Molecular characterization of Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8 strains from Russia

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We report the molecular characterization, with subtyping of both K1 and K14.1/K15 genomic regions, of seven new human herpesvirus-8 (HHV-8) strains from Russian patients with classical Kaposi's sarcoma. Phylogenetic studies, based on the complete K1 gene/protein analysis, indicate that six of these strains belong to the A subtype, with one belonging to the A4 group and exhibiting a unique deletion of 19 amino acids in the VR2 region at position 186–204. PCR-based studies of the K14.1/K15 genomic region indicate that four of the new strains were of the M subtype while three belonged to the P subtype. Our study indicates an important genetic diversity of the HHV-8 strains currently present in Russia, including a new peculiar strain possessing a unique deletion in the VR2 segment, and confirms the absence of correlation between the K1 and K14.1/K15 molecular subtypes, as M and P genotypes can be observed in the A K1 subtype.

Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus-8 (HHV-8), is a new γ2-herpesvirus first identified in a biopsy tumour from an AIDS-related Kaposi’s sarcoma (KS) (Chang et al., 1994). This virus is now considered as the aetiological agent of all clinico-epidemiological forms of KS (Boshoff & Weiss, 1998; Schulz, 1998). Recent molecular epidemiological studies exploiting the significant high genetic variability of the K1 gene, located at the left end of the genome and encoding a highly glycosylated transmembrane protein, were recently reported (Cook et al., 1999; Fouchard et al., 2000; Kasolo et al., 1998; Meng et al., 1999; Zong et al., 1999). Such reports demonstrated the existence of four major molecular subtypes or genotypes of the K1 gene and protein (called A, B, C, D or I, II, III, IV), some of which appear to be geographically related (Cook et al., 1999; Fouchard et al., 2000; Kasolo et al., 1998; Meng et al., 1999; Zong et al., 1999). Thus, KSHV/HHV-8 B strains seem to predominate in Africa and are more distant from groups A and C, found in Europe and the USA, than A and C are from each other (Cook et al., 1999; Kasolo et al., 1998; Meng et al., 1999; Zong et al., 1999). Furthermore, subtype D strains were restricted only in inhabitants of Pacific island heritage (Meng et al., 1999; Zong et al., 1999). The great majority of the K1 strains so far published are from patients originating from the USA (Meng et al., 1999; Zong et al., 1999) and to a lesser extent from western European countries, Italy, Greece and Denmark (Cook et al., 1999; Kasolo et al., 1998), and Africa (Cook et al., 1999; Fouchard et al., 2000; Kasolo et al., 1998; Meng et al., 1999; Zong et al., 1999). To our knowledge, no data are available on the genetic variability of HHV-8 strains from eastern Europe and especially Russia, an endemic area for classical KS.

We report here the molecular characterization, with subtyping of both K1 and K14.1/K15 genomic regions, of seven new HHV-8 strains from Russian patients with classical KS. The main clinical and epidemiological features of the patients are summarized in Table 1. All seven patients (four males, three females; mean age of 74 years) were HIV seronegative and suffered from cutaneous lesions mainly located on the legs. They all lived in and originated from the Moscow area, except for one patient (case 78/48), an 82 year old female who originated from the Chita province in eastern Siberia. DNA was extracted from cutaneous tumour biopsies. The complete 870 bp ORF K1 coding region, from nucleotide position 105 to 974 of the prototype sequence (Russo et al., 1996), was amplified directly from these DNA samples as a 1073 bp PCR product, as previously described (Fouchard et al., 2000), using a single step PCR. The 870 bp sequence was

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The new DNA sequence data of complete K1 genes from 74/8T, 75/10T, 76/42, 77/47, 78/48, 79/55 and 80/56 Russian samples are available in GenBank (accession nos AF201847–AF201853, respectively).
Table 1. Clinical and epidemiological features of the seven patients from Russia with classical KS

