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PATHOGENESIS

HLA-G 14-bp Insertion/Deletion Polymorphism Is a Risk Factor for HTLV-1 Infection

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Abstract

About 95% of HTLV-1 infected patients remain asymptomatic throughout life, and the risk factors associated with the development of related diseases, such as HAM/TSP and ATL, are not fully understood. The human leukocyte antigen-G molecule (HLA-G), a nonclassical HLA class I molecule encoded by MHC, is expressed in several pathological conditions, including viral infection, and is related to immunosuppressive effects that allow the virus-infected cells to escape the antiviral defense of the host. The 14-bp insertion/deletion polymorphism of exon 8 HLA-G gene influences the stability of the transcripts and could be related to HTLV-1-infected cell protection and to the increase of proviral load. The present study analyzed by conventional PCR the 14-bp insertion/deletion polymorphism of exon 8 HLA-G gene in 150 unrelated healthy subjects, 82 HTLV-1 infected patients with symptoms (33 ATL and 49 HAM), and 56 asymptomatic HTLV-1 infected patients (HAC). In addition, the proviral load was determined by quantitative real-time PCR in all infected groups and correlated with 14-bp insertion/deletion genotypes. The heterozygote genotype frequencies were significantly higher in HAM, in the symptomatic group, and in infected patients compared to control ($p < 0.05$). The proviral load was higher in the symptomatic group than the HAC group ($p < 0.0005$). The comparison of proviral load and genotypes showed that −14-bp/−14-bp genotype had a higher proviral load than +14-bp/−14-bp and +14-bp/+14-bp genotypes. Although HLA-G 14-bp polymorphism does not appear to be associated with HTLV-1 related disease development, it could be a genetic risk factor for susceptibility to infection.

Introduction

The human leukocyte antigen-G, a nonclassical HLA class I molecule encoded by the major histocompatibility complex, plays an important role in the regulation of the immune response.[1] This molecule was first identified in a choriocarcinoma cell line and later in placental trophoblast cells[2–5] and plays a relevant role in successful embryo implantation and in the establishment of maternal–fetal symbiosis during human pregnancy.[6,7] The HLA-G gene is characterized by low rate of polymorphisms, and its expression produces both membrane-bound and soluble proteins isoforms, which in turn are highly restricted tissue distribution under normal physiological conditions.[8] On the other hand, HLA-G proteins can be extensively expressed in pathological conditions such as organ transplantation, malignant transformation, inflammatory and autoimmune diseases, and viral infections.[1] There are seven protein isoforms whose expression varies according to the pathological condition, and the mRNA and protein profiles may differ, probably due to post-transcriptional factors, such as alternative splicing.[9] The 14-bp insertion/deletion polymorphism in exon 8 in the 3′ UTR has been found to be associated with the stability and splicing pattern of HLA-G mRNA, which could affect HLA-G protein expression.[10,11] The alternative splicing of mRNA HLA-G containing 14-bp removes 92 bases of 3′ UTR,[12] which in turn may influence the stability of HLA-G transcripts.[11]

Human T-lymphotropic virus type 1 (HTLV-1) is a delta retrovirus. It is related to a form of hematological disease designated adult T-cell leukemia-lymphoma (ATL)[13] and also to a neurologic disease designated HTLV-associated

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Table 1. Demographic Data and Proviral Load of HTLV-1-Infected Patients According to Symptomatology

	Symptomatic				*P values from the comparisons performed:*	
	ATL	*HAM*	*Total*	*Asymptomatic HAC*	*ATL/HAM/HAC*[a]	*Symptomatic/asymptomatic*[b]
Sex	($n=33$)	($n=49$)	($n=82$)	($n=56$)		
Men/women	17/16	17/32	34/48	24/32	0.3344 (MET)	1.0000 (FET)
Age (years)	($n=31$)	($n=48$)	($n=79$)	($n=53$)		
Mean	38.65 ± 16.38	53.125 ± 9.72	47.44 ± 14.51	40.60 ± 13.93	**<0.0001[c] (ANOVA)**	**0.0035[d] (MW)**
Range	14–71	31–73	14–73	15–74		
*Proviral load (copy number/10^5 cells)**	($n=9$)	($n=35$)	($n=44$)	($n=28$)		
$\times 10^1$	0	2	2	3		
$\times 10^2$	0	7	7	15		
$\times 10^3$	2	25	27	8		
$\times 10^4$	2	1	3	2		
$\times 10^5$	3	0	3	0		
$\times 10^6$	2	0	2	0		
Mean	$6.3\times10^5 \pm 8.5\times10^5$	$3.1\times10^3 \pm 3.1\times10^3$	$1.3\times10^5 \pm 4.5\times10^5$	$3.4\times10^3 \pm 7.6\times10^3$	**<0.0001[e] (KW)**	**<0.0005[f] (MW)**
Range	2.4×10^3–2.5×10^6	2.2×10^1–1.7×10^4	2.2×10^1–2.5×10^6	7.6×10^1–3.4×10^4		

*There was not sufficient DNA sample to calculate the proviral loads of all patients.

