59	1.15	0.59–2.34	0.694	22.00	15.75	1.30	0.60–2.95	0.515	16.05	14.05	1.27	0.73–2.23	0.405
UTR4 (DCGCCCAC)															
Others	91.79	86.73	1.00			88.00	93.31	1.00			90.79	89.78	1.00		
UTR 4	8.21	13.27	0.56	0.27–1.20	0.128	12.00	6.69	0.96	0.43–2.17	0.911	9.21	10.22	0.85	0.46–1.62	0.621
UTR5 (ITCCTGAC)															
Others	87.50	91.16	1.00			87.00	91.34	1.00			87.37	91.24	1.00		
UTR 5	12.50	8.84	1.14	0.29–4.66	0.856	13.00	8.66	3.12	0.93–11.00	0.070	12.63	8.76	2.10	1.09–4.20	0.031[c]
UTR6 (DTGCCCAC)															
Others	94.29	92.52	1.00			87.37	91.24	1.00			93.68	91.79	1.00		
UTR 6	5.71	7.48	1.05	0.40–2.95	0.925	12.63	8.76	1.07	0.26–4.11	0.924	6.32	8.21	0.99	0.45–2.25	0.989
UTR7 (ITCATGAC)															
Others	96.43	94.22	1.00			94.00	96.46	1.00			95.79	95.26	1.00		
UTR 7	3.57	5.78	0.44	0.15–1.33	0.131	6.00	3.54	5.98	0.91–34.34	0.054	4.21	4.74	0.80	0.31–2.17	0.654

UTR: (14-bp D/I; +3003C/T; +3010C/G; +3027A/G; +3035C/T; +3142C/G; +3187A/G; +3196C/G). [a, b, c] After the Bonferroni correction, taking into account 7 independent tests (alpha$_c$ = 0.0071), p values remained significant for "a" (p = 0.001) and "b" (p = 0.035), but did not remain significant in "c" (p = 0.215). * Adjusted by sex, skin color, and age. ** Adjusted by sex, skin color, age, and place of origin.

lacking the 14-bpINS and +3010C alleles, i.e., UTR-1, UTR-4, UTR-6, or UTR-14, when present in homozygosis, were associated with protection against development of SLE (p = 0.018; OR 0.54, 95% CI 0.32-0.91). It is notable that when susceptibility and protective haplotypes were observed in the same individual, no statistically significant differences were observed between patients and controls (p = 0.418; OR 0.84, 95% CI 0.56-1.25). These results are shown in Table 4.

Considering that the polymorphic sites observed at the *HLA-G* 3'UTR were in LD, we tried to identify the most relevant ones associated with SLE susceptibility (14-bpINS/+3010C/+3142G/+3187A) by studying haplotypes that presented a partial susceptibility consensus. Regarding the role of the 14-bpINS allele, we observed that a single change of the 14-bpDEL allele in the susceptibility consensus, as observed in UTR-3, UTR-10, UTR-11, UTR-13, did not abolish its contribution to SLE susceptibility. Evidence for this: (1) a double dose of haplotypes combining full or partial susceptibility consensus lacking the 14-bpINS allele was overrepresented in the SLE patient group (p = 0.021; OR 1.60, 95% CI 1.09-2.37) even though UTR-10 and UTR-11 were present only in the control group at low frequency in our study population; and (2) the protection conferred by the double-protection consensus haplotypes was abolished after adding individuals who shared 1 protective haplotype with the susceptibility consensus, but lacking the 14-bpINS (p = 0.202; OR 0.77, 95% CI 0.52-1.13). To study the influence of the +3010 C/G polymorphic site in relation to others associated with SLE susceptibility, we compared the frequency of the haplotypes that lacked the +3010 C allele, but exhibited other suscepti-

Table 4. Comparisons of the frequencies of the *HLA-G* 3' UTR consensus sequences associated with susceptibility to (14-bpIns/+3010C/+3142G/+3187A) or protection against SLE development (14-bpDEL/+3010G/+3142C/+3187G/A) at single (heterozygous) or double (homozygous) dose consensus sequences.

