The Role of the HLA B and C Loci in Human Herpesvirus 8 Replication in a Ugandan Population

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Abstract

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Kaposi sarcoma (KS) is one of the most common cancers among individuals with HIV, and the most common form of cancer in regions of Africa. Human herpesvirus 8 (HHV-8) is the etiologic factor in the development of KS; infection with HHV-8 is necessary, but not sufficient for the progression from primary HHV-8 infection to the development of KS. Since not all HIV-positive individuals infected with HHV-8 develop KS, it has been hypothesized that human genetic variation may play a role in the risk of HIV-associated KS. Human leukocyte antigen (HLA) loci mediate immune responses to viral infection, and genetic variants at HLA loci have been associated with control of oncoviruses in prior studies. We conducted a pilot study to describe the distribution of HLA-B and HLA-C loci in a Ugandan population of families with one family member diagnosed with HIV-related KS. The goals of this study included: (i) a description of the distribution of HLA-B and HLA-C alleles in the overall cohort; (ii) a description of the distribution of HLA-B and HLA-C alleles when the cohort was stratified by KS status and level of HHV-8 viral replication; and (iii) an assessment of potential associations between HHV-8 replication and HLA-B and HLA-C loci using a global transmission disequilibrium test. The sample consisted of 82 individuals in 22 families. HLA-B and HLA-C

loci were highly diverse in this population, with higher heterozygosity at both loci compared with the average loci heterozygosity across populations. HLA-B allele frequencies ranged from 0.01 to 0.10, and HLA-C allele frequencies ranged from .01 to .19. HLA-C*06:02:01G and HLA-C*04:01:01G were overrepresented in both KS-positive and viral replication allele pools, indicating a potential future area of study. No significant association between any HLA-B or HLA-C allele and HHV-8 replication was found by a global transmission disequilibrium test. This study generates hypotheses for further investigation and establishes a knowledge base for future studies.

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INTRODUCTION

Kaposi sarcoma and HHV-8

Kaposi sarcoma (KS) is the most common adult cancer in many parts of Africa, and one of the most common childhood cancers.[1] Multiple forms of KS exist, including classic KS, African KS, iatrogenic KS, and AIDS-associated KS.[2] Although KS was endemic in parts of Africa prior to the onset of the human immunodeficiency virus (HIV) pandemic, HIV infection increases the probability of developing KS more than 1000 fold.[3] KS is one of the most common cancers among individuals with HIV.[1,4]

HIV-associated KS presents more aggressively than other forms of KS, and typically evolves and disseminates throughout the body more quickly.[5] KS has been shown to respond to highly active antiretroviral therapy (HAART).[3] Individuals receiving HAART at the time of KS diagnosis have shown better indicators of host immunity and viral status, with significantly lower median CD4 cell count and RNA viremia levels.[6-7]

Human herpesvirus 8 (HHV-8) is the known etiologic agent for all forms of KS.[3,6] It is believed that immunosuppression in HIV-positive individuals disables immunologic control of HHV-8, leading to an increased risk for KS.[4] However, HHV-8 infection is considerably more prevalent than KS, even in HIV positive populations.[6] Transmission appears to be primarily non-sexual, as HHV-8 frequently appears early in childhood in Africa, and strong evidence exists for horizontal and familial transmission.[5,8] HHV-8 replicates in oral epithelial cells, and in some individuals may be found in the peripheral blood perhaps through the trafficking of immune cells through the oropharynx or infection of the tonsilar bed, leading in turn to more widespread

dissemination.[9] Saliva has been implicated as a vehicle for transmission in multiple studies.[5,8] HHV-8 seropositivity is typically more prevalent in males, and increases with age.[10] Viral replication in the peripheral blood is a significant predictor of progression to KS.[11] Studies in Africa have indicated that the risk of developing KS increases with a higher titer of HHV-8 antibodies independently of HIV status, indicating that biological factors besides HIV infection influence the progression of HHV-8 infection to KS.[6]

HHV-8 infection is prevalent in Africa, and a population-based study found that over 50% of the Ugandan population is HHV-8 seropositive.[10] Virus is found frequently in the saliva, and less commonly in the peripheral blood, of infected individuals.[12] Patterns of viral replication at the two anatomic sites show that detection of virus in individuals is heterogeneous, some HHV-8 infected individuals never have virus detected in saliva or peripheral blood despite frequent sampling, and others having virus present at high quantities on multiple days assessed. No work to date has elucidated the reason for the diversity within populations in the control of HHV-8 replication. Past studies of HHV-8 in Ugandan populations have shown that HHV-8 viral replication is associated with KS and affiliation with the Baganda ethnic group.[12] Because of the high prevalence of HHV-8 infection in Uganda, this population is ideal for studying biological factors associated with HHV-8 viral replication and progression to KS.

The HLA locus and Kaposi sarcoma

Because KS clusters have been observed in families, it has been hypothesized that genetic factors may mediate the progression of HHV-8 infection to KS.[13-15] The major histocompatibility complex (MHC), located on chromosome 6 and extending over $4x10^6$ base pairs, plays a key role

in the human immune system and is a candidate region for association with HHV-8 progression to KS.

