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Human Herpesvirus Type 8 Genotypes in Iatrogenic, Classic and AIDS-associated Kaposi's Sarcoma from Greece

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Abstract. *Background: Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 (HHV-8) is consistently found in almost all observed Kaposi's sarcomas (KS), whether AIDS-associated, iatrogenic or classic. To our knowledge no data are available on the genetic polymorphism of HHV-8 from Greece. We report the study of 15 renal transplant recipients with KS, 5 with AIDS-associated KS, 11 with classic KS and 60 healthy individuals from Greece. Materials and Methods: Polymerase chain reaction (PCR) was carried out on DNA extracted from peripheral-blood mononuclear cells (PBMC) or KS cutaneous biopsies, using specific primers for the HHV-8, KS330 fragment from ORF-26 (233 bp) and the highly variable region (VR1) from ORF-K1 (363 bp). Results: HHV-8 DNA was detected in 30 out of 31 (97%) KS cases, regardless of their clinico-pathological subtype and in 10 out of 60 (16.7%) healthy individuals. Sequencing of the ORF26 fragment demonstrated that the 40 HHV-8 strains were of the A and C sub-types. Furthermore, sequencing of the ORF-K1 showed that these HHV-8 strains of Greek origin were of the A1, A4, C1 or C3 sub-type. Conclusion: Our findings imply a possible link of the C3 subtype of HHV-8 in renal transplant-related KS cases (iatrogenic KS) in Greece, a link of the A4 subtype in AIDS-associated KS cases and a potential involvement of the A1 subtype in Greek classic KS incidences, as HHV-8 strains among healthy individual tested belong to the C1, C3 or A1 subtypes.*

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Kaposi's sarcoma (KS) was first described as an "idiopathic multiple pigmented sarcoma of the skin" by Moritz Kaposi in 1872 (1). KS is now categorized according to four different epidemiological manifestations (2): a) classic, in which the skin lesions have little or no visceral involvement; b) African endemic, which can show systematic involvement and progress rapidly, c) AIDS-associated, which manifests both as cutaneous and visceral lesions and is the most aggressive form of the disease; and d) iatrogenic immunosuppression-related, which develops as a result of immunosuppressive therapy in transplant recipients.

Beral predicted that AIDS-associated KS was caused by an unknown infectious agent other than HIV (3), but it was not until late 1994 that Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), was identified as the probable candidate etiological agent (4). Although its role in pathogenesis has yet to be defined, HHV-8 has been identified as an important cofactor in the development of KS and it has been detected in virtually all KS patients (5).

Recent molecular epidemiological studies have revealed that the KS330 fragment from ORF26 of HHV-8, as well as the highly variable left end of the ORF-K1 gene, are very good markers for HHV-8 genotyping and strain differentiation (6-10). Four subtypes (A, B, C and D) of HHV-8 are classified on the basis of ORF-26 and ORF-K1 (6, 9, 10). HHV-8 subtypes, as defined especially by K1 sequences, have specific geographical distribution patterns worldwide (6, 9, 10). Isolates from Europe, Asia and the United States are generally A and C subtypes, those from Africa are predominantly the B subtype, while the rare D subtype appears exclusively from the Pacific Islands (11).

Although several studies have revealed some patterns of HHV-8 genetic variation, there is limited information on the distribution of HHV-8 genotypes in human populations

Table I. *Epidemiological and clinical data of 31 Kaposi's sarcoma patients from Greece.*

