HLA-G 3’ Untranslated Region 14–Base Pair Deletion: Association With Poor Survival in an HIV-1–Infected Zimbabwean Population

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We aimed to evaluate whether the HLA-G 14–base pair (bp) polymorphism (rs16375) has an impact on human immunodeficiency virus HIV progression and survival in an antiretroviral therapy–naïve Zimbabwean cohort (n = 312). Rs16375 was genotyped using a competitive allele-specific polymerase chain reaction system; CD4 cell counts and HIV RNA were measured with flow cytometry and commercially available polymerase chain reaction; survival was followed up for 4.3 years. The homozygous HLA-G −14-bp genotype is associated with higher viral load (P = .004), lower CD4 cell count (P = .01), and increased mortality (hazard ratio, 1.9; 95% confidence interval, 1.033–3.522; P = .04) compared with HLA-G +14-bp carriers.

Keywords. Zimbabwean population; HIV; HLA-G; polymorphism; survival.

The nonclassic class I HLA-G is involved in the modulation of immunological responses by inhibiting the activity and mediating apoptosis of cytotoxic CD8 T cells and natural killer (NK) cells by interacting with their inhibitory receptors and inhibiting the alloreactivity by CD4 T cells [1]. In viral infections, a natural property of the viruses is to develop strategies to escape the host immune defense. According to findings reported elsewhere, HLA-G might, for certain viruses, be an additional help to support infected cell immune escape by decreasing inflammatory and immune responses of the host immune reaction [2]. By having an anti-inflammatory effect, HLA-G might also protect against damage by immunological conditions in a systemic infection. However, at the same time, HLA-G might exert reduced immunity against the specific infection. For human immunodeficiency virus (HIV)–infected individuals, HLA-G–mediated anti-inflammation might have an important role because excessive inflammation and failing specific cytotoxic control are important features of HIV disease pathogenesis [3].

A 14–base pair (bp) sequence insertion (+14-bp) or deletion (−14-bp) polymorphism in the 3’ untranslated region of exon 8 has been described in HLA-G (rs16375). The +14-bp allele is believed to result in more unstable HLA-G messenger RNA compared with the homozygous HLA-G −14-bp genotype, causing lower soluble HLA-G (sHLA-G) protein expression [4]. A decrease in proinflammatory cytokines has been shown to alter anti–HIV-1 reactivity in vitro [5]. Because HLA-G alters a cytotoxic T-lymphocyte response, the HLA-G 14-bp genotype might also be important for survival among HIV-positive individuals.

Functionally active HLA-G polymorphisms (including the 14-bp polymorphism) have been associated with an altered risk of heterosexual HIV-1 infection in African women [6], perinatal transmission of HIV-1 from infected mothers to their infants [7]. Furthermore, it was reported that the −14-bp homozygous genotype was significantly associated with a reduced risk of HIV vertical transmission in Brazilian children [8]. The objective of this study was to evaluate whether the HLA-G rs16375 14-bp polymorphism has an impact on HIV progression and survival in a cohort (n = 150) of antiretroviral therapy–naïve HIV-infected individuals recruited and followed up in rural Zimbabwe before antiretroviral therapy was available.

METHODS

Samples, DNA Extraction, HIV RNA, and CD4 Cell Counts
This study was conducted in Mupfure and adjacent areas in Shamva District, Mashonaland Central Province, Zimbabwe. Details relevant to the study design and follow-up of the study cohort have been described elsewhere [9, 10]. In brief, the
cohort included 379 individuals, 196 of whom were infected with HIV-1. All of the individuals were naïve to treatment with antiretroviral drugs. DNA was extracted from all blood samples using QIAamp DNA Blood Midi (Qiagen). For the HLA-G genotyping analysis we excluded 67 of the 379 individuals from the parent study cohort owing to poor DNA quality, and the analysis was performed using the remaining 312 samples.

Plasma HIV RNA levels were determined with the Roche Amplicor HIV-1 Monitor test, version 1.5 (Hoffmann-La Roche), and CD4 cell counts were determined with a FACSCalibur (Becton-Dickinson).

