Molecular Evidence for Mother-to-Child Transmission of Kaposi Sarcoma–Associated Herpesvirus in Uganda and K1 Gene Evolution within the Host


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Background. Epidemiological studies of Kaposi sarcoma (KS)–related herpesvirus (KSHV) indicate that having a KSHV-seropositive mother is a risk factor for KSHV infection in children.

Methods. We determined the KSHV K1 sequences in concordantly polymerase chain reaction–positive Ugandan mother-child pairs, to ascertain whether they shared the same viral strain. We also examined sequences amplified from saliva and buffy coat samples from the same subjects, to investigate potential intrasubject sequence differences.

Results. We obtained K1 sequences from 6 of 10 mother-child pairs. In 1 pair, the subtypes differed between mother and child. The mother and child in 2 other pairs shared the same subtype, but the sequences differed. The mother and child in 2 pairs shared KSHV strains with exact (100%) nucleotide homology. The last pair showed evidence of viral strain concordance between mother and child but also showed evidence of evolution of the viral sequence within the child. Of 26 study subjects, 19 showed no evidence of intrasubject K1 sequence variability, but, in 7 subjects, all of whom were children, amino acid variation of 1%–4% was observed.

Conclusions. Our findings are consistent with KSHV transmission from maternal and nonmaternal sources in KS-endemic regions. Our results also provide evidence for ongoing evolution of the K1 gene in KSHV-infected children.

Kaposi sarcoma (KS)–associated herpesvirus (KSHV) is the infectious cause of KS, primary effusion lymphoma, and multicentric Castleman disease [1–4]. The incidence of KS is high in sub-Saharan Africa, intermediate in Mediterranean countries, and low in the general populations of the United States, Asia, and northern Europe [7–11].

KSHV transmission is poorly understood. In areas with low KSHV seroprevalence, infections typically occur in adulthood, perhaps through sexual transmission, especially between men who have sex with men (MSMs) [12, 13]. In regions with high KSHV seroprevalence, KSHV infection occurs in childhood, and detection of anti-KSHV antibodies increases with age [14–16]. Childhood seropositivity is associated with having a seropositive family member, especially the mother and, to a lesser extent, older siblings [16–19].

In previous studies, we showed that the mother’s KSHV-infection status (seropositivity or viral DNA detection) was associated with the infection status of her child, consistent with mother-to-child KSHV transmis-
sion, probably via saliva [16, 20, 21]. Similarly, in South Africa, HIV-seronegative mothers with high anti-KSHV antibody titers were more likely to have KSHV-seropositive children [19], suggesting that the risk of transmission is partly a function of viral load, as reflected by the higher antibody titer. In the present study, we investigated transmission of KSHV between mothers and their children by determining the sequences of the K1 gene from paired saliva and buffy coat samples from mother-child pairs. The K1 gene varies as much as 40% at the amino acid level and is used to distinguish the 5 major KSHV subtypes, A–E, as well as distinct strains within subtypes [22–27].

Infection with multiple strains of Epstein-Barr virus (EBV), a closely related herpesvirus, has frequently been observed both in immunocompromised [28–30] and healthy [31, 32] subjects. The question of whether infection with multiple strains of KSHV occurs is controversial. Several studies have found no evidence of infection with >1 KSHV strain [22, 33–35]. However, 2 recent publications from the same group have reported that infection with >1 strain occurs frequently in patients with KS and their family members in Malawi [36, 37]. Thus, we also investigated whether KSHV superinfection occurs among Ugandan subjects who are concordantly KSHV polymerase chain reaction (PCR) positive in both saliva and buffy coat samples.

Table 1. Amino acid variation in K1 sequences in concordantly infected mother-child pairs.

<table>
<thead>
<tr>
<th>Pair ID (study IDs)</th>
<th>Mother genotype</th>
<th>Child genotype</th>
<th>Clones sequenced, no.</th>
<th>Amino acid changes between mother and child, no.</th>
<th>Age of child, years</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (M19/C11)</td>
<td>A5</td>
<td>A5</td>
<td>4 MBC, 7 MS, 6 CBC, 7 CS</td>
<td>0</td>
<td>10.9</td>
</tr>
<tr>
<td>B (M20/C12)</td>
<td>B</td>
<td>B</td>
<td>8 MS, 4 CBC</td>
<td>0</td>
<td>5.2</td>
</tr>
<tr>
<td>C (M21/C8)</td>
<td>A5</td>
<td>A5</td>
<td>5 MS4, 4 CBC, 6 CS</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>D (M22/C9)</td>
<td>A5</td>
<td>A5</td>
<td>3 MS, 4 CBC</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>E (M24/C1)</td>
<td>B</td>
<td>B</td>
<td>5 MS, 2 CBC, 7 CS</td>
<td>1 (M vs. CBCa), 0 (M vs. CS a³), 1 (M vs. CS b³)</td>
<td>6.5</td>
</tr>
<tr>
<td>F (M23/C10)</td>
<td>C</td>
<td>A5</td>
<td>6 MS, 8 CBC</td>
<td>28</td>
<td>16</td>
</tr>
</tbody>
</table>