The primary purpose of MHC molecules is to bind peptide fragments on the cell surface and display them for recognition by immune cells. The MHC is composed of three subgroup classes: Class I, Class II, and Class III. MHC Class I proteins (including HLA-A, HLA-B, and HLA-C proteins) are found on all nucleated cells, and display peptide fragments derived from intracellular processing. MHC-1 molecules play a role in viral immunity by presenting viral peptides to CD8+ T cells (cytotoxic T-cells), initiating acquired T-cell immunity. Class I molecules also act as ligands for killer cell immunoglobulin-like receptors (KIRs) on the surface of natural killer (NK) cells.[16-17] Although the immune response to many bacterial and parasitic infections does not rely heavily on HLA-mediated T-cell responses, an effective immune response to viruses depends on the effective presentation of viral proteins by Class I molecules and the subsequent T-cell response.[17] Variance in the HLA genes coding for MHC Class I molecules may affect the molecule's ability to effectively bind and present viral proteins, and thus plays a significant role in the immune response to viruses.

HLA genes for MHC Class I molecules are highly polymorphic. More than 500 HLA-B and 120 HLA-C alleles have been found.[18] HLA-B and HLA-C are less than 200 kbp apart and are in strong linkage disequilibrium.[19] While the HLA region is highly polymorphic in all populations, Ugandan populations show higher genetic diversity in the HLA region compared with other studied populations. The heterozygosity of a Ugandan population was 0.95 at the HLA-B locus and 0.91 at the HLA-C locus in a 2004 study; in contrast, another study found the mean

heterozygosity of multiple populations outside of Uganda was 0.901 (SD 0.031) at HLA-B and 0.858 (SD 0.022) at HLA-C.[18,20]

Variability in HLA genes has been associated with protection and susceptibility to the HIV virus, hepatitis B and C (HBV/HCV), human papillomavirus (HPV), and the Epstein-Barr virus (EBV).[21-23] Two studies investigated the role of HLA locus alleles in HHV-8 shedding, and have found associations between particular HLA-A and HLA-B alleles and viral shedding; however, neither result has been replicated.[24-25] To our knowledge, no study has utilized family-based methods to investigate associations between HHV-8 shedding and HLA-locus alleles. This analysis describes the distribution of HLA-B and HLA-C alleles in a Ugandan population, and analyzes associations between HHV-8 viral replication and HLA-B and HLA-C alleles.

Family-based genetic epidemiology methods

Family-based methods are a powerful tool for the identification of genetic variants associated with phenotypic traits. These association studies use family-based controls, rather than unrelated controls found in population-based association studies (such as genome-wide association studies, which tests for statistical associations between a marker and a phenotype across the entire genome in unrelated cases and controls). Compared with population-based methods, family-based association studies have several advantages. Population stratification, the presence of population substructures within a larger population, can reduce the power of population-based association analyses, but does not affect family-based methods. Family-based methods also enrich the sample population for rare variants that may not be detectable in population-based association studies. Furthermore, family-based studies have more power than studies using unrelated subjects for an equivalent number of sample units.[26]

The transmission disequilibrium test (TDT) is a McNemar-type test that uses a trio design and tests for linkage in the presence of association. In a TDT, sampling units consist of a child affected by the trait of interest and his parents. The TDT tests for the overtransmission of particular alleles to the affected child; the null hypothesis is that, for a given marker, the frequency of allelic transmission and non-transmission to the affected child is equivalent, indicating no linkage and no association between the marker and the trait. The TDT method can only be utilized with heterozygous parents, since it depends on the ability to track identical by descent (IBD) alleles.[26] The TDT is robust under deviations from Hardy-Weinberg equilibrium.[27]

While the initial TDT test was designed for biallelic markers and dichotomous traits, a number of family-based association test (FBAT) extensions have been developed to accommodate different genetic models and polymorphic markers. One of these extensions enables TDT methods to be used with multiallelic markers. As testing for associations between the trait and all individual alleles poses multiple testing issues in multiallelic loci, Spielman and Ewens (1996) developed a multiallelic TDT that tests for the presence of significance between any of the alleles and the trait of interest.[28] The global test is useful when no particular allele is hypothesized to be associated with the trait of interest.

This pilot study assessed the distribution of HLA-B and HLA-C alleles in a sample of Ugandan family trios, in which one index parent had previously been diagnosed with HIV-related KS. To generate hypotheses for future studies, individuals were stratified by KS and HHV-8 status, and the distribution of alleles in each group was compared descriptively. Finally, to test for potential

associations between HHV-8 viral replication and HLA-B and C alleles, a global TDT was performed. All tests in this study are exploratory, rather than confirmatory, given the lack of prior data suggesting specific relationships between HLA alleles and HHV-8 shedding.

METHODS

Study setting

The Uganda Cancer Institute (UCI) is the sole cancer treatment facility for the country of Uganda. A long-standing collaboration with the Fred Hutchinson Cancer Research Center ("UCI / HCCA") has allowed for the study of KS through prospective cohort studies. The cohort for this study was established by identifying index patients with KS at the UCI and up to eight family members. Potential index patients were recruited from other UCI studies, referred by clinicians, or approached study coordinators directly. Recruitment flyers were posted at clinical locations, and UCI clinicians were informed about the study and told that those referred may receive expedited diagnostic biopsies and clinical care.

After recruitment, individuals and families were screened in an eligibility visit. Index patients were eligible for the study if they had previously been diagnosed with HIV-associated KS, if they had a stable spouse or partner, and if they had two children between the ages of 6 weeks and 12 years biologically related to both parents. All participants were not eligible for the study if they intended to be away from the study location for prolonged periods of time, if they refused to provide informed consent, if they were involved in another study requiring blood draws, if they intended to get pregnant within the following month, or if they were unable to provide documentation of HIV-positive status or past KS diagnosis. Children were excluded if they were

younger than 6 months or older than 12 years. A total of 107 participants and 29 families were enrolled and completed the entire study protocol.