Haplotypes	Patients, n = 188	Controls n = 281	p	OR (95% CI)[4]
Homozygous for protection[1]	23	58	0.018	0.54 (0.32–0.91)
Other UTR	165	223		
Homozygous for susceptibility[2]	50	50	0.029	1.67 (1.07–2.61)
Other UTR	138	231		
Heterozygous for protection[3]	55	93	0.418	0.84 (0.56–1.25)
Other UTR	133	188		

[1] Combination of UTR-1, -4, -6, -14 haplotypes. [2] Combination of UTR-2, -5, -7, -15, -16 haplotypes. [3] Combination of UTR-1, -4, -6, -14 with UTR2, -5, -7, -15, -16 haplotypes. [4] Fisher's 2-tailed test. SLE: systemic lupus erythematosus; UTR: untranslated region.

bility markers (UTR-8 and UTR-9). Unfortunately, these haplotypes were found only in association with previously detected protective haplotypes (UTR-1, UTR-4, and UTR-14) in the control population, which made analysis more difficult. Nevertheless, increased protection was observed with the inclusion of individuals exhibiting UTR-8 and UTR-9 in the double-protection haplotype group (p = 0.005; OR 0.47, 95% CI 0.28-0.79). In contrast, susceptibility was abolished with the inclusion of the UTR-8 and UTR-9 haplotypes in the double dose susceptibility haplotype group (p = 0.093; OR 1.46, 95% CI 0.94-2.25), suggesting that the susceptibility to SLE conferred by the +3010 C allele was independent of the 14-bpINS allele in the consensus sequence.

Specific HLA-G 3'UTR haplotypes were associated with clinical and laboratory features. The association of specific haplotypes with disease manifestations was examined only for 157 patients with SLE, because 33 cases (17%) had incomplete clinical information. Univariate analysis showed that (1) individuals carrying the UTR-3 haplotype were protected against the development of pericarditis (p = 0.037); (2) those carrying the UTR-5 haplotype exhibited fewer immunological disorders (p = 0.031), as evaluated by the presence of anti-dsDNA, anti-Sm, or antiphospholipid antibodies; and (3) individuals carrying the UTR-4 haplotype presented a 2.7-fold risk to develop oronasal ulcers and were less likely to present antinuclear antibody (ANA; p = 0.010; Table 5). Multivariate analysis adjusted for sex and age confirmed the association of the UTR-5 haplotype with fewer immunological disorders (p = 0.009; OR 0.37, 95% CI 0.17-0.78) and the association of UTR-4 with undetectable ANA (p = 0.010; OR 0.22, 95% CI 0.07-0.76). The protection conferred by the UTR-3 haplotype against the development of pericarditis (p = 0.069) was not confirmed. Comparison of homozygous protective versus homozygous susceptible haplotypes revealed no association with disease severity according to clinical profile at diagnosis, suggesting that the *HLA-G* 3'UTR polymorphisms are related more to susceptibility to SLE than to disease severity. This idea is supported by the lack of associations between disease activity indexes (SLEDAI) and 3'UTR haplotypes (data not shown).

DISCUSSION

A report evaluating single-nucleotide polymorphism density in the MHC region in patients with SLE showed higher densities at the HLA-DRB1, C4, TNF, HLA-B, and HLA-A loci than in other MHC subregions[38]. Considering that the HLA-A and HLA-G loci are in LD[39,40] and that HLA-A alleles are associated with SLE[8], and considering the tolerogenic properties of the HLA-G molecule, the study of the regulatory regions of the HLA-G locus in patients with SLE is pertinent, particularly 3'UTR, which has elements that may post-transcriptionally influence HLA-G expression[41,42]. In our study, we found the same 8 polymorphic sites in the 3'UTR of the *HLA-G* gene, as reported by Castelli, *et al*[18], some of them at different frequencies when compared to controls.

