Molecular Typing of Human Herpesvirus 8 Isolates from Patients with Kaposi's Sarcoma in Hungary

RITA ÖTVÖS[1], ATTILA JUHASZ[1,2,3], ERIKA SZALAI[4], DORINA UJVARI[5], KATALIN ÖTVÖS[6], KATALIN SZABO[7], EVA REMENYIK[2], LASZLO SZEKELY[8], LAJOS GERGELY[1] and JOZSEF KONYA[1]

Departments of [1]Medical Microbiology, [2]Dermatology, [6]Paediatrics, [7]Pulmonology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary; [3]Department of Central Laboratory, Moritz Kaposi General Hospital, Kaposvár, Hungary; [4]Department of Microbiology, St. Laszlo Hospital, Budapest, Hungary; [5]Department of Woman's and Children's Health, Obstetrics and Gynecology Unit, Karolinska University Hospital, Stockholm, Sweden; [8]Department of Microbiology, Tumor and Cell Biology and Center for Integrative Recognition in the Immune System, Karolinska Institute, Stockholm, Sweden

Abstract. *Background: Kaposi's sarcoma (KS) shows a distinct geographical and ethnic distribution. Genes at both ends of the human herpesvirus 8 (HHV-8) genome have been shown to vary considerably. Seven major molecular subtypes of HHV-8 were defined based on the amino acid sequence of the open reading frame K1 (orf-K1). The aim of the present study was to characterize HHV8 isolates from hospitalized patients in Hungary. Materials and Methods: A total of 36 archival paraffin-embedded Kaposi's sarcoma tissue samples were collected. Polymerase chain reaction (PCR) was carried out on the extracted DNA, using specific primers for HHV-8. After identifying the presence of HHV-8 by amplification of its orf26 region, the orf-K1 region was amplified, sequenced and used for phylogenetic analysis. Results: From the 36 orf26-positive cases, orf-K1 was amplified and was analyzed successfully in 12 cases. Phylogenetic studies, based on the complete K1 gene/protein sequences, indicate that all strains belong to the A subtype. Specifically, six of them were related to the A1 subgroup, six to the A2 subgroup and three previously reported to the A3 subgroup. Nucleotide sequence data are reported and are available in the Genbank database under accession numbers KF829938-KF829947.*

Correspondence to: Jozsef Konya, Department of Medical Microbiology, Medical and Health Science Center, University of Debrecen, 4032 Debrecen, Nagyerdei krt. 98., Hungary. E-mail: konya@med.unideb.hu

Key Words: Kaposi's sarcoma, HHV-8 genotypes, PCR, K1, subtype A.

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma (KS) associated herpesvirus (KSHV), is a member of the sub-family gamma herpesviridae that has been associated with all forms of KS (1). As a rare vascular tumor, KS was described initially in elderly men of Mediterranean or Jewish descent [Kaposi, 1872 cited in (2)]. The clinico-epidemiological forms of KS have been classified as classical or sporadic KS (in the Mediterranean region), AIDS-associated, endemic (in Africa), and iatrogenic (in organ and tissue transplant recipients receiving immunosuppressive therapy) (3). HHV-8 is also associated with primary effusion (body cavity-based) lymphoma (PEL/BCBL) and with multicentric Castleman's disease. The HHV-8 genome consists of one single continuous long unique region (LUR) of 140 kb, which is flanked on both sides by terminal repeats of several 801 bp-long repeat subunits. The great majority of the LUR of HHV-8 is highly conserved, while both ends show high variability. Molecular analysis of the genetic variability of the K1 gene can be exploited for epidemiological studies. K1 is located at the left end of the genome and encodes a highly variable membrane glycoprotein of 298 amino acids (4-7). The amino acid sequence of K1 varies from 0.4% to 44%, with the changes concentrated at the two hypervariable regions (VR1 and VR2). Seven major molecular subtypes (designated A, B, C, D, E, F and Z) of HHV-8 have been classified on the basis of K1 sequence analysis (8-11).