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)/sex</th>
<th>HIV</th>
<th>Ethnic group</th>
<th>Place of residence</th>
<th>Type of lesion/location</th>
</tr>
</thead>
<tbody>
<tr>
<td>8T</td>
<td>81/M</td>
<td>–</td>
<td>Russian, Christian</td>
<td>Moscow or province of Moscow</td>
<td>Patches and plaques/whole body</td>
</tr>
<tr>
<td>10T</td>
<td>66/M</td>
<td>–</td>
<td>Russian, Christian</td>
<td>Moscow or province of Moscow</td>
<td>Patches and plaques/lower extremities</td>
</tr>
<tr>
<td>42</td>
<td>83/F</td>
<td>–</td>
<td>Russian, Christian</td>
<td>Moscow or province of Moscow</td>
<td>Single nodes and nodules/lower extremities</td>
</tr>
<tr>
<td>47</td>
<td>70/F</td>
<td>–</td>
<td>Russian, Christian</td>
<td>Moscow or province of Moscow</td>
<td>Nodes, vegetation on the plaques/lower extremities</td>
</tr>
<tr>
<td>48</td>
<td>82/F</td>
<td>–</td>
<td>Russian, Christian</td>
<td>Chita province/eastern Siberia</td>
<td>Patches and plaques/whole body</td>
</tr>
<tr>
<td>55</td>
<td>70/M</td>
<td>–</td>
<td>Russian, Christian</td>
<td>Moscow or province of Moscow</td>
<td>Patches and plaques/forearm and lower extremities</td>
</tr>
<tr>
<td>56</td>
<td>68/M</td>
<td>–</td>
<td>Russian, Christian</td>
<td>Moscow or province of Moscow</td>
<td>Patches and plaques/upper and lower extremities</td>
</tr>
</tbody>
</table>

performed after TA cloning of the PCR product (Invitrogen), using ABI 377 Perkin Elmer apparatus (Eurogentec). Three clones were sequenced on both strands from four amplified samples, while only one clone was sequenced from the three other samples.

All seven newly obtained sequences were different from each other, exhibiting among themselves 0–6–13% nucleotide divergence and 2–21% amino acid divergence. Thus, some strains were closely related, such as the 75/10T and 77/47 with only a five nucleotide difference on 870 nucleotides, while strain 78/48 was the most divergent one, exhibiting with the six others a divergence ranging from 8% to 13% at the nucleotide level.

Phylogenetic studies were performed using the seven new sequences and all the other complete K1 gene sequences (33) available in GenBank. Since tree building algorithms rely on different assumptions, we used two different methods, neighbour joining and DNA maximum parsimony, to increase the reliability of the derived tree topologies. As seen in Fig. 1, the four main already known clades (A, B, C, D) were clearly identified based on the basis of consistent topological associations, and high bootstrap values, in the two phylogenetic analyses. Furthermore, the location of the seven new HHV-8 strains from Russia, with six of them belonging to the A subtype and one to the C subtype, were identical in the two analyses. Moreover, quite similar results were obtained by performing phylogenetic analyses on the nucleotide or on the amino acid sequences (Fig. 1).

As seen in Fig. 1, which shows the phylogenetic tree prepared by the neighbour joining method on protein sequences, among the six A strains, two (75/10T and 77/47) were related to the A1 subgroup (Zong et al., 1999), and one (76/42) was related to the A4 subgroup. These three strains and two related others (74/8T and 79/55), which exhibit an unreported insertion of a single amino acid (histidine or aspartic acid, respectively) at the same position 65, belong to the large A’ subgroup (Cook et al., 1999). The last subtype A strain (80/56) was slightly more distant and was related in some analysis to the A3 subgroup. Regarding the C subtype, the only new C strain (78/48) from Russia, exhibiting the typical five amino acid deletion of subtype C at position 201–205, belongs to the C3 subgroup (Zong et al., 1999), or to the C’ subgroup (Cook et al., 1999). Worth noting is that this 78/48 strain is the only one originating from eastern Siberia.

Sequence analyses of strain 76/42 indicate that this new Russian strain possesses an unusual deletion of 57 nucleotides in the VR2 region. This deletion is located at position 186–204 of the amino acid sequence of the K1 protein and so has only a four amino acid overlap with a five amino acid deletion specific for the C subtype (amino acids 201–205). This peculiar strain also possesses all the specific mutations of the A4 molecular subgroup, including the four amino acid deletion characteristic of the A4 subgroup and located downstream of the large deletion, at position 207–210.

