Project Title: Associations of Human Leukocyte Antigen G with Resistance and Susceptibility to HIV-1 Infection in the Pumwani Sexworker Cohort

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Abstract: HIV/AIDS is currently a global pandemic with an estimated 33.3 million persons living with HIV/AIDS worldwide. One of the best hopes for eliminating the spread of HIV is in developing an effective vaccine. A group of sex trade workers in Pumwani, Kenya are resistant to HIV-1 infection despite frequent exposure and provide an example of natural protective immunity. Human leukocyte antigens (HLA) class I and II molecules have been shown to be associated with resistance/susceptibility in this cohort. HLA-G is a non-classical class I allele that is primarily involved in mucosal and inflammatory defence. In this study, we examined the influence of HLA-G genotypes on the resistance to HIV-1 infection using a sequenced based method. The G*01:01:01 genotype was significantly associated with resistance to HIV-1 infection (p-value 0.002, OR = 2.11, 95% CI 0.259-0.976). The G*01:04:04 genotype was significantly associated with susceptibility to HIV-1 infection (p-value 0.039, OR = 0.502, 95% CI 0.259-0.976). Kaplan-Meier survival analysis correlated with these results. G*01:01:01 genotype was associated with significantly slower rate of seroconversion. Alternatively, G*01:04:04 genotype was significantly associated with an increased rate of seroconversion. Our study showed that functionally active HLA-G alleles play an important role in resistance/susceptibility to HIV-1 infection. Since HLA-G is important in mucosal and inflammatory responses further studies need to be conducted to better understand it's functional significance in HIV-1 transmission.

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Introduction

HIV/AIDS is currently a global pandemic with an estimated 33.3 million persons living with HIV/AIDS and 1.8 million AIDS related deaths annually (1). Currently, there are 22.5 million persons living with HIV/AIDS in Sub-Saharan Africa where the pandemic has hit the hardest. Although effective anti-retroviral therapies have been developed to control progression, there is still no cure. One of the best hopes for eliminating the spread of HIV is in developing an effective vaccine. Attempts have not yet been successful due to the rapid mutations of the HIV retrovirus and insufficient understanding of protective immunity against the virus. Understandings of natural resistance to HIV may lead to knowledge that could aid in developing an effective HIV vaccine or prevention strategies.

The Pumwani Sex Worker Cohort was established in 1985 in Nairobi, Kenya to study risk factors in sexually transmitted infections. A sub-population of women in this cohort have remained PCR and seronegative for HIV-1 despite repeated exposures (2). Investigation of the mechanisms of this natural resistance to HIV infection could provide crucial information for HIV vaccine development. Human Leukocyte antigens (HLA), also known as the Major Histocompatibility Complex (MHC) in humans, are extremely polymorphic. There are two classes of HLA molecules, class I molecules presenting antigens to CD8+ T-cells and class II molecules presenting antigens to CD4+ T-cells. Previous studies of the Pumwani Cohort have shown that both HLA class I and class II alleles have been associated with resistance/susceptibility to HIV (3-8).

HLA-G is a member of the non-classical Class I antigens (9) along with HLA-E and HLA-F. This group of genes can be distinguished from classical Class I antigens HLA-A, HLA-B and HLA-C in having low gene polymorphism, limited spatial distribution and low expression levels. The HLA-G consists of an extra-membrane portion coded by exons 2-4, a transmembrane region coded by exon 5 and a cytoplasmic tail coded by exons 6 and 7. Exons 2 and 3 code for the $\alpha 1$ and $\alpha 2$ domains that form the peptide-binding cleft. There are seven different isoforms of HLA-G protein, four membrane bound (HLA-G1, HLA-G2, HLA-G3, HLA-G4) and three soluble (HLA-G5, HLA-G6 and HLA-G7) (10) due to the alternatively spliced mRNA transcripts that lack one or more exons. There are 47 HLA-G alleles currently in the IMGT/HLA database (11) compared to 1,698 HLA-A and 2,271 HLA-B and 1,213 HLA-C alleles. Much of the allelic variation of HLA-G is synonymous substitution and to date a total of 15 unique HLA-G proteins have been reported. In addition, there are 2 null alleles, G*01:05N carries a deletion at codon 130 resulting in a frame shift, and G*01:13N has a mutation at codon 54, both changes result in a premature stop codon. HLA-G been implicated in fetal-maternal tolerance during pregnancy (12), inflammatory disease, autoimmune disease, infectious disease and tissue transplantation (13).