Data collection procedures

The UCI / HCCA has established teams of physicians, nurses, and research coordinators to collect data for multiple ongoing studies. To collect the data for this study, these teams visited participating families at an enrollment visit and four subsequent weekly home visits. At the initial enrollment visit, investigators collected blood samples for the analysis of genomic and viral data. Parents were provided with swab kits for daily orpharyngeal collection over the course of the next week to collect daily HHV-8 viral DNA data. At each subsequent visit, investigators collected the past week's swab kits and provided parents with a new swab kit for the coming week. Investigators took additional plasma samples at each visit for additional HHV-8 DNA testing.

Clinical data was collected via interviews at the enrollment visit. Participants were asked demographic questions, family history of KS, questions about past and current health conditions, and questions about current medications. Clinicians collected additional information on participants' current health status during a physical exam visit, including information on current KS lesions. When participants completed the study or were terminated, investigators documented reasons for study termination in an additional form.

Data analysis

Sequence-specific oligonucleotide hybridization (SSOH) and other sequencing-based typing methods were used to define exons 2 and 3 of HLA-B and HLA-C genes, and identify each

individual's HLA-B and HLA-C alleles. 82 of the original 107 genetic samples passed quality control procedures for genotyping. Samples that did not pass quality control due to Mendelian inconsistencies, inability to genotype, or sex discrepancies were excluded from the data analysis. Seven families were excluded using these criteria, leaving 22 families remaining in the analysis.

To measure viral replication, HHV-8 DNA was quantified using high-throughput PCR at the Hutchinson Centre Research Institute-Uganda Molecular Diagnostics laboratory in Kampala. For each study participant, HHV-8 DNA levels were quantified from the 28 oropharyngeal samples and 4 plasma samples collected over the month of participation in the study. For the purpose of this analysis, HHV-8 viral replication was transformed into a dichotomous variable of ever or never detected at either plasma or saliva sites.

Descriptive statistics were calculated using Stata 13.0. To determine whether HHV-8 replication was associated with Kaposi sarcoma or Baganda tribal affiliation, as had been observed in prior studies, two non-genetic association tests were performed.

A test for statistical deviation from Hardy-Weinberg equilibrium was performed using the Statistical Genetics Utility Programs package (Ott 1988-2008). To ensure that data for Hardy-Weinberg significance testing was independent, only adult genotypes were included. To assess potential linkage and association of HHV-8 viral replication with HLA-B and HLA-C loci, a global transmission disequilibrium test for multiallelic markers was performed using FBAT 2.0.3 (MacOS). Because only families with children affected by the trait of interest can be assessed in a transmission disequilibrium test, only the 10 families (36 individuals) with children who had

viral replication were included in the association analysis. No covariates were included in this analysis.

Power calculations were performed to estimate the likelihood that an association could be found in this sample. TDT test power was calculated using software developed by Ferreira et al. (2007).[29] In calculating power, the following assumptions were made: (1) The allele frequencies for HLA-B and HLA-C were assumed to range from the smallest to largest found in this sample; (2) Disease prevalence was .5; (3) Locus variance ranged from 0 to 1.

RESULTS

Demographic characteristics of sample

82 individuals and 22 families were included in the study after exclusions. The mean age for index patients was 38.8 (SD 6.9), and the mean age for spouses of index patients was 31.5 (SD 5.4). The mean age for children was 5.3 (SD 2.8). All study participants were Ugandan. Almost all index patients were male (95%), and 53% of children were male. Individuals representing eight different tribes participated in the study, including the Batooro, Baganda, Bagwere, Bakiga, Banyoro, Basoga, Lugbara, and Samia tribes. The majority of both index patients (68%) and spouses of index patients (73%) were from the Baganda tribe. [Table 1]

Clinical characteristics of sample

Per study criteria, all index patients had previously been diagnosed with HIV and HIV-related KS. The majority of index patients presented with KS lesions at the time of the physical exam (68%). While the majority of spouses were HIV-positive (73%), none of the spouses or children presented with KS lesions or had been previously diagnosed with KS. Three children (8%) were

HIV-positive. The majority of index patients (91%) and 41% of spouses were taking highly active anti-retroviral therapy (HAART) at the time of the study. CD4 cell count data was available for HIV positive participants only. The median CD4 cell count in HIV positive index patients (347.5, IQR 52-663) was lower than the median CD4 cell count for spouses (526.5, IQR 232-1708) or for children (1053, IQR 1034-1073).

A total of 1399 oral swabs and 113 blood samples were tested for HHV-8 infection. Overall, HHV-8 was detected in 209 (14.9%) of 1399 oral swabs, including 10 of 15 (66.6%) of persons with KS lesions present and 26 of 67 (38.8%) of persons without KS lesions present. On a participant level, the majority of index patients exhibited HHV-8 viral replication in either plasma or oral samples at any time during the study (81%), while 27% of spouses and 41% of children showed any viral replication. In the sample of all study participants, HHV-8 was detected orally in 36 participants and detected via a plasma sample in 10 participants. HHV-8 was detected orally in 6 out of the 10 individuals with HHV-8 detected via a plasma sample. [Table 1]

HIV Status

Clinical characteristics of HIV positive and HIV negative study participants were also compared. The median age was similar in both HIV positive (35, interquartile range [IQR] 26-53) and HIV negative (36, IQR 25-44) participants, although the age range was larger for HIV positive participants. The majority of HIV-positive study participants had a CD4 cell count above 200 (85%). Seventy-six percent of HIV-positive participants were taking HAART at the time of the study. The median CD4 count was similar in HIV positive study participants that were and were

not taking HAART. HHV-8 was detected in 58% of HIV-positive participants and 39% of HIV-negative participants. [Table 2]

