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Human herpesvirus type 8 variants circulating in Europe, Africa and North America in classic, endemic and epidemic Kaposi's sarcoma lesions during pre-AIDS and AIDS era

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ABSTRACT

Human herpesvirus-8 (HHV-8) variants have been found heterogeneously distributed among human populations living in diverse geographic regions, but their differential pathogenicity in Kaposi's sarcoma development remains controversial. In the present study, HHV-8 variant distribution has been analyzed in classic, iatrogenic, endemic as well as epidemic Kaposi's sarcoma (KS) during pre-AIDS and AIDS period (1971–2008) in countries with different KS incidence rate. DNA samples from cutaneous KS lesions of 68 patients living in Africa ($n=23$, Cameroon, Kenya and Uganda), Europe ($n=34$, Greece and Italy) and North America ($n=11$) have been subjected to PCR amplification of HHV-8 ORF 26, T0.7, K1 and K14.1/15, followed by direct nucleotide sequencing and phylogenetic analysis. Among the 23 African samples, the majority of HHV-8 ORF 26 variants clustered with the subtype R ($n=12$) and B ($n=5$). Conversely, the viral sequences obtained from 45 European and North European tumors belonged mainly to subtype A/C ($n=36$). In general, HHV-8 and K1 variant clustering paralleled that of ORF 26 and T0.7. Genotyping of the K14.1/15 loci revealed a large predominance of P subtype in all tumors. In conclusion, comparison of the HHV-8 sequences from classic or endemic versus AIDS-associated KS showed a strong linkage of the HHV-8 variants with specific populations, which has not changed during AIDS epidemic.

Introduction

Kaposi sarcoma (KS), a mesenchymal tumor involving blood and lymphatic vessels (Safai and Good, 1981;Pyakurel et al., 2006), is characterized by four different clinical and epidemiological settings (Schwartz et al., 2008): (1) classic KS, mainly occurring in elderly men of Mediterranean or Eastern European Ashkenazi origin, with specific geographic foci in Italy, Greece, Turkey and Israel (Iscovich et al., 2000;Guttman-Yassky et al., 2003;Dal Maso et al., 2005;Buonaguro et al., 2003); (2) African-endemic KS which existed for many decades before human immunodeficiency virus (HIV) infection in some equatorial African countries (Buonaguro et al., 2003;Serwadda et al., 1986;Oettlé, 1962;Templeton, 1981); (3) iatrogenic KS, developing in solid organ transplantation recipients following immunosuppressive drug treatment (Buonaguro et al., 2003;Stribling et al., 1978;Penn, 1983;Tessari et al., 2006); and (4) epidemic or AIDS-associated KS, which has been the most frequent neoplasm in homosexual and bisexual men with AIDS before the HAART era (Buonaguro et al., 2003; Biggar et al., 1987;Dal Maso et al., 2009;Beral et al., 1990).

Herpes-type viruses have been suggested to play a role in KS for more than 20 years (Giraldo et al., 1972;Giraldo et al., 1980), but only in the late 1994 specific DNA sequences of a new KS-associated virus, the human herpesvirus type 8 (HHV-8), were identified in AIDS-associated KS (Chang et al., 1994). This virus is now considered as the etiological agent of all clinic-epidemiological forms of this tumor type (Buonaguro et al., 1996;Moore and Chang, 2003;Buonaguro et al., 2003). Phylogenetic studies performed on the well-conserved ORF 26 (minor capsid gene) allowed the identification of eight distinctive subtypes designated as A/C, J, K/M, D/E, B, Q, R or N groups, which are diversely distributed in different geographical regions. In particular, four subtypes (B, N, Q and R) have been found virtually exclusively among Sub-Saharan African samples, one (D/E) has been found only within indigenous South Asian and Polynesian (Pacific Rim) populations and three (A/C, J and K) have been identified almost exclusively

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in Eurasian subjects (European, United States, North Asian and Middle Eastern) (Zong et al., 2007). Sequence analysis of the highly variable ORF K1 region has allowed the identification of seven major HHV-8 subtypes (A, B, C, D, E, F and Z), comprising each several sub-clades, whose distribution in the world parallels that of ORF 26 variants: HHV-8 subtype B predominates in Africa and F has been identified in Ugandan Bantu tribe, subtype D is present in the Pacific islands; subtype E clusters in ancient populations, like Brazilian Amerindians, while A and C predominate in Europe and USA (Zong et al., 1999;Poole et al., 1999;Biggar et al., 2000;Kasolo et al., 1998;Kajumbula et al., 2006). It is still unclear whether different genotypes may have different pathogenic and tumorigenic properties associated with diverse rates of disease progression (Mancuso et al., 2008;Whitby et al., 2004).

Seroepidemiological surveys indicated that HHV-8 is not a ubiquitous virus, in contrast to the only other human γ-herpesvirus Epstein–Barr virus, but its presence is mainly restricted to areas of high endemicity for classic or endemic KS (Boshoff and Weiss, 2001; Dedicoat and Newton, 2003). However, it is noteworthy that the incidence of KS has changed markedly during AIDS epidemic particularly across the African continent: before the HIV epidemic KS was a disease primarily affecting men, with extreme incidence variation among specific populations in different geographical regions. In Uganda from 1954 to 1960 and 1968 to 1970, KS represented 6.4% to 6.6% of all male cancer patients, respectively, with rare female cases (Davies et al., 1965;Templeton et al., 1972). However, in 1989 to 1991 period, KS prevalence in male cancer patients rose to 48.6% (incidence of 30.1/100,000), becoming the most frequently reported cancer in men, while prevalence in female cancer patients climbed to 17.9% (incidence of 11.0/100,000) (Wabinga et al., 1993). Since the HIV epidemic, KS has become as common in women as in men and is prevalent also in many African countries where it was almost unknown but where HHV-8 has been shown to be prevalent (Dedicoat and Newton, 2003). These observations point to a role for other factors in the aetiology of KS including the possibility that different HHV-8 variants spread during HIV/AIDS epidemic.