Sample	HIV-1	HHV-8 DNA		Type of KS
		ORF-26	ORF-K1	
KS1	-	+	+	Iatrogenic (RTR)
KS2	-	+	+	Iatrogenic (RTR)
KS3	-	+	+	Iatrogenic (RTR)
KS4	-	+	+	Iatrogenic (RTR)
KS5	-	+	+	Iatrogenic (RTR)
KS6	-	+	+	Iatrogenic (RTR)
KS7	-	+	+	Iatrogenic (RTR)
KS8	-	+	+	Iatrogenic (RTR)
KS9	-	+	+	Iatrogenic (RTR)
KS10	-	+	+	Iatrogenic (RTR)
KS11	-	+	+	Iatrogenic (RTR)
KS12	-	+	+	Iatrogenic (RTR)
KS13	-	+	+	Iatrogenic (RTR)
KS14	-	+	+	Iatrogenic (RTR)
KS15	-	-	-	Iatrogenic (RTR)
KS16	+	+	+	AIDS-associated
KS17	+	+	+	AIDS-associated
KS18	+	+	+	AIDS-associated
KS19	+	+	+	AIDS-associated
KS20	+	+	+	AIDS-associated
KS21	-	+	+	Classic (patient in dialysis)
KS22	-	+	+	Classic
KS23	-	+	+	Classic
KS24	-	+	+	Classic
KS25	-	+	+	Classic
KS26	-	+	+	Classic
KS27	-	+	+	Classic
KS28	-	+	+	Classic
KS29	-	+	+	Classic
KS30	-	+	+	Classic
KS31	-	+	+	Classic

and the association of specific variations with ethnicity and geographic origins as well as with the different forms of KS such as the AIDS-associated, iatrogenic, African and classic (Mediterranean) KS. To our knowledge no data are available on the genetic variability of HHV-8 from Greece.

We report here the molecular characterization, with subtyping of both ORF-26 and ORF-K1 genomic regions of HHV-8 strains isolated from Greek patients with different KS type (iatrogenic, AIDS-associated and classic) and from healthy individuals.

Materials and Methods

Patient samples. Patients with Kaposi's sarcoma were selected on the basis of clinical and histopathological criteria, from departments of dermatology and transplantation in Greece. All the patients were Greek and only five of them were HIV-1-positive. The specimen set included PBMC or formalin-fixed paraffin-embedded (FFPE) cutaneous biopsy of Kaposi's lesion. Of 31 KS patients, 15 had iatrogenic (renal transplant recipients) KS, 5 AIDS-associated KS and 11 classic KS. DNA isolated from PMBC from 60 healthy individuals was also tested. The specimens were PMBC from renal transplant recipients and healthy individuals and FFPE-tissues from patients with AIDS-associated and classic KS.

DNA isolation. Genomic DNA was isolated from PBMC by using the QIAamp Blood kit (Qiagen, Germany) following the manufacturer's directions. KS FFPE tissue blocks were sectioned and de-paraffinized by boiling in 400 μl lysis buffer (50mM Tris-HCl, pH8.5, 0.5% Tween 20, 1mM EDTA and 200 μg/ml proteinase K), cooling to 4˚C and then removing the resolidified paraffin; the mixture was then incubated at 55˚C until completely digested. The DNA was extracted with phenol-chloroform, precipitated with ethanol and then suspended in water.

Polymerase chain reaction (PCR) amplification and nucleotide sequencing. Each sample was shown to contain amplifiable DNA using standard PCR conditions and primers for human GAPDH (glyceraldehyde-3-phosphatate dehydrogenase) gene (12). HHV-8 ORF-K1 and KS330 subtyping was performed, as previously described (9, 10). DNA sequencing of HHV-8 PCR products was performed using the Big Dye-DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, USA) and ABI Prism 310 Genetic Analyzer automated sequencer (Applied Biosystems). To ensure that the observed mutations were not the result of Taq-generated errors in PCR, sequencing was performed on at least two distinct PCR products generated from each DNA sample, and both strands of each product were independently sequenced.

Alignments and phylogenetic analysis. HHV-8 subtypes based on ORF-26 and ORF-K1 were assigned on the basis of previous reports (9, 10, 13). Phylogenetic analysis of the sequences was performed using the PHYLIP package, version 3.5C. A majority-rule consensus tree was computed from 100 bootstrap replications (14) using that package. The DNA sequence data for the prototype A1(BCBL-R), A4(BCBL-B), A5(Ug374), B1(431KAP), B2(Ug81), B2*(UgD1), BCBL-1, C1(ASM72), C3(BC2), C3(BC3), D1(TKS10) and D2(ZKS3) subtype genes are available from GenBank (accession no. AF133038 to AF133044, AF130289, AF130291, AF13092, AF170531 and U86667).

