Detection of HERV-K(HML-2) Viral RNA in Plasma of HIV Type 1-Infected Individuals

RAFAEL CONTRERAS-GALINDO,1,2 MARK H. KAPLAN,2 DAVID M. MARKOVITZ,2 ERIC LORENZO,1 and YASUHIRO YAMAMURA 1

ABSTRACT

Approximately 8% of the human genome sequence is composed by human endogenous retroviruses (HERVs), most of which are defective. HERV-K(HML-2) is the youngest and most active family and has maintained some proviruses with intact open reading frames (ORFs) that code for viral proteins that may assemble into viral particles. Many HERV-K(HML-2) sequences are polymorphic in humans (present in some individuals but not in others) and probably many others may be unfixed (not inserted permanently in a specific chromosomal location of the human genome). In the present study HIV-1 and HCV-1-positive plasma samples were screened for the presence of HERV-K(HML-2) RNA in an RT-PCR using HERV-K pol specific primers. HERV-K(HML-2) viral RNA sequences were found almost universally in HIV-1/H11545 plasma samples (95.33%) but were rarely detected in HCV-1 patients (5.2%) or control subjects (7.69%). Other HERV-K(HML-2) viral segments of the RNA genome including gag, prt, and both env regions, surface (su), and transmembrane (tm) were amplified from HERV-K pol-positive plasma of HIV-1 patients. Type 1 and type 2 HERV-K(HML-2) viral RNA genomes were found to coexist in the same plasma of HIV-1 patients. These results suggest the HERV-K(HML-2) viral particles are induced in HIV-1-infected individuals.

The human genome harbors numerous retroviral sequences that comprise up to 8% of the host genome, many of which have accumulated lethal mutations that have impaired their ability to replicate.1-3 The human endogenous retrovirus type-K (HERV-K,HML-2) family is represented by many proviruses, some of which possess intact open reading frames (ORFs) for gag, prt, pol, and env genes.4,5 To date, HERV-K(HML-2) is the only endogenous retroviral subfamily with the ability to produce viral particles, apparently not infectious.6-9 However, an intact HERV-K proviral sequence (K113) and perhaps other unidentified unfixed elements might code for replication-competent viruses.10-12

Lower et al. reported the detection of anti-HERV-K antibodies in the plasma of 70% of HIV-1 patients compared to only 3% of healthy blood donors.13 Antibodies to HERV-K were also detectable in drug users, but only after HIV-1 seroconversion.14 However, others have found an association between anti-HERV-K and seminoma, testicular cancer, and teratocarcinoma but not with the HIV-1 infection.15 Anti-HERV-K antibodies formed during the course of HIV-1 infection suggest that HERV-K viral-associated proteins are produced in HIV-1-infected individuals as has been observed for other tumor malignancies.9,15 If HERV-K viral particles are made, it might be expected that they could be protected by viral envelopes in plasma of HIV-1-infected individuals. The RNA genome might be directly amplified from viral RNA extractions of plasma. We selected plasma-derived viral RNA samples from patients infected with HIV-1, HIV-1/HCV-1, and HCV-1 and seronegative control subjects. We screened for plasma-associated HERV-K RNA using HERVK pol-specific primers. We further confirmed the presence of HERV-K(HML-2) using specific primers (Table 1).16,17 Reverse transcription polymerase chain reaction (RT-PCR) was performed using the One-Step RT-PCR kit (Qiagen) according to the instructions of the manufacturer. Briefly, 5 μl of Viral RNA equivalent to 14 μl of plasma was reverse transcribed at 50°C for 30 min. The PCR was performed in 40 cycles, each consisting of 94°C for 1 min; an annealing step
<table>
<thead>
<tr>
<th>Target region</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HERV-K gag</strong></td>
<td>G1: 5'-AGAGGAAAAGGTCCAGATTA-3'</td>
<td>H1: 5'-AGAGGAAAAGGTCCAGATTA-3'</td>
</tr>
<tr>
<td><strong>HERV-K prt</strong></td>
<td>P1: 5'-GAACCTTACACAGGCATTA-3'</td>
<td>H1: 5'-GAACCTTACACAGGCATTA-3'</td>
</tr>
<tr>
<td><strong>HERV-K pol</strong></td>
<td>P3: 5'-TCCCCTTGGAATACTCCTGTTTTYGT-3'</td>
<td>H1: 5'-TCCCCTTGGAATACTCCTGTTTTYGT-3'</td>
</tr>
<tr>
<td><strong>HERV-K su</strong></td>
<td>ES1: 5'-AGAAAAGGGCCTCCACGGAGATG-3'</td>
<td>H1: 5'-AGAAAAGGGCCTCCACGGAGATG-3'</td>
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<tr>
<td><strong>HERV-K tm</strong></td>
<td>ET1: 5'-GCTGTAGCAGGAGTTGCATTG-3'</td>
<td>H1: 5'-GCTGTAGCAGGAGTTGCATTG-3'</td>
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<tr>
<td><strong>HERV-K U5</strong></td>
<td>U5: 5'-AAATCTCTCGTCCCACCTTAC-3'</td>
<td>L2: 5'-AAATCTCTCGTCCCACCTTAC-3'</td>
</tr>
<tr>
<td><strong>HERV-H pol</strong></td>
<td>5'-TTAAGAACCTCTCATTTCCTTTCCATC-3'</td>
<td>5'-TTAAGAACCTCTCATTTCCTTTCCATC-3'</td>
</tr>
<tr>
<td><strong>β-Actin RNA</strong></td>
<td>5'-GCGCGGCTACAGCTTCA-3'</td>
<td>5'-GCGCGGCTACAGCTTCA-3'</td>
</tr>
</tbody>
</table>