[a]Comparisons among the three groups (ATL / HAM / HAC) performed by one-way ANOVA followed by the Tukey multiple comparisons post-test, and by the Kruskal–Wallis test (KW), followed by Dunn's multiple comparisons post-test, or by an exact test that employs the Metropolis algorithm (MET) to obtain an unbiased estimate of the exact p-value for a given RxC contingency table (in the case of significant differences revealed by this last analysis, pair-wise post-test comparisons were performed using the Fisher exact test).

[b]Comparisons between the Symptomatic /Asymptomatic groups performed by the two-sided t-test, two-sided Mann-Whitney test (MW), two-sided Fisher exact test (FET) or by an exact test that employs the Metropolis algorithm (MET) to obtain an unbiased estimate of the exact p-value for a given RxC contingency table.

[c]Comparison of mean age performed by one-way ANOVA followed by the Tukey multiple comparisons post-test revealed significant differences in ATL vs. HAM ($p < 0.001$) and HAM vs. HAC ($p < 0.001$) comparisons; no significant differences were observed in the ATL vs. HAC ($p > 0.05$) comparison.

[d]The unpaired Mann-Whitney Test (MW) for mean age revealed significant differences in the two-tailed P value = 0.0035, considered significant.

[e]The Kruskal-Wallis test (KW) followed by Dunn's multiple comparisons post-test revealed significant differences in proviral loads in ATL vs. HAM ($p < 0.001$) and ATL vs. HAC ($p < 0.001$) comparisons; no significant differences were observed in the HAM vs. HAC ($p > 0.05$) comparison.

[f]The Mann-Whitney Test (MW) revealed significant differences in proviral loads when two-tailed P value <0.0005 was used, considered extremely significant.

myelopathy/tropical spastic paraparesis (HAM/TSP).[14] Other inflammatory diseases have also been associated with HTLV-1 infection, such as chronic arthropathy, polymyositis, uveitis, infective dermatitis, and Sjögren's syndrome.[15–21] Approximately 15 to 20 million people worldwide are believed to be infected,[22] and the majority of them (about 95%) remain asymptomatic. Until now, it is not fully understood why some infected individuals develop the associated diseases, whereas others do not.[23]

Some studies have shown that a high HTLV-1 proviral load is a significant risk factor for the development of HAM/TSP[24,25] and the rate of lysis of the infected cells has a significant negative correlation with proviral load.[26] In addition, Komatsu et al.[27] showed that HLA-G molecules are expressed in MT-2 cells, an HTLV-1-infected cell line, and it appears that HLA-G confers protection against lysis promoted by natural killer cells. So, the stability of HLA-G transcripts could be related to the protective effect of HTLV-1-infected cells and consequently to high proviral load and clinical manifestations.

In this context, the current study analyzed the 14-bp insertion/deletion polymorphism of exon 8 HLA-G gene in an unrelated healthy population and in HTLV-1-infected patients exhibiting or not clinical symptoms. The HTLV-1 proviral load was also studied and correlated with 14-bp polymorphism genotypes.

Materials and Methods

Patients and controls

The study was approved by the Institutional Ethics Committees (Process number 7639/2005), and written informed consent was obtained from all patients and volunteers. A total of 138 HTLV-1-infected patients (followed-up at Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto- HCFMRP-USP and Instituto Nacional do Câncer -INCA-RJ) and 150 unrelated healthy bone marrow donors (registered at the Transplant Unit of HCFMRP-USP, Process number 7581/2007) were included in the study. Eighty-two of the HTLV-1-infected patients were symptomatic (33 ATL and 49 HAM) and 56 exhibited no clinical symptoms (HAC).

Evaluation of HLA-G 14-bp insertion/deletion

DNA was extracted from the buffy coat (EIA-positive samples) using the *Super Quik-Gene-DNA Isolation kit* (Promega, Madison, WI) according to manufacturer's instructions. The 14-bp insertion/deletion polymorphism was identified according to reported conditions,[28] and the polymerase chain reaction products were analyzed by nondenaturing 7% polyacrylamide gel electrophoresis, followed by silver staining. Some samples were submitted to automatic sequencing to confirm the 14-bp insertion/deletion (data not shown).