Results

We studied 31 patients with Kaposi's sarcoma (Table I). As controls, 60 healthy individuals were selected for PCR analysis. All the individuals tested originated in Greece and five of them were HIV-1-positive.

HHV-8 PCR results were considered positive when HHV-8 sequences were detected after ethidium-bromide gel electrophoresis and sequencing of the PCR products. All the 91 samples (31 from KS and 60 controls) were amplified by the specific primers for the human GAPDH gene, demonstrating the integrity of the DNA samples. As presented in Table I, HHV-8 sequences were detected with both set of primers (HHV-8 ORF-26 and ORF-K1) in 30

Table II. *Comparison of polymorphic nucleotide patterns that identify several distinct subtypes of HHV-8 genomes within the 233-bp ORF-26 gene locus (13). The subtype D patterns are indistinguishable from those of subtype A.*

DNA samples	ORF-26 (233 bp)									Subtype
	981	1032	1055	1086	1094	1103	1122	1132	1139	
BCBL-R	T	C	G	C	G	C	G	A	A	A
BCBL-B	T	C	G	C	G	C	G	A	A	A
431KAP	C	C	G	C	G	C	G	G	C	B
ASM72	C	C	G	T	A	C	G	G	C	C1
BC2	C	A	T	T	A	C	G	G	C	C3
BC3	C	A	T	T	A	C	G	G	C	C3
TKS10	T	C	G	C	G	C	G	G	C	A/D
ZKS3	T	C	G	C	G	C	G	A	A	A/D
KS1-KS12, H1-H5	C	A	T	T	A	C	G	G	C	C3
KS13, KS14, KS21, H6-H8	C	C	G	T	A	C	G	G	C	C1
KS16-KS20, KS22-KS31, H9-H10	T	C	G	C	G	C	G	A	A	A/D

out of 31 KS cases (97%). The same sequences were found in only 10 out of 60 healthy individuals (16.7%) with both sets of primers.

To further study the possibility of sequence variation, sequence analysis of PCR-amplified products of the KS330 fragment within the ORF-26 were generated from the HHV-8-positive samples. Comparison of polymorphic nucleotide patterns, identifying several distinct subtypes of HHV-8 genomes within the ORF-26 gene locus, demonstrated that all the HHV-8 isolates from iatrogenic KS (14 cases, one patient was negative for the presence of HHV-8), the isolate from the single patient in dialysis with classic KS and 8 out of 10 isolates from the healthy individuals belonged to the C subtype. From those 23 samples belonging to the C subtype, 17 strains (KS1-KS12 and H1-H5), as seen in Table II, exhibited nucleotide substitutions specific for C3 subtype, and 6 strains (KS13, KS14, KS21 and H6-H8) exhibited nucleotide substitutions specific for C1 subtype. By OFR-26 genotyping, 10 out of 11 cases of classic KS (KS22-KS31), the five AIDS-associated cases (KS16-KS20) and 2 out of 11 isolates from the healthy individuals (H9 and H10) were classified as A/D subtype. Since the D patterns are indistinguishable from those of subtype A patterns (Table II), the use of ORF-26 patterns alone for classification is not recommended.

Regarding the ORF-K1, as presented in Table III, 23 strains belonged to the subtype C. Specifically 17 of them (KS1-KS12 and H1-H5) were related to the C3 subgroup and 6 (KS13, KS14, KS21 and H6-H8) to the C1 subgroup. The isolates KS1-KS10, H2 and H5 exhibited 96.7% identity with the BC2 prototype and 97.5% with the BC3 prototype, while the isolates KS11, KS12, H1, H2 and H3 exhibited 95.8% identity with the BC2 prototype and 99% with the BC3 prototype on an amino acid level. The isolates KS13, KS14, H6 and H7 exhibited 95.8% amino acid identity with the C1 prototype (ASM72), whereas the strains KS21 and H8 exhibited 92.5% of amino acid identity with the C1 prototype (ASM72). The five isolates from AIDS-associated KS (KS16-KS20) exhibited a typical A4 subtype (99% of amino acid identity with the prototype BCBL-B). The strains KS22-KS31 from classic KS, as well as H9 and H10, exhibited 95% amino acid identity with the A1 subgroup (BCBL-R). In view of the analysis of ORF-K1 variability, there was a molecular aggregate reflecting the clinico-epidemiological type of KS (Figure 1).