**Annealing temperature:** The annealing step in the PCR reaction was performed 5–8°C below the lowest T<sub>m</sub> of the subset of primers for each reaction.

<table>
<thead>
<tr>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Size (bp)</th>
</tr>
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<tbody>
<tr>
<td>55</td>
<td>437</td>
</tr>
<tr>
<td>58</td>
<td>805</td>
</tr>
<tr>
<td>50</td>
<td>1390</td>
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<td>1390</td>
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</tr>
<tr>
<td>57</td>
<td>126</td>
</tr>
<tr>
<td>57</td>
<td>126</td>
</tr>
</tbody>
</table>
TABLE 2. DETECTION OF HERV-K RNA IN PLASMA FROM CLINICAL PATIENTS

<table>
<thead>
<tr>
<th>Source of plasma tested</th>
<th>Number tested</th>
<th>Number positive</th>
<th>Percentage positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1-positive patients</td>
<td>184</td>
<td>193</td>
<td>95.33</td>
</tr>
<tr>
<td>HIV-1/HCV-positive patients</td>
<td>15</td>
<td>15</td>
<td>100.00</td>
</tr>
<tr>
<td>HCV-positive patients</td>
<td>1</td>
<td>19</td>
<td>5.20</td>
</tr>
<tr>
<td>Seronegative blood donors</td>
<td>1</td>
<td>13</td>
<td>7.69</td>
</tr>
</tbody>
</table>

*HERV-K viral pol RNA was amplified by RT-PCR using 5 μl of RNA extractions equivalent to 14 μl of plasma. Positive results consisted of at least two of three positive PCR replicates.

5–8°C below the Tm of the primers for 1 min and an extension step of 1 min per 0.5 kb (see Table 1).

HERV-K pol was positive by RT-PCR in 95.33% of the HIV-1 cases, but was rarely detected in HCV-1+ and HIV-1/HCV-1 seronegative control plasma samples (Table 2). The authenticity of the PCR products was confirmed by sequencing. Neighbor-joining phylogenetic analysis of 30 HERV-K pol clonal sequences amplified from six different plasma samples confirmed the existence of the subfamily HERV-K(HML-2) (Fig. 1). The subfamily HERV-K(HML-3) was also coamplified in all HIV-1-positive plasma samples studied. All the pol sequences amplified corresponding to HERV-K(HML-2) have intact open reading frames (ORFs).

FIG. 1. Phylogenetic dendogram of 244-bp HERV-K pol sequences amplified from HIV-1 patients (black circles), together with reported HERV-K subfamilies (HLM1 to HLM10) and type A, B, C, and D retroviruses. Queries sequences are available upon request.
To rule out the possibility of that only short pol RNA transcripts were present in plasma, we amplified different gene segments of the HERV-K(HML-2) viral RNA genome using the set of primers described in Table 1. We used six plasma samples taken from HIV-1+; HIV-1+/HCV-1+, HCV-1+, and seronegative patients. All HERV-K genes were amplified from HIV-1-seropositive patients but not from HCV-1+ patients or control subjects (Fig. 2). We also performed an amplification reaction without the reverse transcription step to eliminate the possibility of DNA contaminants in plasma samples. β-Actin primers that span spliced mRNA regions did not amplify in six HIV-1 RNA extractions, suggesting that the HERV-K amplified is not a product of cellular RNA contamination. In addition, primers specific for HERV-H pol sequences,18 previously found in plasma from rheumatoid arthritis patients,19 did not amplify in HIV-1 RNA extracts. The authenticity of the RT-PCR products was confirmed by sequencing. The size of the amplification product obtained with the env (su) primers was used to determine the type of HERV-K(HML-2) present in the amplification reactions. A 292-bp deletion in type 1 viruses gives raise to an ~1105-bp amplification product. On the other hand, HERV-K type 2 genomes are characterized by an ~1397-bp amplicon. The amplification of env (su) showed both type 1 and type 2 HERV-K(HML-2) genomes to be present in plasma samples from HIV-1 patients (Fig. 1).20–21