The participants were followed up until death or last follow-up (up to 4.3 years after inclusion). The study was approved by the Medical Research Council of Zimbabwe (MRCZ/A/918) and the Central Medical Scientific Ethics Committee of Denmark (624-01-0031). Oral and written informed consent was obtained from all participants.

**HLA-G 14-bp Genotyping**

All 312 DNA samples were genotyped for the rs16375 14-bp polymorphism by competitive allele-specific polymerase chain reaction using the KASPar method (KBioscience). Each sample was analyzed in duplicate in 384-well plates. Cycling conditions were as follows: 15 minutes at 94°C; 10 cycles of 10 seconds at 94°C and 1 minute at 61°C; and 26 cycles of 10 seconds at 94°C and 1 minute at 55°C. The following primers were used: HLA-G sense primer, 5′-cctgttgtggactagttggcca-3′, and antisense primer, 5′-FAM-gtcccttttgcattagc-3′; and 14-bp insertion antisense primer, 5′-VIC-tcccttttgactcagaac-3′. Only samples in which identical genotypes were obtained for both duplicate samples were used (it was not possible to obtain a genotype for 4 samples, which were excluded from the study). We reached a genotype success rate >98%, resulting in 308 (150 HIV-positive and 158 HIV-negative) individuals. All obtained genotypes agreed between duplicate samples. The allele and genotype frequencies were in Hardy-Weinberg equilibrium for all individuals.

**Statistical Analyses**

To ensure that no clinical or demographic differences were found between the 71 excluded individuals and the 308 included individuals, we used Student’s t test to evaluate CD4 cell counts and HIV-1 RNA levels among the HIV-1–infected individuals. The allele and genotype frequencies were calculated by direct gene counting from the genotyping results. The significance of differences in allele and genotype frequencies between groups was calculated by a χ² test.

The survival among HIV-1–infected individuals was examined with a Kaplan-Meier analysis of HIV-1–infected individuals stratified into 2 groups according to genotype. These 2 groups were formed on the basis of earlier findings that the homozygous −14/−14-bp genotype for the HLA-G 14-bp polymorphism has a significant effect on HIV transmission. We therefore stratified group 1 to include the −14/14-bp and +14/14-bp genotypes and group 2 to include the −14/−14-bp genotype. The 2 groups were compared by means of a log-rank test. Multivariate Cox proportional hazards regression analysis was adjusted for age (continuous data), sex (binary data [0 or 1]), CD4 cell count (linear), and HIV-1 RNA level (logarithmic) at the beginning of the study (baseline). Differences in CD4 cell count and HIV-1 RNA level according to genotype group among the HIV-1 infected were evaluated with a 2-way analysis of variance.

Statistical analyses were performed using SAS statistical software (version 9.1.3). The results are presented as P values and 95% confidence intervals (CIs). Differences were considered statistically significant at P < .05 (2 sided). We calculated the statistical power to detect genetic effects on survival among the 150 HIV-infected individuals by assuming a significance level of 95% and a 0–3.54-year follow-up interval with

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<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV-1 Positive (n = 150)</th>
<th>HIV-1 Negative (n = 158)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−14/−14 (n = 58)</td>
<td>+14/−14 (n = 92)</td>
</tr>
<tr>
<td>Frequency of genotype, %</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>Age, mean (95% CI), y</td>
<td>34 (30–37)</td>
<td>34 (30–37)</td>
</tr>
<tr>
<td>CD4 cell count, mean (95% CI), cells/µL</td>
<td>307 (259–354)</td>
<td>401 (351–450)</td>
</tr>
<tr>
<td>Plasma HIV-1 RNA load, mean (95% CI), log_{10} copies/mL</td>
<td>4.8 (4.61–4.99)</td>
<td>4.4 (4.21–4.59)</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus.
RESULTS