HHV-8 Replication

Clinical and demographic characteristics were compared for adults with HHV-8 ever detected, HHV-8 detected by oral swabs, HHV-8 detected by plasma samples, and HHV-8 never detected. Demographic and clinical characteristics did not differ substantially when comparing individuals with oral HHV-8 detection, plasma HHV-8 detection, and HHV-8 detected by any means. The mean age of adults with any viral replication observed was 37.6 (SD 7.5), and the mean age of adults with no replication was 32.2 (SD 5.5). The majority of adults with any viral replication were male (71%), and only 25% of adults with no viral replication were male. The majority of adults with viral replication observed were not taking HAART at the time of the study (25%). The median CD4 cell count was 413 in individuals where viral replication was observed and 432 in individuals where no viral replication was observed; the majority of individuals had a CD4 cell count above 200, regardless of HHV-8 presence. [Table 3]

The majority of individuals with active cutaneous KS had viral replication observed (93%), and only one individual with KS had no viral replication. However, a substantial proportion of adults with viral replication did not have KS (42%). To ascertain whether a prior association between viral replication and KS existed in this sample, a chi-squared test was performed. Viral replication was significantly associated with the presence of KS ($p<0.001$). [Table 4]

Exactly 50% of individuals with low CD4 cell count (less than 200) were observed to have viral replication. Eighty-eight percent of individuals with viral replication also had normal CD4 cell counts. Viral replication was not significantly associated with CD4 cell count (p=0.588). [Table 5]

An association between Bagandan tribal affiliation and HHV-8 replication had been found in prior studies. To determine whether this association was present in the sample population, a chi-squared test was performed. Baganda tribal status was not significantly associated with HHV-8 replication (p=0.469). [Table 6]

Hardy-Weinberg

Forty-four adult genotypes were included in Hardy-Weinberg calculations. The observed heterozygosity at the HLA-B locus was 100%, and the observed heterozygosity at the HLA-C locus was 91%. No deviation from Hardy-Weinberg equilibrium was found for HLA-B (X^2=147.661, df=378, p=1.0) or HLA-C (X^2=77.605, df=153, p=1.0). Given the small sample size, Hardy-Weinberg estimates may not be reliable.

HLA-B Allelic Distribution

A total of 26 HLA-B alleles were found in the 44 adult study participants. Allele frequencies ranged from 0.01 to 0.1, with no predominant allele in this population. The most frequently found alleles were B*58*02 (AF=0.10) and B*15:10:01 (AF=0.91). Other relatively common alleles included B*42:01:01 (AF=0.068), B*58:01:01G (AF=0.08), and B*53:01:01 (AF=0.08).

These five alleles account for 42% of the HLA-B allele pool for the adult sample population. [Table 7]

HIV-positive adults were stratified by KS status, and the HLA-B allele distribution in each group was calculated. The most common HLA-B allele in KS-positive individuals was B*58:02 (AF=0.18), which had an allele frequency of .03 in KS-negative individuals. Allele B*58:02 was 15% more frequent in individuals with viral replication compared with individuals where no viral replication was seen. B*07:02:01G and B*15:10:01 were the most frequently found alleles in KS-negative individuals (AF=0.18 and AF=0.16, respectively). B*07:02:01G was not found in KS-positive populations. [Table 8]

The sample population was also stratified by HHV-8 replication level. The most frequent HLA-B allele in individuals with viral replication was B*15:10:01 (AF=0.119), although this allele was also frequently found in individuals with no viral replication (AF=0.088). B*58:02 was also frequently found in both individuals with no viral replication (AF=0.125) and viral replication (AF=0.095). Alleles B*45:01:01G and B*49:01:01G were 6% more common in the viral replication allele pool than in the allele pool from individuals with no viral replication. Allele B*07:02:01G was 7% more common in the no viral replication allele pool. [Table 9]

The HIV+ adult population was stratified by low (<200) and high (>200) CD4 count. Because only 12 individuals (17% of HIV+ adults) had low CD4 count, no more than 2 individuals with low CD4 count had any particular HLA-B allele, and no pattern in allele distribution was detected. [Table 10]

HLA-C Allelic Distribution

Seventeen HLA-C alleles were present in the adult study population. The most frequent alleles in the allele pool were HLA-C*06:02:01G (AF=0.21), HLA-C*04:01:01G (AF=0.17), and HLA-C*03:04:02 (AF=0.10). These three alleles composed 48% of the adult allele pool. [Table 11]

When HLA-C allele distributions in KS-positive and KS-negative adult populations were calculated, HLA-C*06:02:01G was the most commonly found allele in KS-positive populations (AF=0.34). This allele was not commonly found in KS-negative populations (AF=0.03). HLA-C*04:01:01G was also found at higher allele frequencies in the KS-positive population compared with other HLA-C alleles in this population (AF=0.23) and allele frequencies in the KS-negative population (AF=.06). HLA-C*07:02:01G and C*03:04:02 were commonly found in KS-negative populations (AF=0.19 and 0.16, respectively). C*07:02:01G was 17% more common in the KS-negative allele pool than the KS-positive allele pool, and C*03:04:02 was found 11% more frequently. [Table 12]

When the population was stratified by viral replication level, HLA-C*06:02:01G was the most frequently found allele in the HHV-8 replication pool (AF=0.275), and was found 12% more frequently than in the no HHV-8 replication pool (AF=0.155). C*04:01:01G was also frequently found in the replication allele pool (AF=0.238) and was also less common in the replication allele pool (AF=0.131). C*17:01:01G and C*03:04:02 were also found commonly in the no replication allele pool (AF=0.119). [Table 13]

When HIV-positive adults were stratified by CD4 count, C*06:02:01G and C*04:01:01G were the most frequently found alleles in both the low CD4 and high CD4 count allele pools. However, the number of individuals with low CD4 levels was small enough (17% of the adult HIV-positive population) that it is not possible to ascertain patterns in allele distribution in this population. [Table 14]

Familial HHV-8 Replication Patterns and Global Transmission Disequilibrium Test

The total population consisted of 22 families, including 17 families with four individuals and 5 families with three individuals. Four families had no member with HHV-8 replication present. In every other family, the KS-positive index patient showed HHV-8 replication. Children with HHV-8 replication were present in 10 families (45% of families), and both children showed HHV-8 replication in six out of the 17 families with four individuals. Every member of the family showed HHV-8 replication in three families.