Discussion

This is the first study to report on the genetic diversity of HHV-8 genomes originating from renal post-transplantation iatrogenic, AIDS-associated and classic KS, as well as from healthy individuals of Greek origin.

The results of the present study support and extend the findings of Zang *et al.* (11), Zong *et al.* (13), Nicholas *et al.* (15) and DiAlberti *et al.* (16), indicating the existence of variants of HHV-8. The data from the aforementioned studies supported a geographical correlation with strain variation in HHV-8, as samples originating from Europe, Asia and the United States were of A or C subtypes, whereas those from Africa were predominantly of the B subtype, and the rare D subtype appeared exclusively from the Pacific Islands.

Here, in order to determine which molecular subtype of HHV-8 was present in the KS patients as well as among healthy individuals originating from Greece, we performed sequence analysis of fragments of genes coding for the

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

HHV-8 ORF-K1 protein:												
BCBL-R	MFLYVVCSLA	VCFRGLLSLS	LLSSPNLCPG	VISTPYKLTC	LSNASLPISW	YCNNTRLFRP	TETTLFPVTI	ACNFTCVEQS	GHRQSIWITW	HAQPVLQTLC	AQPSNTVTCG	QHVTLYCSTS	A1
BCBL-B	----------	----------	----------	----------	----------	---D-----L	--R-------	P---------	----------	----------	----------	----------	A4
Ug374	----------	----------	----------	----------	----------	---D---W-L	-DQSFTVA--	T---------	----------	N---------	----------	----------	A5
431KAP	-L-CI----L	---PK----H	-P-F-H----	------T---	P--R------	---G-Q-H-I	-ASN-TVSSL	T-----MTT-	-PTH----Q-	YT--------	-------S--	-P-----D--	B
Ug81	-L-CI----L	---PK----H	-P-F-H----	------T---	P_-R------	---G---H-I	-ASN-TVSLL	T-----MTT-	-PTH----E-	YT--------	-------S--	-P-----D--	B2
UgD1	-L-CI----L	---PK----H	-P-F-H----	------T---	P_-R------	---G-Q-R-I	RGSN-TVSLL	T-----MTA-	-PTH----E-	YT--------	-------S--	-P-----D--	B2*
ASM72	----------	---------Y	-Q--------	------T---	P-AT---T--	---D-----L	-HD-FTV-NF	I---S--G--	---H-L-M--	YG--------	G_A-------	----------	C1
BC2	----------	----------	-Q--------	------T---	P--T---T--	---D---L-L	-QQ-FTV-AL	I---S--G--	---H-L----	YP--------	G---------	----------	C3
BC3	----------	----------	-Q-------R	-S----T---	P--T---T--	---D---L-L	-QP--TVSNL	I---S--G-F	---H-L----	YP--------	G---------	----------	C3
TKS10	----------	---P-----H	-SV--QF--A	-L--S-T---	P-D-------	---G---L-I	-GA--TIPSL	TG-----DH-	-LSH----QR	YPP-------	----T-----	-R-S-H----	D1
ZKS3	-------N--	---P-----H	-PAF-P----	-L--N-T---	P-D-------	---G-L-M-Y	HR---TLMNL	-A-W---N--	-ISH----Q-	YTE-------	----------	-R---H----	D2
KS1-KS10,H2,H5	----------	----------	-Q-------R	-S----T---	P--T---T--	---D---L-L	-QQ-FTV-NL	I---S--G-F	---H-L----	YP--------	G---------	----------	C3
KS11-KS12, H1,H3,H4	----------	----------	-Q-------R	-S----T---	P--T---T--	---D---L-L	-QQ--TVSNL	I---S--G-F	---H-L----	YP--------	G---------	----------	C3
KS13,KS14,H6,H7	----------	---------Y	-Q--------	------T---	P--T---T--	---D-----L	-QD-ITV-NL	I---S--G--	---H-L-M--	Y---------	G-A-------	----------	C1
KS21,H8	----------	---------Y	-Q--------	------T---	P-AT---T--	---D---L-L	-QG-ITV-SL	V---S--G--	---H-L-M--	S---------	G-A-------	----------	C1
KS16-KS20	----------	---Q------	----------	----------	----------	---D-----L	--R-------	P---------	----------	----------	----------	----------	A4
KS22-KS31,H9,H10	----------	---Q------	----------	------N---	----------	---------L	--K-VI----	----------	----------	----------	----------	----------	A1