The distribution of HHV-8 isolates varies with geography and ethnicity (12, 13). Compared to other human herpesviruses, HHV-8 appears to be infrequent in the general population of North America and Northern and Western Europe, but is more prevalent in several Mediterranean countries, in particular in Italy and Greece, and is widespread in many parts of Africa (14-17). Among individuals of Western countries at increased risk of HIV infection, HHV-8 is more

Table I. *Epidemiological and clinical data of seven patients with Kaposi´s sarcoma from Hungary.*

Patient	Age (years)	Gender	KS status	Type of KS	Specimen		K1 type	Accession. No.
					Type	Sequence ID		
1	87	F	Cutaneous	Classic	Skin tumor biopsy 1	Hun30a	A1	KF829938
					Skin tumor biopsy 2	Hun30b*	A1	KF829939
					Skin tumor biopsy 3	Hun30b*		
2	85	M	Cutaneous	Classic	Skin tumor biopsy 1	Hun31a	A2	KF829940
					Skin tumor biopsy 2	Hun31b*	A1	KF829941
					Skin tumor biopsy 3	Hun31b*		
3	84	M	Cutaneous	Classic	Skin tumor biopsy	Hun32	A1	KF829942
4	82	M	Cutaneous	Classic	Skin tumor biopsy	Hun33	A1	KF829943
5	39	M	Visceral and cutaneous	Classic	Skin tumor biopsy 1	Hun34a	A1	KF829944
					Skin tumor biopsy 2	Hun34b	A2	KF829945
6	78	M	Cutaneous	Classic	Skin tumor biopsy	Hun35	A2	KF829946
7	88	M	Cutaneous	Post-transplant	Skin tumor biopsy	Hun36	A2	KF829947

*Identical sequences.

prevalent among homosexual men than among patients with hemophilia, injecting drug users and those who contracted HIV heterosexually (18-20). Subtypes A and C have been found in Europe (21), the USA (22), Middle East and Asia (23). Subtype B is found mainly in Africa (4, 24) and French Guiana (5, 25). Subtype D was first reported in Taiwan, in some Pacific islands (24) and Australia (26), and subtype E has been found among Amerindian population of the Brazilian (22) and Ecuadorian Amazon regions (27). Subtype Z has been found in a small cohort of Zambian children (6) and a new subtype F has been identified in Uganda (28, 29). So far, no K1-based phylogenetic analysis has been performed on HHV-8 isolates in hospitalized patients in Hungary.

We here report the molecular typing of HHV-8 strains isolated from Hungarian patients based on orf-K1 genomic regions.

Materials and Methods

Patient samples. A total of 36 paraffin-embedded biopsies were tested in 17 patients. (We have more samples from the same patients, which were taken different times.) 21 specimens from 6 patients were described by Juhasz *et al*. (30). Further 15 specimens from 11 patients were also paraffin-embedded, formaldehyde-fixed biopsies of 'classic' (HIV-negative) KS. The main clinical and epidemiological features of the patients are summarized in Table I.

DNA extraction. At least three 20 μm sections were obtained from each of the 15 formalin-fixed and paraffin-embedded KS tissue samples. The sections were deparaffinized with xylol for 3 X 5 min, washed five times with ethanol and air-dried. The deparaffinized samples were incubated with 200 μl lysis buffer (100 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 8.0, 1% sodium dodecyl sulfate, 25 mmol/l EDTA, pH 8.0 and 200 μg/ml proteinase K) at 55˚C for one hour, followed by heat inactivation of proteinase K at 95˚C for 10 min. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). After centrifugation, the water-phase was transferred to a new tube with 1 ml isopropanol and kept at - 20˚C overnight. The pellet was washed in 70% ethanol and resuspended in 30 μl Tris-EDTA.

Polymerase chain reaction (PCR). We have optimized the temperature and cycling conditions of PCR amplification of the K1 sequences. We used serial dilution of DNA isolated from BCBL-1 cell line. For all assays stringent precautions against PCR contamination were taken. The DNA extraction process was validated by amplifying the β-globin gene using the PCO3 and PCO4 primers (31). The presence of HHV-8 was detected by amplification of the conserved orf26 gene. The orf26 primers amplified a 172 bp-long amplicon (data not shown) (1, 32). (All patient samples contained the virus.)