Ten families with children testing positive for viral replication were included in the global transmission disequilibrium test. Viral replication was not significantly associated with any individual HLA-B or HLA-C allele (HLA-B: $p=0.523$; HLA-C: $p=0.693$).

Power

To demonstrate the potential power to find linkage and associations, power calculations were performed. Given the allele frequencies found in this population, the power was calculated assuming allele frequencies ranging from 0.006 to 0.11 for HLA-B, and 0.01 to 0.21 for HLA-C. Power was greatest under the assumption that all variance in disease liability was due to the test

locus (total locus variance=1, background additive genetic variance=0, shared environment variance=0; 57%-87% power). Power significantly dropped with lower proportions of total locus variance and higher proportions of background additive genetic variance and shared environment variance (5%-19% power). [Table 15]

DISCUSSION

This pilot study was designed to assess the HLA-B and HLA-C allele pools for a Ugandan population, describe the distribution of HLA-B and HLA-C alleles in HIV-related KS and non-KS individuals, and evaluate the evidence for an association between HHV-8 replication and HLA-B and HLA-C alleles. Because this was a pilot study and the sample size was small, the goal was to generate hypotheses for future studies, rather than test hypotheses. HHV-8 is a relatively recently-identified virus that has not been as extensively studied as other similar viruses, but studies of the closely related Epstein-Barr virus provide some indication that studying HHV-8 may provide insight into oncogenic viruses.

One major goal of this study was to look at the HLA-B and HLA-C allele distribution in a Ugandan population. A study by Cao (2004) examining the HLA-B and HLA-C alleles in five different African populations, including a Ugandan population, provides a basis for comparing the allele frequencies found in this study.[18] Similar to results from Cao, the HLA-B and HLA-C loci are highly heterogeneous in this sample. The heterozygosity for this sample was 1.0 at HLA-B and 0.91 at HLA-C; similarly, the heterozygosity in Cao's sample was 0.97 at HLA-B and 0.92 at HLA-C. Allele frequencies at both loci were slightly higher than those reported for the Ugandan population in Cao. HLA-B allele frequencies ranged from less than 0.01 to 0.1 and

HLA-C allele frequencies ranged from less than 0.01 to 0.21. In comparison, Cao found HLA-B frequencies ranging from less than 0.01 to 0.06, and HLA-C allele frequencies ranging from less than 0.01 to 0.16. The population in Cao was sampled from two different cities, and likely represented a wider variety of populations than in this study sample.

The HLA-B alleles most frequently represented in Ugandans in the study by Cao were B*0801, B*1503, and B*0702. In contrast, this study found that B*5802 and B*1501 were most frequently represented. However, the B*0801, B*1503, and B*0702 alleles were present at frequencies around 5-6%, consistent with the allele frequencies found in Cao. It is possible that the overrepresentation of the B*5802 and B*1501 alleles is specific to this Ugandan subpopulation, or that the small sample size led to the chance observation of higher allele frequencies for these alleles. These alleles may also be more frequent in populations with a Baganda majority. While the Baganda ethnic group is approximately 17% of the population,[30] the sample population in this study was 70% Baganda.

The study by Cao found the highest HLA-C allele frequencies for alleles C*0701 (AF=0.160), C*0401 (AF=0.141), and C*0602 (AF=0.117), while this study found the highest allele frequencies for C*0602 (AF=0.205), C*0401 (AF=0.17), and C*0701 (AF=0.114). While the specific allele frequencies differed substantially between the two studies, the most common three alleles remained the same in both studies. The HLA-C locus is considerably less heterogeneous than the HLA-B locus, which might explain the consistent frequency of particular alleles in both the Cao study and this sample; different populations within Uganda may not diverge as much at this locus. Because of the small sample size, it is difficult to ascertain whether the difference in

allele frequencies may be related to the specific population studied here, or whether it occurred by chance.

No *a priori* association between a particular allele and progression to KS had been hypothesized prior to this study. While one study found some associations between classical KS and HLA alleles, the study's sample size was small and lacked controls.[31] Because *post hoc* analyses are prone to Type 1 errors, and because this study's sample size and highly polymorphic loci of interest limit the potential for finding associations, no association testing was performed to assess associations between HLA loci and KS.

Descriptive statistics show a possible overrepresentation of the HLA-C*06:02:01G and HLA*C04:01:01G alleles in the KS-positive and viral replication allele pools. HLA-C*06:02:01G, in particular, composed 34% of the allele pool in KS-positive adults and only 3% of the allele pool in KS-negative adults. (Tables 10, 13-14) Given the heterogeneity of the HLA-C locus, an allele frequency of 0.34 is quite high. HLA-C*06:02 has also been significantly associated with increased risk of cervical intraepithelial neoplasia (CIN) in HPV-16 positive individuals in Sweden.[32] Like Kaposi sarcoma, which is associated with a lack of control of HHV-8 viral replication, CIN and the development of cervical cancer are associated with the lack of HPV viral control. The prior association of HLA-C*06:02 with an oncovirus-related condition establishes some corroborating evidence that supports further investigation of this allele.