Viruses have evolved numerous mechanisms by which they avoid the human immune system. Since HLA class I proteins bind viral antigens and present them to cytotoxic T-lymphocytes, this is a target by which a virus could escape host immune defence. However, HLA class I proteins inhibit natural killer cells and those cells that lack class I antigens may be targets for lysis. The HIV-1 Nef protein has been shown to downregulate both HLA-A and HLA-B, a plausible means for avoiding host detection (14). HIV-1 also induces upregulation of interleukin-10 (IL-10)

which selectively up regulates HLA-G expression while downregulating HLA class I and II antigens. Importantly, HLA-G is known to interact and inhibit NK cells through specific KIR complexes. Thus it is plausible that HLA-G may play a role in allowing the HIV-1 virus to avoid detection by the immune system through inhibition of lysis by NK cells (15).

For this reason HLA-G alleles have been studied in association with heterosexual transmission of HIV-1. However two studies examining the role of HLA-G alleles and resistance or susceptibility to HIV-1 obtained conflicting results. In a study of an African population the G*01:05N null allele had a protective effect against HIV-1 infection (15). However a study on an Italian Caucasian population showed that the G*01:05N was associated with risk for HIV-1 infection (16). The G*0105N null allele has a stop codon in exon 4 which terminates the translation of the HLA-G1 and HLA-G5 isoforms. The study of the African population sought to explain the results by suggesting the G*01:05N produced less soluble G isoforms and lead to less tolerant natural killer cells, thus better in detecting HIV-1 infected cells. It is difficult to resolve this conflict as both studies have several limitations. For one, they did not use high resolution HLA-G typing and therefore they were not able to detect all possible HLA-G alleles. Neither study included exon 4 in their analysis. Furthermore both studies compared HIV positive and negative patients, and the biological phenotype of study populations has not been well defined. Lastly, the sample size of these two studies is relatively small. All of these limitations warranted a more thorough investigation of HLA-G in the Pumwani Cohort.

Thus the aim of this study was to further elucidate the role of HLA-G alleles in association with either resistance of susceptibility to HIV-1 infection in the Pumwani cohort. Individuals in the cohort were genotyped using a novel sequence based typing method and allele frequencies were tabulated. Comparisons were then made using SPSS statistical software to determine the role of HLA-G and HIV-1 infection. Identification of alleles enriched in the HIV-1 resistant or HIV-1 infected women by cross-sectional analysis was made and the influence of these alleles on seroconversion was examined.

Materials and Methods

Study population

The study was conducted among 937 individuals enrolled in the Pumwani sex worker cohort, a prospective cohort established in 1985 in Nairobi, Kenya to study risk factors in sexually transmitted diseases. The majority of the women were from South-Central and South-West Kenya, and regions of Tanzania and Uganda around Lake Victoria. More than 95% of them are Bantus and 5% of them are Nilotes. The sexual transmitted disease status was not the requirement for the enrolment. Criteria for enrolment, sample collection and sexually transmitted infection testing have been described elsewhere (2). Ethics committees at the University of Manitoba and the University of Nairobi have approved this study. Informed consent was obtained from all women enrolled in the study.

DNA isolation

Genomic DNA was extracted from whole blood, buffy coat, B-cells, peripheral blood mononuclear cells and peripheral blood lymphocytes using either the QIAamp DNA Mini Kit (Qiagen Inc., Mississauga, ON) or the BioRobot EZ1 (Qiagen Inc., Mississauga, ON) following manufacturer's instructions. DNA concentration and optical density (A260/A280) were determined using the NanoDrop (NanoDrop Technologies Inc., Wilmington, DE) and NanoDrop 3.0.1 software (Coleman Technologies Inc., Glen Mills, PA).

PCR and sequencing primers

PCR and sequencing primers were designed based on HLA-G genomic DNA sequences obtained from NCBI's Genome Biology website (http://www.ncbi.nlm.gov/Genomes, Homo sapiens genome build 35.1) using PrimerSelect (Lasergene, DNASTAR, Madison, Wisconsin, USA) and Sequencher 4.5 (Gene Codes). The PCR primer set HLAGPCRF and HLAGPCRR designed to amplify a 994 bp fragment which covers partial intron 1, exon 2, intron 2, exon 3, and partial intron 3 of the HLA-G gene. The primer set HLAGEX4PCRF and HLAGEX4PCRR produce a 463 bp fragment, which covers partial intron 3, exon 4, and partial intron 4. Relevant information for PCR primer sequences, annealing temperatures and fragment sizes can be found in Table 1.