Proviral load

To quantify the proviral load, 500 mg of genomic DNA was added to a PCR mixture consisting of SYBR® Buffer, 3.5 m*M* $MgCl_2$, 50 mM dNTP, 1.25 U Ampli Taq Gold, 100 U AmpErase® UNG, and 10 pmol of each primer described by Miley *et al.*[29] Human β-actin was used as endogenous control. The human β-actin primers used were β-actin F (5′-CTG AGG CAC TCT TCC A-3′) and β-actin R (5′-CCA GGG CAG TGA TCT CCT TCT-3′). Real-time PCR was performed in duplicate for each sample using the ABI Prism 7500 system (Applied Biosystems, Foster City, CA) with the following cycle conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 64°C. Proviral load was calculated using the following formula: average of tax/average of human β-actin$\times 2\times 10^5$. The values obtained (copy number$\times 10^5$) were transformed to Log_{10} scale.

Statistical analysis

Allele and genotype frequencies were computed by the direct counting method. The frequency of each allele or genotype was compared between patients and controls by the two-sided Fisher exact test, with the aid of the GraphPad Instat 3.06 software, which was also used to estimate the odds ratio and its 95% confidence interval (*CI*). GENEPOP 3.4 software was used to observe the adherences of genotypic proportions to expectations under Hardy–Weinberg equilibrium (*HWE*) by the complete enumeration method.[30] The parametric Student *t*-test or the two-sided nonparametric Mann–Whitney test was used

Table 2. Comparison of Genotype and Allele Frequencies Among the Groups

	Genotypes			*Alleles*
Groups	*+14bp/+14bp*	*+14bp/−14bp*	*−14bp/−14bp*	*+14bp or −14bp*
ATL×HAM	1.0000	1.0000	0.3351	0.2779
ATL×HAC	0.3543	0.6467	0.6320	0.6302
ATL×Control	0.2083	0.2475	0.8427	0.6786
HAM×HAC	0.2677	0.5499	0.6591	0.5778
HAM×Control	0.1345	0.1570	0.1296	0.4338
Symptomatic×HAC	0.1955	0.8535	1.0000	0.8967
Symptomatic×Control	0.0624	**0.0199**[a]	0.6560	0.8436
Infected×Control	0.1552	**0.0135**[b]	0.1310	0.8006
HAC×Control	1.0000	**0.0221**[c]	0.3889	1.0000

All comparisons were performed using two-sided Fisher exact test, considering significant the p values <0.05.
[a]Odds ratio = 1.936 and 95% Confidence Interval: 1.119–3.349.
[b]Odds ratio = 1.814 and 95% Confidence Interval: 1.136–2.897.
[c]Odds ratio = 2.202 and 95% Confidence Interval: 1.137–4.277.

Table 3. Samples Sizes, 14-bp Polymorphism Allele and Genotype Frequencies and Hardy–Weinberg Equilibrium (HWE)

		Alleles[a]			*Genotypes*[a]		
Groups	*N*	*+14bp*	*−14bp*	*pHWE*[b]	*+14bp/+14bp*	*+14bp/−14bp*	*−14bp/−14bp*
ATL	33	0.3788	0.6212	0.2828	0.0909	0.5758	0.3333
HAM	49	0.4643	0.5357	0.0748	0.1786	0.5714	0.2500
Symptomatic	82	0.4024	0.5976	**0.0230***	0.0976	0.6098	0.2927
HAC	56	0.4184	0.5816	0.4292	0.1020	0.6327	0.2653
Infected	138	0.4275	0.5725	**0.0240***	0.1304	0.5942	0.2754
Control	150	0.4167	0.5833	0.3220	0.1933	0.4467	0.3600

[a]Allele and genotype frequencies computed by the direct counting method.
[b]Probability of adherence to the Hardy–Weinberg equilibrium expectations.
*Significant difference in the *HWE* test in symptomatic and infected patients ($p = 0.023$, $S.E. = 0.0012$ and $p = 0.024$, $S.E. = 0.0018$ respectively), indicating deviation from the *HWE*.

to compare data between two groups, and ANOVA (parametric) and the Tukey post-test or Kruskal–Wallis test (nonparametric) followed by the Dunn post-test were used to compare data among three groups. The normality Kolmogorov–Smirnov test was used to decide about the correct test to apply.