To amplify K1 sequences, we used K1-N (nt 105 to 121) - K1-C (nt 971 to 955) outer primers in combination with nK1-2B (nt 603 to 584) primer for the semi-nested round. The genomic positions correspond to genome of BC-1 strain (33). Detailed descriptions of the primers are shown in Table II. Briefly, a 25 μl reaction volume containing 12 μl JumpStart REDTaq ReadyMix Reaction Mix (Sigma, Sigma-Aldrich, Saint Louis, MO, USA), 0.5 μl primers and 100-500 ng DNA in 12 μl. Thermalcycling conditions were set at 95˚C denaturation for 3 min, followed by 30 cycles of 95˚C for 30 s, and 1 min at 58˚C at the appropriate annealing temperature, 72˚C for 1 min, and 4 min extension at 72˚C. Simultaneously, each PCR run contained several negative controls (distilled water instead of DNA templates) and positive controls (HHV-8 DNA isolated from BCBL-1). The reaction products were checked on 1.5% agarose gels, observed by a gel imaging system.

Sequencing. Sequencing reaction of the nested PCR products of the *orf-K1* PCR was performed using the BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). Sequencing products were detected by ABI Prism 310 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, California, USA). To ensure that the observed mutations were not the result of errors in PCR, sequencing was performed on at least two distinct PCR

Table II. *Human β-globin and HHV-8 primers.*

Primer	PCR round	Sequence (5'-3')	Amplicon (bp)	Nucleotide position*
β-Globin				
PCO3		CTTCTGACACAACTGTGTTCACTAGC	110	
PCO4		TCACCACAACTTCATCCACGTTCACC		
orf-K1				
K1-N	1st	CGCGAATTCATGTTCCTGTATGTTGT	866	105 to 121
K1-C		CGCCTCGAGCGTACCAATCCACTGGTT		971 to 955
orf-K1				
K1-N	2nd	ACGCCTTACACGTTGACCTG	498	105 to 121
K1-2B		TTGACAGGCGAGACGACGAC		603 to 584

*The genomic positions correspond to genome of BC-1 strain.

products generated from each DNA sample, and both strands of each product were independently sequenced.

Phylogenetic analysis. With phylogeny.fr, we created a phylogenetic tree of the partial K1 amino acid sequences (34, 35). Nineteen reference sequences from GenBank and published reports were included in the analysis to aid subtype identification. All sequences were first aligned in ClustalW (Kyoto University Bioinformatics Center). Radial and unrooted linear phylogenetic trees were drawn by the program DRAWTREE (version 3.66, University of Washington).

Results

The diagnosis of patients with KS was based on clinical and histopathological characterizations of skin lesions. The main clinical and epidemiological features (age, sex, type of sample, KS status, type of KS) of these patients are listed in Table I. Out of the 36 paraffin-embedded tissue specimens, the orf26 regions was detected in 36 (100%) by nested PCR. Out of the 36 orf26-positive samples, orf-K1 region was detected in 22 (61%) by semi-nested PCR. The orf-K1 PCR bands were suitable for sequencing and sequence analysis in 12 biopsies. PCR amplification of the remaining 10 biopsies did not result in K1 amplicons of proper quantity or quality for bi-directional sequence analysis.

The lengths of the partial sequences of HHV-8 isolates from this study (GenBank accession numbers KF829938-KF829947) ranged from 281 to 358 nucleotides. There was a common 167-bp region (nt 169 to 336) revealing nucleotide sequence from each isolate. This common region was used to analyze the phylogenetic relationship. Into the phylogenetic analysis, we inserted other known Hungarian sequences (36). We used two different sets of sequences for the phylogenetic studies. The first one involved all K1 subtype nucleotide sequences, while the second set, based on amino acids, comprised all the corresponding subtype-A sequences (Figures 1 and 2).

Regarding the orf-K1, as presented in Table III, all tested strains belonged to the subtype A. Specifically, six were related to the A1 subgroup, four to the A2 subgroup and three to the A3 subgroup. The isolates Hun30a, Hun30b, Hun31b, Hun32, Hun33 and Hun34a exhibited 100% identity with the K1/E25 and K1-77/47 prototype (accession number: AY204654 and AF201850) (25), while the isolates Hun31a, Hun34b, Hun35 and Hun36 96,5%, 94,8% and 93% identity with the K1/E41 prototype on an amino acid level (accession number: AY204657) (25). The isolates Hun14a and Hun13 exhibited 100% and Hun14b 93% amino acid identity with the A3 prototype BCBL-1 (accession number: JN800483) (36).