PCR Reactions

The 50 µl final PCR reaction mixture consists of 60 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 15 mM (NH4)2SO4, 25 µM of each dNTP, 0.1% gelatin, 6.25 pmol of each primer, 1.25 Unit of Taq DNA polymerase (Gibco/ BRL, Life Technologies, Burlington, Ontario, Canada) and 50-100 ng DNA. The cycle parameters used in the PTC-200 Peltier Thermal Cycler (MJ Research, Inc., Watertown, MA, USA) was 45 cycles of 1 min at 96°C, 1 min at 63°C (exon 2 and 3) or 60°C (exon 4), and 2 min at 72°C. This was followed by a 10 min incubation at 72°C. To confirm the successful amplification of the gene, 1% agarose gel electrophoresis was used to examine the correctly sized PCR products. The PCR products were then purified using Multi-well Filter Plates (Pall Corp., Ann Arbor, MI).

Sequencing

The purified PCR products reaction was sequenced using BigDye v3.1 from the ABI Prism BigDye Cycle sequencing kits (Applied Biosystems, Foster City, CA). Sequencing primers were developed based on genomic sequences to sequence each particular exon. The specific primers sequences as well as optimal annealing temperatures are listed in Table 1. The sequencing products were analyzed on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

HLA-G genotyping and data analysis

The HLA-G alleles were typed using computer software CodonExpress (University of Manitoba, Winnipeg, MB, Canada) developed based on a taxonomy-based sequence analysis method (17, 18). The HLA-G database was downloaded from IMTG/HLA Database (http://www.ebi.ac.uk/imgt/hla/new.html). HLA-G allele frequencies were calculated by direct counting. Phenotypic frequencies were expressed as the percentage of individuals bearing the corresponding allele specificity. Python for populations-32-0.7.0 was used to test for deviations

from Hardy-Weinberg equilibrium (19). Chi-square testing and logistical regression was used to determine whether any significant associations exist between HLA-G and resistance or susceptibility to HIV.

Results

Allele Frequency Distribution of HLA-G

Using a sequence-based typing method I genotyped exon 2, 3 and 4 of HLA-G of 936 patients enrolled in the Pumwani sexworker cohort in Nairobi, Kenya. These exons encode the alpha 1, 2 and 3 domains of HLA-G. 17 unique HLA-G alleles were identified in this population (Table 2). The 17 unique HLA-G alleles encode 6 different proteins (G*01:01, G*01:03, G*01:04, G*01:06, G*01:10 and G*01:11) and 1 null allele (G*01:05N). Five HLA-G alleles were detected at the frequencies above 5% in the population with G*01:01:01 (39.1%) and G*01:01:02 (20.09%) as the most frequently observed alleles. Together with G*01:03 (9.03%), G*01:04:04 (9.19%) and G*01:05N (6.09%), the five HLA-G alleles are accounted for 83.5% of all HLA-G alleles in the population.

The observed heterozygosity was slightly lower at 74.7% than expected at 78.2%, but this was not significant ($p = 0.2188$). The observed homozygosity was higher at 25.3% than the expected 21.8% ($p = 0.0197$). Overall, the frequency distribution is slightly out of Hardy-Weinberg expectations ($p = 0.0225$). This is due to two common phenotypes containing G*01:01:02 allele. The G*01:01:02 homozygote was found to be significantly higher than expected (5.8% versus 4.0%, $p = 0.0082$), while the frequency of the common genotype G*01:01:02/G*01:01:01 was significantly lower than expected (12.5% versus 15.7%, $p = 0.0133$).

Association of human leukocyte antigen G genotypes associated with resistance and susceptibility to HIV

HLA-G*01:01:01 was found in higher frequency in the HIV resistant group in both heterozygous (48.42%) and homozyogous (25.26%) than in the HIV positive heterozygous (41.15%) or homozygous (15.90%). The G*01:01:01 genotype was significantly associated with resistance to HIV (Table 3) with a p value of 0.002. The genotype has a protective effect, as those with it are 2 times less likely to be HIV positive (OR = 2.11, 95% CI 0.259-0.976).