Results

A total of 138 HTLV-1-infected patients (58 men and 80 women) aged 14–74 years were genotyped for 14-bp HLA-G polymorphism. The mean age of the HAM group was significantly higher than that of the HAC group ($p < 0.0001$) and ATL group ($p < 0.0001$). The mean age of the symptomatic group (HAM + ATL) was higher than that of the HAC group ($p = 0.0035$) (Table 1).

We also evaluated the proviral load of all groups. The proviral load of the ATL group was significantly higher than that of the HAM ($p < 0.0001$) and HAC ($p < 0.0001$) groups. Accordingly, the proviral load of the symptomatic group (HAM + ALT) was statistically higher than that of the HAC group ($p < 0.0005$) (Table 1).

The heterozygote genotype frequencies were significantly higher in the HAM group ($p = 0.0221$, *Odds ratio*: 2.202 and 95% *CI*: 1.137–4.277), in the symptomatic group (HAM + ATL) ($p = 0.0199$, *Odds ratio:* 1.936 and 95% *CI*: 1.119–3.349), and in HTLV-1-infected patients (HAC + HAM + ATL) ($p = 0.0135$, *Odds ratio:* 1.814 and 95% *CI*: 1.136–2.897) when compared to control group (uninfected individuals). No significant differences in allele frequencies were found among the groups studied (Table 2).The allele and genotype frequencies of 14-bp insertion/deletion and *HWE* were determined in infected patients and control groups. Adherences of genotypic proportions were found in all groups, except in the symptomatic and infected groups ($p = 0.0230$, $S.E. = 0.0012$ and $p = 0.0240$, $S.E. = 0.0018$, respectively) (Table 3).

The comparison between proviral load and genotype frequencies showed that the −14-bp/-14-bp genotype had a higher proviral load than the +14-bp/-14-bp (*ratio*: 9/1) and +14-bp/+14-bp (*ratio*: 41/1) genotypes, although the difference was not statistically significant (Fig. 1). On the other hand, when the median was observed, the proviral load of −14-bp/−14-bp genotype was lower than others (Table 4).

Discussion

In the current study, we evaluated the role of the 14-bp HLA-G polymorphism in HTLV-1 infection. We found no association between the HLA-G 14-bp polymorphism and the presence or absence of symptoms in HTLV-1 infected patients. However, a significant difference was observed in heterozygote genotype (+14-bp/-14-bp) between HAM and uninfected groups. In addition, when symptomatic (HAM + ATL) was compared to same genotype, a significant difference was found. Likewise, a statistical significance was observed when infected patients group (HAC + HAM + ATL) was compared to uninfected patients group. Therefore, these results indicate that this genotype could be a genetic risk factor for susceptibility to HTLV-1 infection.

HLA-G molecules exert immunosuppressive roles by interacting with receptors differentially expressed on NK cells, T lymphocytes, and APCs. Indeed, the tolerogenic properties might be beneficial to pregnancy outcome, transplantation, autoimmunity, and inflammatory diseases by negatively

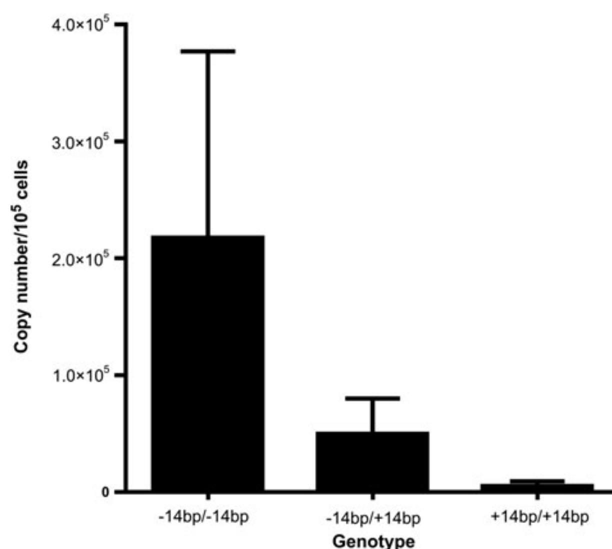

FIG. 1. Comparison of proviral load (copy number/105 cells) among the three genotypes of the HTLV-1 patient groups (−14pb/−14pb, +14pb/−14pb, and +14pb/+14pb). The differences did not reach the statistical significance.