The 14-bp deletion allele was the predominant allele, with a frequency of 65% in the study cohort. There were no significant differences in the frequencies of 14-bp HLA-G alleles between HIV-positive and HIV-negative individuals (P = .56, Table 1). The baseline characteristics and results from Pearson’s χ² test are summarized in Table 1. The sex distribution did not differ according to HIV-1 status (P = .88). The mean CD4 cell count was lower among HIV-infected individuals homozygous for the HLA-G −14/−14 genotype (HLA-G −14/−14, 307 cells/µL [95% CI, 259–354]; HLA-G −14/+14 and +14/+14, 401 cells/µL [95% CI, 351–450]; P = .01). Moreover, the mean plasma HIV-1 RNA level was higher among HIV-infected individuals homozygous for the HLA-G −14/−14 genotype (HLA-G −14/−14, 4.8 log₁₀ copies/mL [95% CI, 4.61–4.99]; HLA-G −14/+14 and +14/+14, 4.4 log₁₀ copies/mL [95% CI, 4.21–4.59]; P = .004).

Survival was compared between the HIV-infected individuals stratified by the log-rank test into 2 groups according to the HLA-G 14-bp genotype (Figure 1). There was a significant difference in survival between the 2 groups (P = .04). Individuals who carried the +14-bp insertion allele had a lower mortality risk resulting in a higher 4-year survival rate (80%) than in individuals homozygous for the −14-bp allele (66%).

In the unadjusted Cox proportional hazards regression analysis, mortality was higher among HLA-G −14/−14 homozygous individuals than among carriers of HLA-G +14-bp (hazard ratio = 1.9, 95% CI, 1.033–3.522, P = .04). This difference remained significant after adjustment for age and sex (hazard ratio, 2.0; 95% CI, 1.070–3.702; P = .03). However, when CD4 cell count and HIV-1 RNA at baseline were included in the multivariate Cox model, the significance disappeared (hazard ratio, 1.5; CI, .753–2.578; P = .29). This result did not differ when adjusted for baseline HIV-1 RNA level, CD4 cell count, age, and sex (P = .22; hazard ratio, 1.5; 95% CI, .792–2.760).

DISCUSSION

Our findings suggest that the mechanism by which HLA-G influences the survival of HIV infected-individuals may be impaired immunological control. Moreover, the CD4 cell count was significantly lower in the −14-bp homozygous group, in agreement with the hypothesis. Our results did not reveal any difference in genotype frequencies between healthy and HIV-infected individuals. However, the time of seroconversion was unknown for our study participants, and we cannot exclude the possibility that HLA-G 14-bp deletion/deletion genotype carriers exert a negative selection pressure on HIV-infected individuals. This topic should be further investigated in a different study design that includes individuals at risk of HIV infection and those with known seroconversion dates.

Fabris et al found that HIV-exposed uninfected children had an HLA-G 14-bp deletion/deletion genotype frequency of 69%; whereas the unexposed uninfected healthy controls of the same genotype had a frequency of 34%. In our study, the uninfected controls had a 14-bp deletion/deletion genotype frequency of 42%. However, our study design is not directly comparable with the vertical mother-to-child transmission studies, because we do not have information on whether or not our healthy control group had been exposed to HIV virus.

If individuals are already HIV infected, some studies have shown that the opposite genotype (presence of 14-bp insertion polymorphism) is more protective for the further disease progression. This might be explained by a shift in relation to the balance between membrane-bound HLA-G expression and sHLA-G expression. This balance might be regulated by the proinflammatory cytokine interleukin-10 which is up-regulated during HIV infection and at the same time related to HLA-G expression. A recent study demonstrated an up-regulation of sHLA-G in persons with progressive HIV infection and showed that HLA-G increases the ability of dendritic cells to secrete interleukin-12p70, a proinflammatory cytokine that can activate effector cells of the immune system [11].

Although the heterozygous genotype and the homozygous deletion/deletion genotype have been shown to cause the same elevated sHLA-G levels in serum and plasma blood samples,
we observed an effect on survival only among the homozygous 14-bp deletion/deletion genotype carriers. Therefore, our findings suggest that the role of HLA-G in HIV disease, as in earlier studies on HIV, may have an underlying mechanism other than elevated sHLA-G levels. However, prior studies have actually shown that heterozygosity at other HLA class I loci variants is strongly associated with slower HIV disease progression [12]. In conclusion, within the genetic context of our study population, our results show that the HLA-G rs16375 14-bp insertion/deletion polymorphism is associated with survival in an HIV-infected Zimbabwean population.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

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**References**