Limitations of this study preclude reaching conclusions about associations between these HLA-C alleles and the traits of interest. Because the sample size is small, the absolute number of these alleles does not differ substantially between the populations with and without viral replication. The small overall allele pool could have led to an overestimation of the proportion of this allele in the population. Although the consistency of overrepresentation in both "affected" pools is interesting, KS-positive status was significantly associated with viral replication in this study. Hence, the consistent overrepresentation in both the KS-positive and viral replication pools may result from the relationship between those two traits. Future studies using larger samples should be performed to assess whether there may be an association between HLA-C*06:02:01G and either viral replication or HIV-related KS.

No linkage was found between HHV-8 viral shedding and any HLA-B or HLA-C allele in a global TDT test. Given the small sample size (10 families with virus ever detected), this result is unsurprising. Under a conservative estimate of 50% variance in disease liability due to the test locus, the maximum power for finding an association between an allele and HHV-8 replication was 15% for HLA-B and 19% for HLA-C. (Table 15) Other aspects of the global TDT might have limited the potential for finding linkage. TDT tests only have power in the presence of an association, and are usually performed after associations have already been found between alleles and traits. Although the global TDT was an imperfect methodology for testing an association between the HLA loci and HHV-8 viral replication, given that no known association between HLA-B or HLA-C and HHV-8 replication existed, it was the most appropriate association test for this sample. Other analyses using generalized estimating equations (GEE) had been originally proposed for finding associations, as this methodology could take into

account correlated data. However, GEE methods are biased in small sample sizes, and so were not appropriate for this sample. Furthermore, the global TDT minimizes Type 1 error that could occur with multiple allele testing at the heterogeneous HLA-B and HLA-C loci. No *a priori* knowledge of an association between a particular allele and the trait of interest is necessary for performing a global TDT, making it a more appropriate test of significance for a hypothesis-generating pilot study.

Several other limitations existed within this study that could have affected the results. Because of the small sample size, and because approximately 50% of individuals showed no viral detection during the study period, individuals were considered to have "viral replication" if they had any viral DNA present in their blood or saliva samples. Dichotomization in prior studies of HHV-8 took into account viral replication levels and the number of days that replication was found, and hence the "viral replication" phenotype was more specific. Because phenotype specification is critical to finding associations in genetic epidemiology studies, future research should take into account the prevalence of this phenotype when ascertaining the needed sample size. Another potential limitation was the distribution of the traits of interest among male and female adults. All but one of the adults with KS was male. While HHV-8 is found much more frequently in males in Uganda,[10,33] the incidence of HIV-associated KS in Uganda is nearly equal in men and women.[34] If potential associations between HHV-8 replication and HLA loci are mediated by gender-related factors, the uneven distribution of gender in KS-positive participants could have affected the results of this study.

Despite the limitations, this pilot study identifies two potential alleles of interest for further investigation and provides relevant information for designing future genetic studies in Ugandan populations. Future research in this area should utilize larger sample sizes and address the other limitations of this study to clarify the role of HLA loci in the development of HIV-related KS.

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TABLES AND FIGURES

Table 1. Demographic and clinical characteristics of sample population

Characteristic	Index patient (n=22)	Spouse (n=22)	Children (n=38)
Mean age (years)	38.8	31.5	5.3
% Male	95%	5%	53%
% Ugandan*	100%	100%	-
% Baganda*	68%	73%	-
% with active cutaneous KS**	68%	-	-
% with HHV-8 ever detected	81%	27%	42%
% with oral detection	63%	27%	42%
Mean log copies/ml	1.13 (SD 1.99)	0.415 (SD 1.40)	0.407 (SD 1.218)
% with plasma detection	36%	5%	3%
Mean log copies/ml	2.11 (SD 1.42)	-	0.441 (SD 1.290)
% with HIV	100%	73%	8%
% taking HAART*	91%	41%	-
Median CD4 (range)***	347.5 (52-663)	526.5 (232-1708)	1053.5 (1034-1073)
% CD4 <200	27%	-	-

*Data collected for adults only

**Lesions found at time of the physical exam; all index patients had previously been diagnosed with KS

***CD4 data was only available for HIV+ individuals

Table 2. Characteristics of HIV+ adult study participants

Characteristic	HIV+ (n=)	HIV- (n=)
Median age in years (range)	35 (26-53)	36 (25-44)
Median CD4 (range)	416 (52-1708)	-
Taking HAART	405 (52-957)	-
% CD4 <200	15.4%	-
% taking HAART	75.6%	-
% with HHV-8 ever detected	57.5%	39.0%

Table 3. Characteristics of adult study participants, by HHV-8 status

Characteristic	HHV-8 detected (n=24)	Oral detection of HHV-8 (n=20)	Plasma detection (n=9)	No viral replication (n=20)
Mean age (years)	37.6	37.4	38.6	32.2
% Male	71%	65%	78%	25%
% Baganda	75%	75%	66%	65%
% Family history of KS	8%	5%	11%	7%
% taking HAART	75%	70%	100%	38%
Median CD4 (range)	413 (52-1708)	349 (52-1708)	433 (194-663)	432 (145-957)
% CD4 <200	13%	16%	22%	20%

Table 4. HHV-8 replication in adult participants with and without current KS lesions

	Viral replication	No viral replication	X^2	p-value
KS-positive*	14	1	13.8103	< 0.001
KS-negative	10	19		