Table 4. Comparison of Proviral Load (Copy Number/10^5 Cells) Among the Three HTLV-1 Genotype Groups (+14bp/+14bp,+14bp/−14bp, and −14bp/−14bp)

	Genotypes			P values from the comparisons performed:
	+14bp/+14bp (n = 12)	+14bp/−14bp (n = 43)	−14bp/−14bp (n = 17)	+14bp/+14bp /+14bp/−14bp /−14bp/−14bp[a]
Mean/SD	$5.3\times10^3 \pm 1{,}3\times10^5$	$5.0\times10^4 \pm 2.0\times10^5$	$2.2\times10^5 \pm 6.6\times10^5$	0.1768 (KW)
Range	$2.2\times10^1 - 4.7\times10^4$	$2.8\times10^1 - 9.7\times10^5$	$1.1\times10^2 - 2.6\times10^6$	
Median	3.7×10^3	2.2×10^3	7.7×10^2	

[a]Comparisons among the three genotype groups (+14bp/ + 14bp, + 14bp/−14bp, and − 14bp/−14bp) performed by the Kruskal–Wallis test (KW) followed by Dunn's multiple comparisons post-test.

regulating the immune reaction. However, HLA-G might be deleterious by allowing the tumor or virus-infected cells to escape from immune system defense.[31]

Studies on tumor cells show that HLA-G can be expressed on the cell surface, secreted, incorporated into exosomes, and, as recently described, the tumor cells can transfer membranes containing HLA-G molecules to NK cells in a mechanism called trogocytosis.[32–34] Functionally, NK cells that acquire HLA-G reduce cell proliferation, are no longer cytotoxic, and are capable of inhibiting the cytotoxic functions of other NK cells by working as suppressor cells.[32] Like tumor cells, virus-infected cells develop strategies to escape the antiviral defense of the host, and the level of HLA-G expression may be one of them. Komatsu and Yoshida[27] showed the relationship of HLA-G expression and HTLV-1 infection whereby the elimination of HLA class I molecules (including HLA-G) in MT-2 infected cells increased the lysis induced by NK cells. Although the results indicate a protective effect of the HLA-G molecule against the lysis of HTLV-1 infected cells, the mechanism is not completely understood.

The 14-bp insertion/deletion polymorphism in exon 8 of the 3′UTR region of HLA-G gene is related to the mRNA splicing pattern and, consequently, to mRNA stability and protein levels.[10,11] Thus, the role of the 14-bp polymorphism in the modulation of HLA-G expression may be involved in protection of HTLV-1-infected cells from the immune system and in the proviral load levels. Previous studies in HIV-1 have shown that the −14-bp/−14-bp genotype and −14-bp allele are related to reduced risk of vertical transmission and that +14-bp/+14-bp is related to HIV-1 infection in sex workers by reducing soluble HLA-G expression [35–37]. Some authors have observed that the −14-bp/−14-bp genotype appears to be a risk factor for vertical transmission of HCV, while no association was found between genotype and susceptibility to HCV infection in sickle-cell disease patients.[8,38] Another study showed that both the −14-bp/−14-bp genotype and the −14-bp allele could be related to susceptibility to CMV infection in children.[39] These discrepancies between genotype and susceptibility to transmission are strongly related to virus type, ethnic background, and route of infection.

The proviral load is considered to be the main risk factor for development of symptoms. It has previously been shown that HAM patients had higher proviral load compared to HAC patients.[25] Concordantly, in the present study we observed that the proviral load was significantly higher in the ATL than in the HAM group ($p < 0.0001$) and the HAC group ($p < 0.0001$). In addition, the symptomatic group (HAM + ATL) presented higher proviral load than in the asymptomatic (HAC) group ($p < 0.0005$). However, the proviral load levels of symptomatic patients and HACs are widely variable and overlapping. Thus, it is not clear if only a high proviral load is necessary to cause symptoms.[24] Different factors, including HLA-G profile, may be related to the onset of symptoms. Based on mean value, our results show that patients with the +14-bp/+14-bp genotype had a lower proviral load compared to patients with the +14-bp/−14-bp and −14-bp/−14-bp genotypes. This finding may be explained by the fact that the insertion of 14-bp supports the production of HLA-G transcripts with lower stability. Thus, this instability may result in poor HLA-G expression and less protection of infected cells from lysis by NK cells and cytotoxic T lymphocytes, causing a decrease of proviral load.

In conclusion, the present data show that the HLA-G 14-bp polymorphism does not appear to be associated with development of HTLV-1 diseases (HAM or ATL). On the other hand, differences in proviral load levels can be observed between genotypes. Despite the lack of association with this specific gene polymorphism, we do not exclude a possible role of HLA-G in the pathogenesis of HTLV-1. Further studies evaluating other HLA-G polymorphic sites should be conducted to elucidate the role of this HLA molecule in HTLV-1 pathogenesis.

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Author Disclosure Statement

No competing financial interests exist.

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