HLA-G*01:04:04 was found in higher frequency in the HIV positive group in both heterozygous (20.08%) and homozygous (0.60%) than the HIV resistant heterozygous group (11.58%). When analyzed using cross-tab chi-square analysis we determined the G*01:04:04 genotype was significantly associated with susceptibility to HIV (Table 3) with a p-value of 0.039. It was marked by a 2 fold increased risk of susceptibility to HIV (OR = 0.502, 95% CI 0.259-0.976).

Association of human leukocyte antigen G genotypes with reduced or increased risk of HIV seroconversion

Kaplan-Meier survival analysis was used to identify genotypes that may influence seroconversion. G*01:01:01 genotype was associated with significantly lower rate of seroconversion (Fig. 1a). Alternatively, G*01:04:04 genotype was significantly associated with an increased rate of seroconversion (Fig. 1b). These findings were consistent with the results from the crosstab analysis, which provides further confirmation.

Multivariate analysis

Binary logistic regression analysis was performed to determine whether either of the allele associations was dependent or independent of previously identified significant alleles. G*01:01:01 genotype was compared to other resistant alleles such as DPA1-01:03:01, DQB1-05:03:01, and DRB1-01 but was found to be independent of all of them. G*01:04:04 was compared to alleles associated with susceptibility such as DRB1503/05 haplotype, DPB1-04:02 and found to be independent, but was found to be dependent of HLA-A*23:01. When a cross tabulation was performed neither G*01:04:04 or A*23:01 was found to be significant without the other, indicating that they are co-dependent.

Discussion

Overall the HLA-G allele frequencies in our population were similar to that of another African population (20). That said, overall the population fell outside the Hardy-Weinberg equilibrium due to the G*01:01:02 allele. With such a large sample size, it is unlikely that due to typing error. Hardy-Weinberg equilibrium assumes large population size, random mating, no migration, equal fitness of alleles, and equal or no mutation. Such assumptions may not perfectly conform to our study population. Multiple factors, such as migration, pathogen selection, and especially the Wahlund effect, might affect Hardy-Weinberg equilibrium factors and will need to be further studied. It is important to note that the Wahlund effect was speculated as the most plausible hypothesis for observed heterozygote HLA-G allele deficiency in a Brazilian population (21). Importantly, there was no association between G*01:01:02, the allele responsible for the significant result, and HIV resistance or susceptibility. This suggests that the significant result is likely not due to HIV selective pressure on the population.

HIV-1 resistance in the Pumwani Sex Worker cohort is an important model of natural immunity. HLA-G is a non-classical class I antigen with important immuno-tolerant properties. Our study demonstrates that functionally active HLA-G alleles are associated with resistance or susceptibility to HIV-1.

G*01:01:01 was found to be significantly associated with resistance to HIV-1 (Table 3). This has not been found to be associated with resistance in other populations (15,16). G*01:01:01 is the original reference sequence for HLA-G (9) and is the most common allele in the Pumwani Cohort at 39.74% (Table 2). Survival analysis strongly correlated with the chi-square results, demonstrating a significantly protective effect for those with the G*01:01:01 genotype (Fig. 1a). One explanation for these observed results is thatG*01:01:01 has a moderate level of plasma soluble HLA-G compared to other alleles which have either more or less (22). This might be an ideal amount in order to balance inhibition of NK cells and their activation, allowing an appropriate immune response to HIV-1 initial infection.

We found that G*01:04:04 was significantly associated with susceptibility to HIV (Table 3). This is a new finding reported not found in other studies. G*01:04:04 differs from the primary G*01:01:01 lineage by a mutation at codon 110 (CTC → ATC) resulting in an amino acid change of leucine to iso-leucine. This mutation is located in exon 3 and thus would change the protein configuration of the antigen binding pocket. More interestingly, the G*01:04 lineage is associated with a high level of soluble HLA-G when compared with G*01:01:01 (22). This may explain why these individuals are susceptible to HIV-1 infection. With a high level of soluble HLA-G there would be greater inhibition of NK cells, allowing HIV-1 infected cells to avoid host immune detection. G*01:04:04 was also found to be co-dependent with A*23:01. At the present we are unable to distinguish which allele is responsible of the susceptibility or whether it is both. They may well be linked, as HLA-G and HLA-A are very closely situated on chromosome 6 when compared to HLA-B or HLA-C.