Discussion

Hayward *et al*. hypothesized that HHV-8 is an evolutionary old human virus distributed worldwide along broad ethnic and geographic lines and acts as a marker for ancient human migration (37). The first expansionary migration was into Sub-Saharan Africa starting 100,000 years ago (B subgroup), then into South Asia and Australia beginning 60,000 years ago (D subgroup). Finally, two major branches migrated into Europe and North Asia approximately 35,000 years ago (A and C subgroups) (38).

HHV-8 DNA is present in all KS tumor samples and in the peripheral blood mononuclear cells (PBMCs). Serological evidence obtained by LANA immunofluorescent antibody assay also indicates that the infection is widespread in the world. The prevalence of the virus is variable, lower in Northern Europe and the USA (0-3% seropositivity), higher in Southern Italy (5 to 20% seropositivity) and very high in Central and Southern Africa (40% to 60% seropositivity), where endemic and classic KS have the highest incidence rates, reaching up to 1.0 and 10 per 100,000 person-years, respectively (17). Similarly, patients with KS and male homosexual patients with AIDS, human immunodeficiency virus (HIV)-positive intravenous drug users

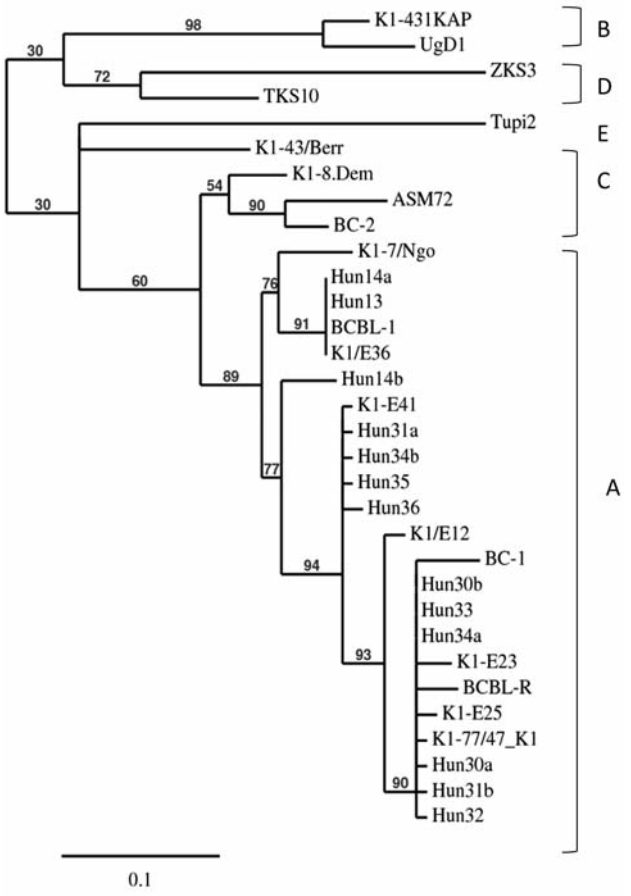

Figure 1. *Phylogenetic analysis of orf-K1 amino acid sequences of HHV-8 isolates studied and of prototype strains available in GenBank. The length of each branch and location indicates genetic distance with the size scale for 10 (10% difference). Confidence levels for the branching pattern were estimated by bootstrap re-sampling of the data. Hun13, Hun14a, Hun14b sequences are published by Szalai et al. (34).*