We further amplified a longer region of the HERV-K viral genome. By using a forward primer that spans the U5 RNA segment and a reverse primer that anneals to pol, we detected an ~5135 full length HERV-K genome in four of six HIV-1-positive plasma samples (Fig. 1). The results strongly suggest that full-length HERV-K RNA genomes are present in HIV-1+ individuals. To protect these RNA genomes from abundant serum RNases, retroviruses have preserved the gag gene to encode the matrix, capsid, and nucleocapsid structures, which is a prerequisite for particle formation.22 Our results suggest the presence of HERV-K viral particles in the circulating blood of HIV-1-infected individuals and provide a rationale for the detection in plasma of antibodies reactive to HERV-K.13,14 Even though the difference in sensitivity between an immunoassay and an RT-PCR may be responsible for the variation in the percentage of HERV-K/HIV-1-positive patients, it is also conceivable that the difference between patients with detectable HERV-K plasma RNA and patients exhibiting an anti-HERV-K response de-

![FIG. 2. Amplification of HERV-K viral RNA from HIV-1+ plasma samples. (A) Genomic organization of HERV-K viral RNA of type 1 and type 2 viruses. HERV-K type 1 lacks a 292-bp nucleotide boundary (▲) that fuses the viral genes pol and env. The 292-bp segment in type 2 viruses has nucleotide sequences that code for the first exon of rec. On the other hand, type 1 HERV-K viruses code for the accessory protein, np9, whose viral function is unknown. In the illustrations between the HERV-K genomes are the primers that were used in this study; they are located in perspective to the regions they anneal. (B) Amplification of HERV-K genes in HIV-1 patients. RT-PCR was performed on 5 μl of viral RNA extractions (from plasma samples) as described in Materials and Methods. Shown are the amplifications of gag, prt, pol, env, and the U5-pol segment representing (a) the six HIV-1+ patients, (b) the six HIV-1+/HCV+ patients, (c) the six HCV+ patients, (d) the six healthy volunteers, and (e) the negative controls: dH2O. L1: Biomarker low (Bioventures, Inc.), L2: 1 kb Ladder (Promega). As observed in the picture of the env SU amplification, the lower band represents type 1 viruses (~1100 bp) and the upper band represents type 2 viruses (~1397 bp).]
tected is due to HERV-K antigen titers that are sufficient to trigger an antibody response.

To date, the only HERV-K subfamily that can produce viral particles is HERV-K(HML-2).6–9 We have provided evidence that HERV-K(HML-2) RNA genomes are present in HIV-1-infected plasma samples. Sequencing analyses of the proviruses that are expressed in HIV-1-positive patients indicated the activation of 32 of 128 HERV-K(HML-2) members with sequence similarities between 98.5% and 100%. Notably, these proviruses all have flanking LTRs and none of them is merely an HERV-K(HML-2 fragment). Compared to the 18 type 2 elements expressed in HIV-1 patients, many sequences were highly similar to the most recent K108, K109, K115, and K113 viruses.4,10 However, some sequences were more than 2% divergent from these proviruses, which might indicate unixed HERV-K elements may have formed in recent times. With the discovery of K113, and perhaps other replication-competent unixed viruses,12 the pathogenic roles of HERV-K in human disease must be explored further. Recent evidence suggests that humans may retain a pool of replication-competent viruses.12

In addition, almost intact HERV-K polymorphisms might be functional in a fraction of individuals. Pseudotyping of HERV-K particles with functional envelopes may also yield infectious viruses, which could have a pathogenic potential.

Besides the pathogenic impact that a replication-competent virus may produce, abnormal expression of HERV-K particles and associated proteins may provoke an autoimmune response. Overexpression of HERV-K has been linked to autoimmune diseases, cancer, and neuropathological conditions, such as HIV-1-associated dementia.23–27 Further, the expression of the Np9 herpesvirus, cancer, and neuropathological conditions, such as HIV-1-associated dementia.23–27 Further, the expression of the Np9 related proteins may provoke an autoimmune response. Overexpression of HERV-K has been linked to autoimmune diseases, cancer, and neuropathological conditions, such as HIV-1-associated dementia.23–27 Further, the expression of the Np9

ACKNOWLEDGMENTS

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