Our findings are in conflict with those previously reported in a West African (Benin population (15) and an Italian Caucasian population (16). In the West African population a significant association was found between G*0105N and protection from HIV-1 infection (p-value 0.0083, OR 0.51, 95% CI 0.31-0.85) and an association with susceptibility for two haplotypes, G*01:01:08/01:04:01 (p-value 0.0009, OR 23.6, 95% CI 1.39–401.7) and G*01:01:01/01:01:08 (p-value 0.012, OR 5.6; 95% CI 1.24–25.3). Whereas, in the Italian population G*0105N was associated with an increased risk for HIV infection (p-value 0.005, OR 4.35, 95% CI 1.38–18.07). In our study the G*01:05N null allele was not found to be significantly associated with either resistance or susceptibility to HIV-1 infection, even though it had a frequency of 6.09% in the population. It had been postulated by Matte et al (15) that the lack of expression of HLA-G protein due to the G*01:05N null allele might mean less inhibition of NK cells and thus the better elimination of HIV-1 infected cells. However, this needs to be confirmed with functional studies. Functional studies should be carried out for the identified associations of HLA-G*01:01:01 with resistance to HIV-1 infection and HLA-G*01:04:04 with susceptible to HIV-1 infection in the Pumwani sex worker cohort. Given the size of the population and the well defined phenotype in our study population, we expect to illustrate functional aspect of the identified association in the future studies.

Another important aspect to consider is the level of soluble HLA-G in the vaginal mucosa. To date all soluble HLA-G study has been for the plasma samples, and the mucosal soluble HLA-G level could be very different. Ultimately this is the place where initial HIV-1 infection takes place. If HLA-G was found to be important for protection from HIV-1 infection due to it's expression at the vaginal mucosa, it could be a good candidate for therapies designed to prevent HIV-1 transmission. Perhaps by partially inhibiting soluble HLA-G expression in the vaginal mucosa we might be able to enhance the activity of NK cells to eliminate HIV-1 infected cells. Toward this end future research is needed to elucidate the functional role of HLA-G in HIV-1 transmission. Particularly the role of soluble and membrane bound HLA-G in the mucosal tract and in blood should be further explored to better our understanding.

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Table 1. HLA-G PCR and sequencing primers

Name	Exon Specificity	Primer Sequence	Location	Annealing Temperature	fragment length (bp)
HLAGPCRF	2 and 3	5'-CGGCCCCTGCGCGGAGGAGGGAGGGG-3'	intron 1 to intron 3	65	994
HLAGPCRR	2 and 3	5'-TCAGGACCAGAGGGAGGGCGATATTC-3'	intron 1 to intron 3	65	994
HLAGEX4PCRF	4	5'-AGGTGCTGCTGGAGTGTC-3'	intron 3 to intron 4	60	463
HLAGEX4PCRR	4	5'-TCTGGGAAAGGAGGTGAAG-3'	intron 3 to intron 4	60	463
HLAGEX2SEQ	2	5'-TCGTGATCTGCGCCCTG-3'	intron 1 to intron 2	53	408
HLAGEX3SEQ	3	5'-CTTTACCAAAATCCCYGCGGGT-3'	intron 2 to intron 3	59	471
HLAGEX4SEQ	4	5'-GTGCTTGAATTTTCTGACTCTT-3'	intron 3 to intron 4	59	429

Table 2. HLA-G genotype and phenotype counts in the Pumwani Cohort.

Allele	Allele Count (2n=1872)	Allele Frequency	Phenotype Count (n=936)	Phenotype Frequency
G*01:01:01	732	39.10	573	61.22
G*01:01:02	376	20.09	322	34.40
G*01:01:04	1	0.05	1	0.11
G*01:01:06	12	0.64	12	1.28
G*01:01:08	19	1.02	19	2.03
G*01:01:09	28	1.50	27	2.88
G*01:01:15	46	2.46	46	4.91
G*01:01:17	9	0.48	9	0.96
G*01:01:19	64	3.42	64	6.84
G*01:03	169	9.03	160	17.09
G*01:04:01	65	3.47	60	6.41
G*01:04:03	4	0.21	4	0.43
G*01:04:04	172	9.19	168	17.95
G*01:05N	114	6.09	110	11.75
G*01:06	50	2.67	49	5.24
G*01:10	6	0.32	6	0.64
G*01:11	5	0.27	5	0.53