*KS-positive includes only individuals with KS lesions during physical exam; all index patients had previously been diagnosed with KS

Table 5. HHV-8 replication in adult participants, by CD4 count

	Viral replication	No viral replication	X^2	p-value
<200	3	3	.2941	0.588
>200	21	13		

Table 6. HHV-8 replication in adult participants, by Baganda tribal affiliation

	Viral replication	No viral replication	X^2	p-value
Baganda	18	13	.5241	0.469
Non-Baganda	6	7		

Table 7. Distribution of HLA-B alleles in adults

HLA-B Allele	Alleles (n=88)	
	n	%
B*07:02:01G	5	5.7
B*08:01:01G	5	5.7
B*13:03	1	1.1
B*15:03:01G	4	4.5
B*15:10:01	8	9.1
B*15:17:01G	2	2.3
B*18:01:01G	3	3.4
B*35:01:01G	2	2.3
B*39:10:01	1	1.1
B*42:01:01	6	6.8
B*42:02	1	1.1
B*44	1	1.1
B*44:03:01G	3	3.4
B*44:15	2	2.3
B*45:01	1	1.1
B*45:01:01G	5	5.7
B*49:01:01G	5	5.7
B*51:01:01G	2	2.3
B*53:01:01	7	8.0
B*53:23	1	1.1
B*53 (new allele)	1	1.1
B*57:02:01	3	3.4
B*57:03:01	1	1.1
B*58:01:01G	7	8.0
B*58:02	9	10.2
B*81:01:01G	2	2.3

Table 8. Distribution of HLA-B alleles in Kaposi's sarcoma patients and HIV-positive adult controls

	Patients (n=22)	Controls (n=16)
HLA-B	**Allele n (%)**	**Allele n (%)**
B*07:02:01G	-	6 (18%)
B*08:01:01G	3 (7%)	2 (6%)
B*15:03:01G	-	3 (9%)
B*15:10:01	1 (2%)	5 (16%)
B*15:17:01G	2 (5%)	-
B*18:01:01G	2 (5%)	1 (3%)
B*35:01:01G	2 (5%)	-
B*39:10:01	-	1 (3%)
B*42:01:01	1 (2%)	3 (9%)
B*42:02	-	1 (3%)
B*44	-	1 (3%)
B*44:03:01G	2 (5%)	1 (3%)
B*44:15	-	2 (6%)
B*45:01:01G	4 (9%)	-
B*49:01:01G	4 (9%)	1 (3%)
B*51:01:01G	2 (5%)	-
B*53:01:01	5 (11%)	1 (3%)
B*53:23	1 (2%)	-
B*53:new	1 (2%)	-
B*57:02:01	1 (2%)	1 (3%)
B*57:03:01	1 (2%)	-
B*58:01:01G	3 (7%)	3 (9%)
B*58:02	8 (18%)	1 (3%)
B*81:01:01G	1 (2%)	-

Table 9. Distribution of HLA-B alleles in sample, by HHV-8 viral replication level

	Viral replication (n=80)	No viral replication (n=84)
HLA-B	**Allele n (%)**	**Allele n (%)**
B*07:02:01G	1 (1.3%)	7 (8.3%)
B*08:01:01G	3 (3.8%)	5 (6.0%)
B*13:03	1 (1.3%)	1 (1.2%)
B*15:03:01G	1 (1.3%)	5 (6.0%)
B*15:10:01	7 (8.8%)	10 (11.9%)
B*15:17:01G	2 (2.5%)	2 (2.4%)
B*18:01:01G	3 (3.8%)	2 (2.4%)
B*35:01:01G	5 (6.3%)	1 (1.2%)
B*39:10:01	1 (1.3%)	-
B*42:01:01	5 (6.3%)	8 (9.5%)
B*42:02	1 (1.3%)	2 (2.4%)
B*44	1 (1.3%)	-
B*44:03:01G	5 (6.3%)	1 (1.2%)
B*44:15	-	4 (4.8%)
B*45:01	-	1 (1.2%)
B*45:01:01G	7 (8.8%)	2 (2.4%)
B*49:01:01G	6 (7.5%)	1 (1.2%)
B*51:01:01G	1 (1.3%)	3 (3.6%)
B*53:01:01	6 (7.5%)	6 (7.1%)
B*53:23	1 (1.3%)	-
B*53:new	2 (2.5%)	-
B*57:02:01	2 (2.5%)	5 (6.0%)
B*57:03:01	1 (1.3%)	-
B*58:01:01G	6 (7.5%)	7 (8.3%)
B*58:02	10 (12.5%)	8 (9.5%)
B*81:01:01G	2 (2.5%)	3 (3.6%)

Table 10. Distribution of HLA-B alleles in population, by CD4 count

	CD4 >200 (n=68)	CD4 <200 (n=12)
HLA-B	**Allele n (%)**	**Allele n (%)**
B*07:02:01G	6 (8.8%)	-
B*08:01:01G	5 (7.4%)	-
B*13:03	-	-
B*15:03:01G	3 (4.4%)	-
B*15:10:01	6 (8.8%)	-
B*15:17:01G	2 (2.9%)	-
B*18:01:01G	2 (2.9%)	1 (8.3%)
B*35:01:01G	2 (2.9%)	-
B*39:10:01	1 (1.5%)	-
B*42:01:01	3 (4.4%)	1 (8.3%)
B*42:02	1 (1.5%)	-
B*44	1 (1.5%)	-
B*44:03:01G	2 (2.9%)	1 (8.3%)
B*44:15	2 (2.9%)	-
B*45:01:01G	2 (2.9%)	2 (16.7%)
B*49:01:01G	4 (5.9%)	1 (8.3%)
B*51:01:01G	1 (1.5%)	1 (8.3%)
B*53:01:01	4 (5.9%)	2 (16.7%)
B*53:23	1 (1.5%)	-
B*53:new	1 (1.5%)	1 (8.3%)
B*57:02:01	3 (4.4%)	1 (8.3%)
B*57:03:01	1 (1.5%)	-
B*58:01:01G	6 (8.8%)	-
B*58:02	8 (11.8%)	1 (8.3%)
B*81:01:01G	1 (1.5%)	-