To minimize the chance of making a type I error in the analysis of allele, genotype, or haplotype association with SLE, we used the Bonferroni-adjusted p level. After applying this strategy, we observed only fifth-class genetic associations ($p \leq 0.05$), as classified by Manly[43], i.e., these associations may be due to variation in statistical power, LD, and population stratification, and may also be due to the influence of other genetic or environmental factors. To further investigate possible limitations of the study due to small sample size, we used the confidence intervals and corresponding frequencies from data that reached significance in the whole population to calculate the power of our study, and we found a power ranging from 70% to 80%, which is considered quite good.

Regarding the 14-bp INS/DEL, our results corroborate the findings of an Italian study that showed increased frequency of the 14-bp INS allele and 14-bp INS-INS genotype in patients with SLE[26]. The same study reported that lower soluble levels of HLA-G (sHLA-G) were more commonly associated with the 14-bp INS-INS genotype than with the 14-bp INS-DEL and 14-bp DEL-DEL genotypes in Italian and Danish patients with SLE. However, the expression of sHLA-G and the presence of the 14-bp INS-INS genotype were not related to disease activity as evaluated by SLEDAI[26]. Because the 14-bp INS/DEL polymorphism is usually in LD with other polymorphic sites, it is also possible that the influence of the INDEL polymorphism may depend on other nearby genetic variations.

Different studies have shown high LD between the 14-bp and +3142 loci[18,23], which we also observed. A guanine at position +3142 increases the affinity of the *HLA-G* mRNA 3'UTR for several microRNA, negatively regulating the *HLA-G* gene expression by inducing mRNA degradation or translation suppression[18,23]. Before correcting p values, we observed an association of the +3142 G allele (p = 0.006), the +3142 G-G genotype (p = 0.021), and the double homozygosis 14-bp INS-INS/+3142G-G with SLE. A study of patients with SLE from southeastern Brazil[29] also showed a significant increase in the +3142 G allele (0.58 vs 0.47; p = 0.011) and of the +3142 G-G genotype (OR 1.90, 95% CI 1.08–3.42). In addition, the double heterozygosis 14-bp INS-DEL/+3142 C-G was associated with milder disease[29]. Therefore, downregulation of *HLA-G* and the presence of the *HLA-G* +3142 G allele are apparently more associated with susceptibility to SLE. Indeed, after Bonferroni correction, the association of the +3142 G allele with SLE remained significant; however, the association with the 14-bp INS allele did not, indicating that disease susceptibility may be due to the +3142 polymorphic site.

HLA-G mRNA stability has also been associated with the

Table 5. Comparison of the *HLA-G* 3' untranslated region (UTR) haplotypes associated with clinical and laboratory features of patients with systemic lupus erythematosus (univariate logistic regression).