Table 3. Association of HLA-G alleles with resistance and susceptibility

HLA-G allele	No. Of positive n = 503	No. Of resistant n = 95	P value	Odds ratio	95% CI
G:01:01:01 genotype	287 (57.06%)	70 (73.68%)	0.002	2.107	1.292-3.438
G:01:04:04 genotype	104 (20.68%)	11 (11.58%)	0.039	0.502	0.259-0.976

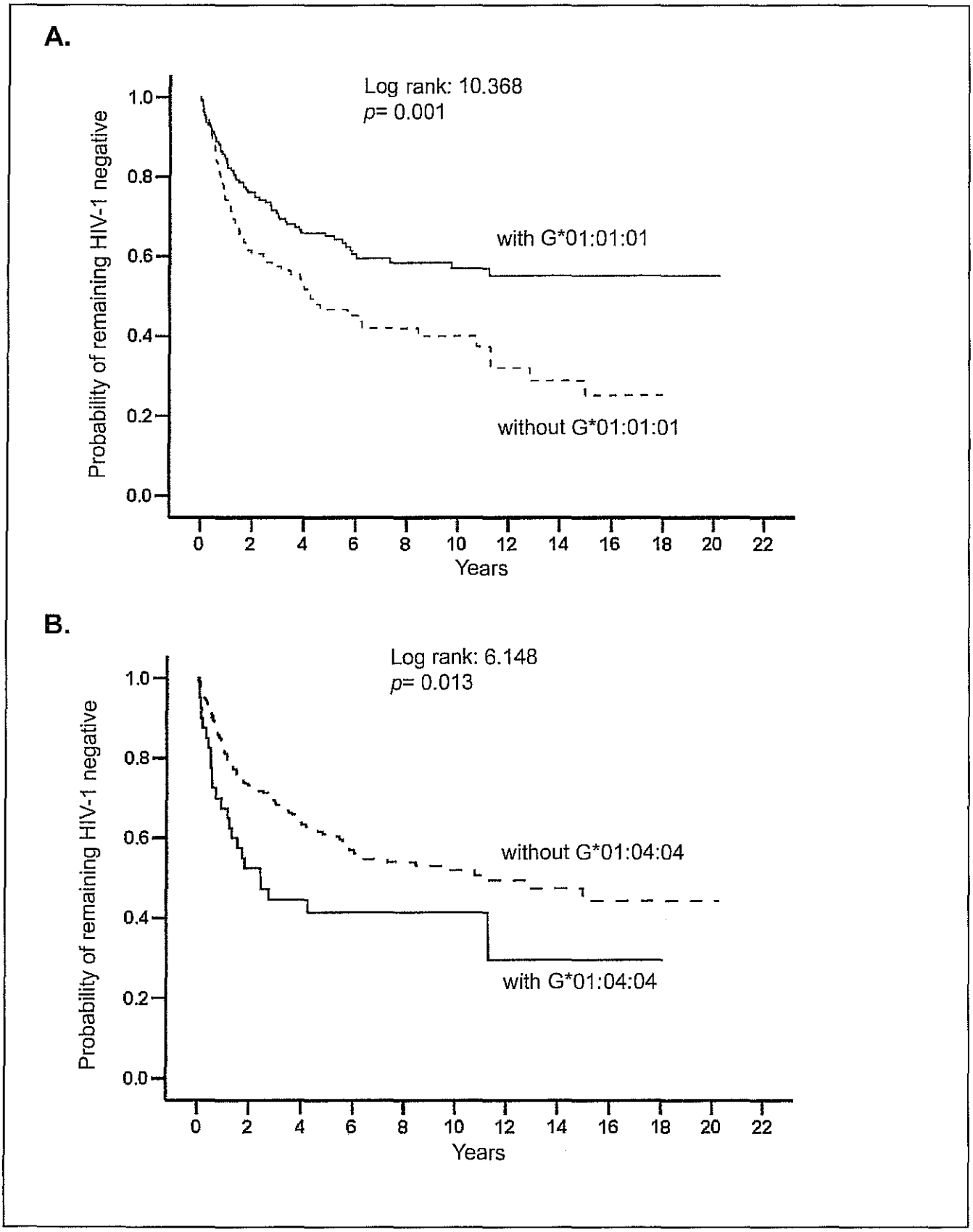

Figure 1. Probability of remaining HIV-1 negative with G*01:01:01 and G*01:04:04