Table 11. Distribution of HLA-C alleles in adults

	Alleles (n=88)	
HLA-C Allele	**n**	**%**
C*02:02:02G	1	1.1
C*02:10	3	3.4
C*03:02:01G	2	2.3
C*03:04:01G	1	1.1
C*03:04:02	9	10.2
C*04:01:01G	15	17.0
C*04:07	2	2.3
C*05:01:01G	1	1.1
C*06:02:01G	18	20.5
C*06*87	1	1.1
C*07:01:01G	10	11.4
C*07:02:01G	7	8.0
C*07:04:01G	2	2.3
C*07:30	1	1.1
C*12:03:01G	1	1.1
C*16:01:01	3	3.4
C*17:01:01G	7	8.0
C*18:01:01G	4	4.5

Table 12. Distribution of HLA-C alleles in Kaposi's sarcoma patients and HIV-positive adult controls

	Patients (n=22)	Controls (n=16)
HLA-C	**n (%)**	**n (%)**
C*02:02:02G	1 (2%)	-
C*02:10	-	3 (9%)
C*03:02:01G	-	2 (6%)
C*03:04:01G	1 (2%)	-
C*03:04:02	2 (5%)	5 (16%)
C*04:01:01G	10 (23%)	2 (6%)
C*04:07	-	2 (6%)
C*05:01:01G	1 (2%)	-
C*06:02:01G	15 (34%)	1 (3%)
C*07:01:01G	6 (14%)	2 (6%)
C*07:02:01G	1 (2%)	6 (19%)
C*07:04:01G	1 (2%)	1 (3%)
C*16:01:01	3 (7%)	-
C*17:01:01G	1 (2%)	4 (13%)
C*06:87	-	1 (3%)
C*07:30	-	1 (3%)
C*12:03:01G	-	1 (3%)
C*18:01:01G	2 (5%)	1 (3%)

Table 13. Distribution of HLA-C alleles in population, by HHV-8 viral replication level

	Viral replication (n=80)	No viral replication (n=84)
HLA-B	**Allele n (%)**	**Allele n (%)**
C*02:02:02G	1 (1.3%)	-
C*02:10	1 (1.3%)	4 (4.8%)
C*03:02:01G	-	3 (3.6%)
C*03:04:01G	1 (1.3%)	1 (1.2%)
C*03:04:02	8 (10.0%)	10 (11.9%)
C*04:01:01G	19 (23.8%)	11 (13.1%)
C*04:07	-	4 (4.8%)
C*05:01:01G	1 (1.3%)	1 (1.2%)
C*06:02:01G	22 (27.5%)	13 (15.5%)
C*07:01:01G	10 (12.5%)	6 (7.1%)
C*07:02:01G	2 (2.5%)	8 (9.5%)
C*07:04:01G	2 (2.5%)	2 (2.4%)
C*16:01:01	2 (2.5%)	4 (4.8%)
C*17:01:01G	6 (7.5%)	10 (11.9%)
C*06:87	1 (1.3%)	1 (1.2%)
C*07:30	-	1 (1.2%)
C*12:03:01G	1 (1.3%)	-
C*18:01:01G	3 (3.8%)	5 (6.0 %)

Table 14. Distribution of HLA-C alleles in population, by CD4 count

	CD4 > 200 (n=68)	CD4 <200 (n=12)
HLA-B	**Allele n (%)**	**Allele n (%)**
C*02:02:02G	-	1 (8.3%)
C*02:10	3 (4.4%)	-
C*03:02:01G	2 (2.9%)	-
C*03:04:01G	1 (1.5%)	-
C*03:04:02	7 (10.3%)	-
C*04:01:01G	9 (13.2%)	3 (25.0%)
C*04:07	2 (2.9%)	-
C*05:01:01G	1 (1.5%)	-
C*06:02:01G	14 (20.6%)	3 (25.0%)
C*07:01:01G	7 (10.3%)	1 (8.3%)
C*07:02:01G	8 (11.8%)	-
C*07:04:01G	2 (2.9%)	-
C*16:01:01	1 (1.5%)	2 (16.7%)
C*17:01:01G	4 (5.9%)	1 (8.3%)
C*06:87	1 (1.5%)	-
C*07:30	1 (1.5%)	-
C*12:03:01G	1 (1.5%)	-
C*18:01:01G	1 (1.5%)	1 (8.3%)

Table 15. Power calculations

Locus	Allele frequency	Total locus variance	Background additive variance	Shared environmental variance	TDT test power
HLA-B	.11	1	0	0	74%
		0	1	0	5%
		0	0	1	5%
		.4	.2	.3	15%
	.01	1	0	0	87%
		0	1	0	5%
		0	0	1	5%
		.4	.2	.3	6%
HLA-C	0.21	1	0	0	57%
		0	1	0	5%
		0	0	1	5%
		.4	.2	.3	19%
	0.01	1	0	0	87%
		0	1	0	5%
		0	0	1	5%
		.4	.2	.3	6%