Variables	Yes	No	p	OR	95% CI	Variables	Yes	No	p	OR	95% CI	Total
Renal involvement[b]						Arthritis						
UTR-1[a]	41	27	0.106	1.57	0.91–2.73	UTR-1[a]	39	29	0.901	1.04	0.60–1.79	68
UTR-2	40	44	0.395	0.80	0.49–1.33	UTR-2	53	31	0.167	1.44	0.86–2.42	84
UTR-3	27	30	0.481	0.81	0.46–1.45	UTR-3	27	30	0.118	0.63	0.35–1.12	57
UTR-4	13	15	0.567	0.80	0.36–1.74	UTR-4	14	14	0.456	0.74	0.34–1.63	28
UTR-5	22	20	0.913	1.04	0.54–2.00	UTR-5	22	20	0.546	0.82	0.43–1.58	42
UTR-6	10	11	0.706	0.84	0.34–2.06	UTR-6	13	8	0.618	1.26	0.52–3.27	21
UTR-7	9	4	0.204	2.18	0.69–8.17	UTR-7	9	4	0.357	1.76	0.56–6.60	13
Total	162	152				Total	178	136				314
Pericarditis						Convulsion/psychoses						
UTR-1[a]	18	50	0.214	1.49	0.78–2.74	UTR-1[a]	17	51	0.057	1.88	0.97–3.57	68
UTR-2	18	66	0.914	1.03	0.55–1.88	UTR-2	17	67	0.389	1.32	0.69–2.47	84
UTR-3	6	51	0.037	0.39	0.14–0.88	UTR-3	10	47	0.939	1.03	0.46–2.12	57
UTR-4	5	23	0.668	0.80	0.26–2.04	UTR-4	4	24	0.669	0.79	0.22–2.15	28
UTR-5	10	32	0.634	1.21	0.53–2.52	UTR-5	4	38	0.165	0.47	0.14–1.23	42
UTR-6	4	17	0.819	0.88	0.25–2.47	UTR-6	2	19	0.344	0.49	0.07–1.75	21
UTR-7	5	8	0.126	2.46	0.72–7.64	UTR-7[†]	0	13	0.000			13
Total	66	248				Total	54	260				314
Skin lesion						Rash						
UTR-1[a]	26	42	0.709	1.11	0.63–1.93	UTR-1[a]	27	41	0.576	0.86	0.49–1.47	68
UTR-2	24	60	0.086	0.62	0.36–1.06	UTR-2	37	47	0.766	1.08	0.65–1.79	84
UTR-3	22	35	0.691	1.13	0.62–2.04	UTR-3	27	30	0.429	1.26	0.71–2.25	57
UTR-4	8	20	0.375	0.68	0.27–1.54	UTR-4	12	16	0.984	1.01	0.45–2.20	28
UTR-5	18	24	0.344	1.38	0.70–2.65	UTR-5	17	25	0.757	0.90	0.46–1.73	42
UTR-6	10	11	0.269	1.65	0.67–4.05	UTR-6	8	13	0.661	0.82	0.31–2.00	21
UTR-7	5	8	0.869	1.10	0.33–3.38	UTR-7	6	7	0.796	1.16	0.37–3.57	13
Total	114	200				Total	134	180				314
Photosensitivity						Oronasal ulcers						
UTR-1[a]	27	41	0.106	0.64	0.37–1.10	UTR-1[a]	10	58	0.458	1.34	0.59–2.84	68
UTR-2	34	50	0.090	0.65	0.39–1.07	UTR-2	9	75	0.649	0.83	0.36–1.78	84
UTR-3	34	23	0.062	1.74	0.98–3.15	UTR-3	4	53	0.201	0.50	0.14–1.31	57
UTR-4	18	10	0.083	2.04	0.93–4.74	UTR-4	7	21	0.034	2.74	1.01–6.72	28
UTR-5	21	21	0.824	1.08	0.56–2.07	UTR-5	5	37	0.966	0.98	0.32–2.47	42
UTR-6	12	9	0.409	1.46	0.60–3.67	UTR-6	2	19	0.709	0.75	0.12–2.74	21
UTR-7	5	8	0.467	0.65	0.19–2.01	UTR-7	1	12	0.622	0.59	0.03–3.15	13
Total	152	162				Total	38	276				314
Immunological disorders[c]						Hematological disorders[d]						
UTR-1[a]	50	18	0.097	1.66	0.93–3.08	UTR-1[a]	51	17	0.451	1.27	0.70–2.39	68
UTR-2	58	26	0.360	1.28	0.76–2.22	UTR-2	60	24	0.983	1.01	0.58–1.77	84
UTR-3	38	19	0.766	1.10	0.60–2.05	UTR-3	39	18	0.591	0.84	0.46–1.60	57
UTR-4	15	13	0.189	0.59	0.27–1.31	UTR-4	20	8	0.991	1.01	0.44–2.51	28
UTR-5	21	21	0.031	0.49	0.25–0.94	UTR-5	30	12	0.989	1.01	0.50–2.13	42
UTR-6	14	7	0.866	1.08	0.44–2.94	UTR-6	15	6	0.992	1.01	0.39–2.90	21
UTR-7	8	5	0.791	0.86	0.28–2.90	UTR-7	8	5	0.429	0.63	0.20–2.13	13
Total	204	110				Total	224	90				314