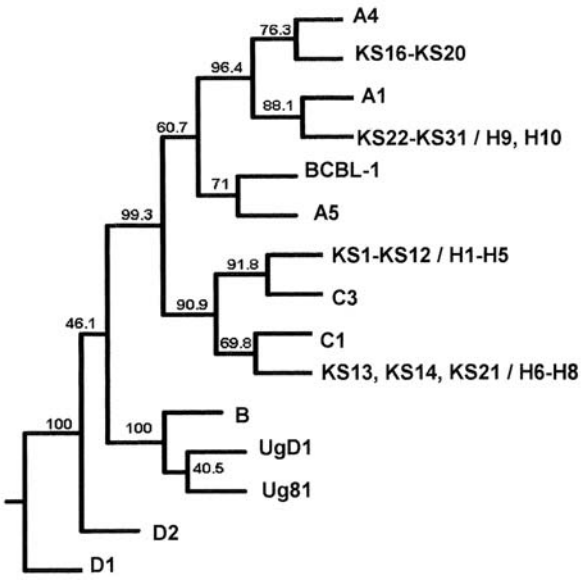

Figure 1. *Phylogenetic analysis of ORF-K1 amino acid sequences of HHV-8 isolates studied and of prototype strains deposited by Zong et al. (9). The consensus tree was computed from 100 bootstrap replications with Phylip Prodist and the neighbor program of the PHYLIP package, based on PAM distances.*

capsid protein (ORF-26) and the highly variable (VR1) domain of ORF-K1 protein. ORF-26 was originally used by Zong *et al.* (11) to propose a classification of HHV-8 genomes in 3 main molecular subtypes A, B and C. By studying the KS330 region within ORF-26, we found that all the analyzed strains of HHV-8 belong to either the A or the C subtype. Sequence analysis of the VR1 domain within the ORF-K1 revealed that the strains distributed to A1, A4, C1 and C3 subtypes, with several minor variants from the prototypes (Table III), indicating that the use of more variable genomic regions, such as ORF-K1 instead of regions with a low degree of sequence nucleotide variation such as ORF-26, is more informative and will permit new insights into the origin, genetic evolution and disease association of HHV-8 (7, 9). In general our findings are in agreement with previous studies reporting that the A and C subgroups are common in Europe (6, 7).

Recent observations suggest that each of the HHV-8 strains may possess different biological properties and cell tropism (17-19), but there is still debate about whether different HHV-8 subtypes have significantly different clinical associations (9, 20). Surprisingly, we observed that a molecular aggregate reflects the clinico-epidemiological types of KS. Specifically our findings imply a possible link of the C3 subtype of HHV-8 to renal transplant-related KS cases (iatrogenic KS) in Greece, a link of the A4 subtype to AIDS-associated KS cases and a potential involvement of the A1 subtype in Greek classic KS incidences, as HHV-8 strains among healthy individual tested belong to the C1, C3 or A1 subtypes. Nevertheless, the available samples within the studied group were limited in number, so we can not make a general claim about the association of a specific viral genotype with clinical presentation and further studies are ongoing to clarify this issue.

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