NOTE. C, child; CBC, child’s buffy coat; CS, child’s saliva; M, mother; MBC, mother’s buffy coat; MS, mother’s saliva.

a 2/2 CBC clones.
b 4/7 CS clones.
c 3/7 CS clones.
Figure 2. Alignment of mother-child pair K1 amino acid sequences. Each panel corresponds to a mother-child pair (A–F) analyzed in this study. Variable regions 1 and 2 (VR-1 and VR-2) are highlighted. Amino acid changes between variants are boxed. A and B, Mother-child pairs with no variation between subjects or sample types. C and D, Mother-child pairs with strain variation between mother and child. E, Mother-child pair with strain variation between mother and child and between sample types. F, An alignment in the mother-child pair with different genotypes.

SUBJECTS AND METHODS

Study population. Between November 2001 and April 2002, 600 children without KS who were attending a sickle cell clinic at Mulago Hospital in Kampala, Uganda, were recruited for the study. Mothers gave written, informed consent for themselves and their children; children 7 years of age or older gave witnessed assent. The study was approved by ethics boards at the National Cancer Institute and the Uganda National Institute for Science and Technology. Mothers or guardians answered a questionnaire that elicited data, pertaining to themselves and their children, on age, sex, environmental risk factors, and general health, as described elsewhere [20, 21, 38]. Both mother...
and child provided venous blood and saliva samples for KSHV serological and molecular testing. The samples were processed and stored at −80°C until KSHV testing was performed. The HIV infection status of mothers and children was not determined for this study; however, none of the subjects were known to be HIV infected, and the expected HIV infection prevalence is low.

Serological testing and selection of subjects. Two ELISAs were used to detect antibodies specific to the K8.1 structural glycoprotein and open reading frame 73–encoded latency-associated nuclear antigen, as described elsewhere [20, 21]. DNA was extracted from saliva and buffy coat samples from children who were KSHV seropositive or indeterminate in one or both ELISA assays, as well as from 50 randomly chosen KSHV-seronegative children, by use of a Qiagen blood and body fluids kit in accordance with the manufacturer’s instructions. DNA samples were then tested for KSHV load by TaqMan quantitative real-time PCR. The mothers of these children were also tested, regardless of their KSHV serostatus [21]. For the present study, we amplified and sequenced the K1 gene region in samples from subjects who were PCR positive for KSHV both in saliva and buffy coat samples and those from mother-child pairs in which at least 1 sample from each member had detectable KSHV DNA.

KSHV genotype nested PCR. We used previously reported nested PCR primers [22, 24] to amplify an 840-bp fragment of the K1 gene. All nested reactions were performed using Jumpstart ReadyMix containing RedTaq DNA polymerase (Sigma). Approximately 100–200 ng of DNA were used in the first-round nested PCR procedure, and 1–5 μL of the resultant amplification was added to the second-round assay. First-round PCR cycling conditions consisted of 1 min 45 s at 95°C and 35 cycles of 1 min at 96°C, 45 s at 51°C, and 1 min at 72°C. The annealing temperature for the second-round assay was 58°C for 30 cycles. Both assays ended with a 5-min hold at 72°C.

DNA samples were grouped for processing into 4 batches, according to subject and material type. Within each batch, samples were grouped for PCR amplification according to whether the estimated sample KSHV load was high or low, to reduce the chances of cross-contamination. Amplicons were cloned and sequenced before proceeding to the next batch. Standard precautions were implemented to prevent contamination and non-specific amplification. Counter tops, hoods, and pipettes were cleaned with Clorox and/or UV irradiated before each assay. Reagents were prepared in a dedicated room free of DNA and PCR amplicons. Different rooms were used for first- and second-round assay plate constructions, as well as for gel analysis and purification. Samples were added to assay plates interspersed between 2 control wells that contained only molecular-grade water. When batches with high viral loads were processed, the samples were also separated by distance on the assay plate in a checkerboard fashion, so that no sample was directly adjacent, horizontally or vertically, to another. Assays were repeated if any control wells showed evidence of contamination. In >50 96-well PCR amplifications, K1 DNA was detected in a negative control well on only 1 occasion. This may have occurred because of...
contamination or because DNA was erroneously placed in the wrong well or gel lane. This plate was discarded, and the amplification was repeated.