[a] UTR-1 (DTGCCCGC); UTR-2 (ITCCCGAG); UTR-3 (DTCCCGAC); UTR-4 (DCGCCCAC); UTR-5 (ITCCTGAC); UTR-6 (DTGCCCAC); UTR-7 (ITCATGAC). [b] Proteinuria > 0.5 g or cell cylinders; [c] Anti-DNA and/or anti-Sm and/or antiphospholipid antibodies (anticardiolipin, lupus anticoagulant, or false VDRL serology); [d] Hemolytic anemia with reticulocytosis and/or leukopenia < 4000 and/or lymphopenia < 1500 and/or thrombocytopenia < 100,000 twice or more. [†] Null value.

+3187A/G polymorphism. The presence of adenine at this position is associated with lower mRNA stability *in vitro*, leading to decreased expression of HLA-G[24]. We showed an association between SLE and the presence of the +3187 A allele and the +3187 A-A genotype that was not maintained after Bonferroni correction owing to the influence of population stratification (p_c = 0.002 for the northeastern study population). Once again, a lower *HLA-G* mRNA

availability due to the presence of the +3187 A allele may confer susceptibility to SLE. However, it is worth mentioning that both polymorphisms, i.e., +3142 and +3187, are somehow associated with each other, because almost all patients carrying the +3187 G allele also carried the +3142 C allele, and the influence of the 2 polymorphisms on HLA-G availability may be codependent. To exemplify such interaction, the only haplotype carrying both alleles associated with a more stable mRNA or less mRNA decay is UTR-1 (Table 3), and this haplotype was found to be protective against SLE development ($p = 0.005$; $p_c = 0.035$), i.e., a greater HLA-G availability is apparently protective against SLE development. Notably, UTR-1 has recently been associated with high production of soluble HLA-G[44].

We also found a higher frequency of the +3010 C allele ($p = 0.008$) and +3010 C-C genotype ($p = 0.033$) in patients with SLE, which lost significance after Bonferroni correction; however, the +3010 C allele still presented a trend to increased frequency ($p_c = 0.064$). Although this polymorphic site has not yet been studied in terms of its post-transcriptional influence on mRNA production, this SNP may influence the binding of several microRNA[25], thus influencing the final mRNA availability. On the other hand, the higher frequency of the +3010 C allele in SLE may reflect the low frequency of the UTR-1 among patients, because this haplotype (usually the most frequent among Brazilians and usually associated with the presence of the +3010 G allele) is quite underrepresented among patients with SLE. However, haplotypes bearing +3010 G (UTR-1, UTR-4, UTR-6, UTR-8, UTR-9, and UTR-14), regardless of the presence of 1 substitution of the 14-bp DEL, +3142 C, or +3187 G alleles, were associated with SLE protection, suggesting a particular relevance of the +3010 C allele in susceptibility to SLE.

Our data indicate that a 3'UTR *HLA-G* polymorphic site associated with lower *HLA-G* mRNA availability (+3142G) was associated with development of SLE, and a 3'UTR haplotype (UTR-1) that has been associated with high production of HLA-G was associated with protection against development of SLE. These findings are in agreement with the idea that the presence of such a tolerogenic molecule would prevent development of SLE. Together, these findings indicate that the 3'UTR of the *HLA-G* gene had a role in SLE development in 2 Brazilian populations that differ regarding ancestry admixture.

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