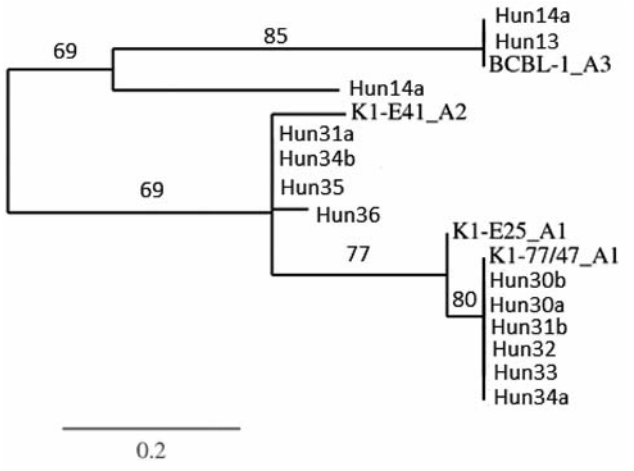

Figure 2. *Phylogenetic tree based on amino acid sequences of orf-K1 region for HHV-8 isolates and reference strains. The scale bar corresponds to 0.2 substitutions/site. References and GenBank gene identifications numbers for the sequences used in orf-K1 regions comparisons A strains. Hun13, Hun14a, Hun14b sequences are published by Szalai et al. (34).*

and hemophiliacs have extremely high seropositivity rates of around 85% (39, 40). The prevalence in Hungary is 2.3-3.5% among blood donors (30, 41), and 31.5% among Hungarian HIV-positive patients (42) (36). Among our patients 1/7 was a female, consistent with previous evidence that KS is more frequent in males than in females (43).

Since limited data are available on HHV-8 molecular epidemiology in Hungary, the aim of the present work was to investigate the distribution of HHV-8 DNA sequences among hospitalized individuals in Hungary. We analyzed the genetic variation and polymorphism of a genomic region of HHV-8 (orf-K1) located at the left-hand side of the viral genome of 36 KSHV strains originating from Hungarian patients with KS.

We attempted to develop a typing system based on amplification and sequencing of the K1 region, altogether 12 sequences were amplified and analyzed successfully, probably because of the G-C content, predicted hairpin structures in the DNA, and the highly variable sequence. Other studies also obtained sequences suitable for analysis in a proportion similar to ours (29, 36).

From one patient (Hun14), different variants of HHV-8 were isolated from serum and PBMCs (36). We have two different samples from another three patients, obtained at different times. The elapsed time between samplings is a minimum of three years. For two patients, we identified different K1 strains, the differences are 3% at the nucleotide level and causes five amino acid changes (Figure 2). This could be the result of detecting more variants of the HHV-8 virus in the same patient (36).

Thirteen specimens were analyzed and clustered to subtype A. This genotype is considered to originate from Europe and North Asia (44). The C subtype is very common in this region as well, but we could not identify it among the isolates, where the K1 section was successfully amplified.

The main outcome is that it provides useful data for molecular epidemiological studies in Hungary. It is necessary to continue the surveillance of cancer such as KS for designing more effective diagnostic and treatment procedures. Nevertheless, the available samples within the studied group were limited in number, so we cannot make a general claim about the association of a specific viral genotype with clinical presentation and further studies are ongoing to clarify this issue.

Acknowledgements

This is to express our remembrance of gratitude to the late Dr. Ágnes Bégány for the histological examinations.

Table III. *Amino acid alignment of the proteins encoded by HHV-8 orf-K1. The complete amino acid sequence is given only for the prototype A1 (K1-E25) on the top line, with amino acid identities indicated by dashes for the other genomes. Subtype designations are given at far right.*

K1-E25	LFRLTERTVF	PVNIACNFTC	VEQSGHRQSI	WITWHAQPVL	QTLCAQPSNT	VTCGQH	A1
K1-77/47	----------	----------	----------	----------	----------	------	A1
Hun30a, Hun30b	----------	----------	----------	----------	----------	------	A1
Hun31b, Hun2	----------	----------	----------	----------	----------	------	A1
Hun33, Hun34a	----------	----------	----------	----------	----------	------	A1
K1-E41	-L------FL	G-N-------	----------	----------	----------	------	A2
Hun31a	--------LL	G-N-------	----------	----------	----------	------	A2
Hun34b, Hun35	------K-LL	G-N-ACNFTC	----------	----------	----------	------	A2
Hun36	-----KK-LL	G-N-ACNFTC	----------	----------	----------	------	A2
BCBL-1	-W---KP-LT	IDI-T-----	----------	----------	----------	------	A3
Hun14a, Hun13	-W---KP-LT	IDI-T-----	----------	----------	----------	------	A3
Hun14b	-L---KP-LT	D-A-------	----------	-----P----	----------	------	A3

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Received November 22, 2013
Revised December 13, 2013
Accepted December 17, 2013