**Cloning and sequencing.** PCR products were isolated from agarose gels by use of the Qiagen QIAquick kit. Purified DNA fragments were visually verified by gel examination before cloning. The K1 gene fragment was cloned into vectors by use of the pGEM-T Easy Vector System (Promega). Five to 10 clones of each amplicon were sequenced. Clones were sequenced using M13 forward and reverse primers, as well as either the forward or reverse K1 nested inner primer. Sequencing was performed using an ABI 3100 sequence detection system (Applied Biosystems).

**Sequence analysis.** Initial evaluation of each sequence was performed using the nucleotide search engine BLAST (National Library of Medicine), to confirm that the amplified product was K1. Nucleotide sequences for each subject and sample type were aligned separately, using ClustalX (version 1.81; Genetics Computer Group). Sequence inspection to ensure alignment accuracy was completed in GeneDoc (version 2.6; K. Nicholas and H. Nicholas, Pittsburgh Supercomputing Center). Nucleotide changes between clones were inspected carefully, so that only those changes present in at least 2 clones were included. Nucleotide sequences were then translated into amino acid sequences. Nucleotide changes present in >2 clones were always nonsynonymous, resulting in changes in the translated amino acid sequence. To determine the K1 subtype of each subject’s strain(s), amino acid sequences were aligned in ClustalX (version 1.8), along with 44 reference strains obtained from GenBank. A neighbor-joining tree was constructed in MEGA (version 2.1; available at: http://www.megasoftware.net/) and visualized using TreeView (version 3.2; available at: http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

**RESULTS**

**K1 nested PCR.** Of 25 children (age range, 2.7–16 years) and 13 mothers (age range, 22–52 years) with evaluable samples, K1 sequences were successfully obtained from 13 of 21 saliva samples and 16 of 24 buffy coat samples from children and from 8 of 11 saliva samples and 2 of 9 buffy coat samples from mothers. Six of 10 mother-child pairs had sequences obtained from both subjects. After a K1 neighbor-joining phylogenetic tree (figure 1) was constructed using all strains identified for each subject, 5 (63%) of 8 mothers and 14 (78%) of 18 children were shown to have genotype A5. Genotypes B and C were identified in 2 (25%) of 8 and 1 (13%) of 8 mothers, respectively. The remaining children’s sequences were all of genotype B.

**KSHV infection in mother-child pairs.** Table 1 summarizes the genotype designations, the number of amino acid changes between subjects and/or sample type for each mother-child pair, and the age of the children. Two mother-child pairs (A and B) shared viral strains with 100% homology between each other and between sample types, suggesting that both mother and child were infected with the same KSHV strain—genotype A5 in one pair and genotype B in the other (figure 2A and 2B).

In 2 other mother-child pairs (C and D), the mother and child both had subtype A5 KSHV. In these pairs, the mothers’ viral sequences displayed no variation between clones from saliva, and amplification was unsuccessful from buffy coat. In mother-child pair C, the child’s viral sequence had no variation between clones from saliva and buffy coat, but there were 8 amino acid changes present between the mother and child (figure 2C). Similarly, the child of mother-child pair D showed no evidence of intrasubject variation (buffy coat only), but 6 amino acid changes differed between the mother and child (figure 2D).

Mother-child pair E had the most complex sequence results. No diversity was seen within the mother’s sequences, but variation was noted between the sequences in the mother and some sequences in the child. The child had sequence variation, albeit of <1%, between clones from saliva and buffy coat and between sequences cloned from saliva. Three distinct but closely related sequences were present in the child, 1 of which was 100% identical to the sequence in the mother (figure 2E).

In mother-child pair F, the mother’s sequence was genotype C and the child’s subtype was A5. This suggests that the child did not acquire the infection from the mother (figure 2F).

**KSHV strain variability within subjects.** Of 26 subjects examined in this study, 24 were concordantly positive for KSHV DNA by PCR in saliva and buffy coat. K1 sequences were obtained from both sample types from 15 subjects (2 mothers and 13 children). Multiple clones were sequenced of each sample type, enabling us to examine intrasubject variation in all 26 subjects. Nineteen subjects had nucleotide (and amino acid) homology of 100% across all clones sequenced. Seven subjects, all of whom were children, had confirmed viral sequence variation of 1%–4% at the nucleotide and amino acid level. Three subjects had variants only in saliva, 2 subjects had variants only in buffy coat, and 2 subjects had variants in both (table 2).