Comparison with all the 160 complete or partial K1 sequences either available in GenBank or in tables of the
Fig. 1. Predicted phylogenetic relationships of the seven new Russian KSHV/HHV-8 isolates comparing ORF K1 nucleotide or amino acid sequences. (a) Linear phylogenetic tree using the TKS10 sequence as an outgroup was generated with Phylip, ProtDist (with the Dayhoff PAM matrix option) and the neighbour program of the PHYLIP package (Felsenstein, 1993), based on PAM distances. The tree contains 45 complete K1 protein sequences including the seven Russian sequences generated in this work (marked with an asterisk) as well as the prototype samples deposited by Zong et al. (1999), the complete K1 sequences reported by Cook et al. (1999), the K1 sequence of BC-1, BCBL-1 and BBG-1 cell lines (GenBank accession nos U75698, U86667 and AF042370, respectively) and one KS strain (GenBank accession no. U93872) named K1-Fr.N here. The numbers at some nodes (bootstrap values) indicate frequencies of occurrence for 100 trees. A1–A5, C1–C5, D1 and D2 correspond to subgroups within the subtypes described by Zong et al. (1999). A‘, C‘ and C“ refer to subtypes described by Cook et al. (1999). The branch lengths are proportional to the evolutionary distance. (b) Linear phylogenetic tree using the TKS10 sequence as an outgroup was generated, once the nucleotide sequences were aligned using the Clustal W program, using SEQBOOT, DNAPARS and CONSENSE of the PHYLIP package. The tree contains 40 complete K1 nucleotide sequences including the seven Russian sequences generated in this work (marked with an asterisk) and all the available K1 nucleotide samples deposited in GenBank; branch lengths are non-informative. The new DNA sequence data of complete K1 genes from 74/8T, 75/10T, 76/42, 77/47, 78/48, 79/55 and 80/56 Russian samples are available in GenBank (accession nos AF201847–AF201853, respectively).
previously quoted papers indicate that none of these sequences possesses the deletion found in the 76/42 Russian strain. The GK18 strain originating from a patient from Greece with classical KS possesses an amino acid deletion located at position 197–206 in the VR2 fragment which overlaps four amino acids with the deletion of the 76/42 strain, but this GK18 isolate was otherwise a typical C subtype. Such data suggest that this region of the VR2 fragment, which contains neither glycosylation sites nor cysteine residues, may be prone to genomic rearrangements.

In four samples, we analysed the complete K1 sequences of three different clones, one obtained in a first PCR, the two others in a second PCR performed 4 months later. The genetic variability within the three clones was very low for three patients (0–2 changes both at the nucleotide and amino acid level), while for one patient, the variability was slightly higher (2–5 changes at both nucleotide and amino acid level). These data thus indicate that the intra-strain K1 variability in a given patient, at a given time, is very low at least for a KS tumour sample (range from 0 to 0.6% nucleotide difference). Furthermore, a phylogenetic analysis was performed using the three clones from these patients. The location of the sequences was identical to when only one clone was analysed (data not shown).

Molecular subtype characterization of the right hand side (RHS) of the KSHV/HHV-8 genome was determined as
previously described (Poole et al., 1999). The extreme RHS genomic region comprises K14.1 and K15 genes. The latter codes for an integral membrane protein related to the LMP2 latency protein of Epstein–Barr virus. Two different PCR strategies were used, a single PCR using a pair of primers unique to the M subtype of ORF K15, and a triple primer PCR set of ORF K14.1 covering the divergent junction of the two subtypes of ORF K15 genes. Specific amplification of ORF K15 subtype M gave a positive result for samples 74/8T, 76/42, 79/55 and BC-1 (subtype M control) and a negative result for 75/10T, 77/47, 78/48, 80/56 and BCBL-1 (subtype P control) (data not shown). The second strategy gave a 450 bp product specific for subtype M for samples 74/8T, 76/42, 79/55 and BC-1 and a 362 bp product specific for the P subtype for the other samples, 75/10T, 77/47, 78/48, 80/56 and BCBL-1 (Fig. 2). Thus, a perfect correlation was obtained between the two strategies used to characterize the M or P subtypes of the RHS genomic region of the seven new Russian HHV-8 strains.

In conclusion, our study, which reports the first molecular characterization of HHV-8 strains from Russia, indicates an important genetic diversity of the HHV-8 strains present in Russian patients suffering from classical KS. Indeed, such HHV-8 strains belong to at least two K1 subtypes (A and C), with several molecular variants within subtype A, and to the two molecular subgroups (M and P) of K14.1/K15. We furthermore confirm the absence of correlation between the K1 and K14.1/K15 molecular subtypes, as M and P genotypes can be observed in strains of the A K1 subtype. Moreover we describe a new peculiar HHV-8 strain possessing a unique deletion in the VR2 segment. Further studies, including molecular characterization of HHV-8 strains infecting patients with a different geographical origin within Russia as well as patients with different clinico-epidemiological forms of KS, are ongoing.

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