**DISCUSSION**

Subtype A5 was the most frequently amplified genotype in our study. This result contrasts with findings of previous studies from Uganda and elsewhere in Africa that reported approximately equal numbers of A5 and B sequences [23, 24, 26, 27, 34]. Those studies, in contrast to ours, examined samples obtained from patients with KS, whereas the subjects in our study did not have KS. It is possible, therefore, that infection with subtype B is more likely than infection with subtype A5 to cause KS, resulting in the overrepresentation of subtype B sequences in tissues from patients with KS, compared with the population as a whole. However, A5 was reported to be found in 10 children with childhood KS in Zambia [39]. In our study, A5 was detected
more frequently in children than in mothers, possibly suggesting that A5 may be more efficiently transmitted.

We demonstrated that, of 6 mother-child pairs from whom we obtained sequences, 2 shared exact KSHV sequence homology, 3 were infected with highly similar strains, and 1 was infected with different genotypes (figure 2). For mother-child pair E, 3 distinct sequences were present in the child that differed from each other by <1%, and one of these sequences was 100% identical to the sequence found in the mother (figure 2E). This indicates that the child acquired KSHV from the mother and suggests that K1 was evolving under selective pressure in the child. Evidence that K1 evolution was being driven by selective pressure is provided by the observation that all of the nucleotide changes resulted in amino acid changes. In mother-child pairs C and D, mother and child shared the same A5 genotype, but the sequence present in the child differed from that of the mother by <3% (figure 2C and 2D). Because little variation is observed between the sequences of A5 strains from unrelated and geographically distant subjects, it is difficult to determine whether these children acquired an independent but similar A5 strain from another family member or the community or whether they acquired KSHV from the mother and the sequence had since evolved in the child. Thus, only in mother-child pair F can we be certain that a child had acquired KSHV from a source other than her mother, and the child in this pair was 16 years of age at the time of enrollment into the study. These findings are, thus, consistent with previous reports that the KSHV infection status of mothers is a major risk factor for KSHV infection of children [16–19, 21].

A limitation of our study is the cross-sectional design, which prevented us from formally demonstrating mother-to-child KSHV transmission. It is possible that the children infected their mothers. We have previously reported that children frequently shed KSHV in saliva [21], indicating that children may be a significant source of KSHV infection, particularly to their younger siblings or playmates. A second limitation of our study is the lack of samples from other family members. Such samples may have enabled us to identify the source of infection for children in the discordant mother-child pairs. Although our study is small, the use of molecular methods to study KSHV transmission in a previously well characterized study population is a major strength.

We also investigated the occurrence of intrasubject strain variation. Of note, all clones sequenced from mothers, regardless of sample type, showed no evidence of sequence variation. However, slight sequence variation was demonstrated in viral K1 sequences in 7 of 18 children. Also of note, all nucleotide changes we report resulted in amino acid changes. The number of clones sequenced per subject and sample type in this study provided a unique window for the observation of cross-sectional intrasubject K1 sequence variation in saliva and peripheral blood. We speculate that the mothers in our study had been infected with KSHV for many years and had established a tightly latent infection with little active KSHV replication and, thus, little opportunity for the immune system to exert selective pressure on the K1 gene sequence. By contrast, the children were more likely to have been recently infected and to have more-active viral replication and high viral load. We suggest that this resulted in selective pressure on the K1 gene. The K1 gene has cytotoxic T lymphocyte epitopes, which have been implicated in the evolution of K1 [40, 41]. This explanation for the intrasubject sequence variation seen in children is consistent with previous observations that children are more likely to have higher levels of KSHV DNA in both saliva and peripheral blood, compared with their mothers [21]. Taken together, our results suggest that KSHV replication results in evolution of the K1 gene, presumably under selective pressure from K1-specific cytotoxic T lymphocytes.

Previous studies of intrasubject K1 sequence variation defined strain differences of ≥5% within a 246-bp fragment in variable region 1 of the K1 gene as being indicative of superinfection [36, 37]. In this study, we sequenced 840 bp, representing most of...
the K1 gene, and sequence variations were confirmed as being present in at least 2 clones. We did not see any variation within any one subject that was ≥3%. We suggest, therefore, that superinfection, if it occurs, is rare in Ugandans with asymptomatic KSHV infection and that intrasubject variability is more likely explained by ongoing evolution of the K1 gene under selective pressure from the immune system. Longitudinal studies of K1 sequence variation within infected subjects are needed to clarify these alternate hypotheses.

Acknowledgments

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References