The aim of the present study was to analyze variations of HHV-8 genomes in tumor biopsies collected before and in the course of HIV epidemic, from patients with classic, iatrogenic, endemic as well as epidemic KS living in Africa, Europe and North America. The identification and characterization of HHV-8 variants has been focused on the highly conserved ORF 26 and T0.7, the hypervariable ORF K1 as well as on the analysis of P and M alleles of the K14.1/15 locus. Furthermore, sequence alignments and phylogenetic analyses have been performed to identify variant clusters and to determine the relationship between the HHV-8 variants identified in this study with those described previously.

Results

Epidemiological and clinical parameters of patients

This study included a total of 68 cases of cutaneous KS lesions. Fourteen cases (all in males) were endemic and 9 cases (3 in females and 6 in males) were epidemic tumor types diagnosed in Black patients from sub-Saharan African countries during 1971 to 1975 and during 1990 to 1991, respectively; the median age at the diagnosis was 49 years (mean ± SD = 49.5 ± 13.8) for endemic and 27 years (mean ± SD = 29.2 ± 8.2) for epidemic KS patients ($p<0.001$). Thirty-five cases (7 in females and 28 in males) were non-epidemic (including 33 classic and two iatrogenic tumors) and 10 cases (all in males) were epidemic KS diagnosed in Caucasian patients from Europe and North America during 1975 to 2008 and during 1990 to 1991, respectively; the median age was 75 years (mean ± SD = 70.4 ± 13.5) for classic and 40.5 years (mean ± SD = 43.0 ± 12.1) for AIDS-associated KS ($p<0.0001$).

ORF 26 and T0.7 genetic variability

PCR amplimers obtained from the 68 DNA samples were sequenced across the ORF 26 locus (nt 604 to 1602) (Chang et al., 1994) encompassing the promoter as well as the whole coding region. All differences between sample sequences are summarized in Table 1. There were 22 sequence patterns of which 12 can be distinctly identified as molecular HHV-8 subtypes because of their presence in at least three samples of this study and/or their identification in previous studies. Sequences from African patients mainly belong to the R (12 out of 23, 52.2%), B (5 out of 23, 21.7%) and J (4 out of 23, 17.4%) subtypes. Only two samples (8.7%) fall into Q subtype. Nine of the 12 samples within the subgroup designated R, coming from Africa, show an additional novel nucleotide change at position 647 (G to A). Furthermore, three out of the four B2 African samples and all samples in the J group show three novel nucleotide changes consisting of T-to-C transitions at nucleotide positions 663, 683 and 686. Only one Ugandan sample, UG-111 belongs to B1 group. Furthermore seven DNA amplimers, which presented novel sporadic nucleotide changes in the ORF 26 locus, fell into the K subclass (GR-100, G to C at nt 1055; GR-101, G to C at nt 1294, C to T at nt 1397 and C to A at nt 1735), the R subclass (UG-85, A to G at nt 1057, C to T at nt 1754; UG-107, C to A at nt 1177; UG-124, C to T at nt 1158, T to G at nt 1631; UG-915, A to G at nt 1141) and the subtype J (GR-94, G to T at nt 704 and A to C at nt 1676). Thirty-six out of 45 (80%) ORF 26 sequences obtained from European and North American patients belong to the A/C class, following the recent nomenclature by Zong et al. (2007). Only 9 out of 45 (20%) sequences clustered within other classes: one sequence from an American patient (US-217) belongs to R; one from an Italian patient (IT-226) belongs to B2; two from Greece (GR-92 and GR-94), one from Italy (IT-220) and two from USA (US-133 and US-215) belong to J class; and two from Greece (Gr-100 and GR-101) belong to K class. Notably, only 3 out of 7 (42.9%) sequences from Greek patients clustered into the A/C subtype. Forty-eight samples have been subjected to sequence analysis of a region extending 307 nucleotides beyond the stop codon within the ORF 26 locus, as recently described by Zong et al (2007). All non-A/C and non-J samples showed several additional nucleotide changes which correlate with nucleotide signature patterns identified within the ORF 26 coding region (Table 1).

Moreover, 22 DNA samples were amplified and sequenced across the T0.7 locus (648 bp). Among the 12 sequences from African patients, the majority belongs to the B1 (6 out of 12, 50%), B2 (4 out of 12, 33.3%) and B3 (1 out of 12, 8.3%) subtypes. Only one (8.3%) falls into C4 subtype. Nine of the 10 samples (90%) from Caucasian patients fall within the subgroup designated A/C, and only one (10%) belongs to the A3 class (Table 2). The GenBank accession numbers of the ORF 26 sequences are GQ252654 to GQ252673 and GU097394 to GU097415, and those of the ORF T0.7 are GU238289-GU238311.

Variability of ORF K1 and genetic linkage to ORF 26 and T0.7

Forty-two DNA samples were amplified with primer pairs specific for the K1 promoter and coding sequence (1035 bp) and PCR products were subjected to direct sequencing analysis. The complete K1 sequence was obtained from 39 samples, while a partial sequence sufficient to perform phylogenetic analysis was obtained from three samples. All K1 sequences were different from each other, exhibiting among themselves 0.3–35.3% nucleotide divergence. Phylogenetic analysis of the K1 gene was performed comparing the newly identified 42 K1 sequences with K1 reference sequences of several HHV-8 subtypes available in GenBank, including several K1 sequences of Ugandan samples from patients attending the Uganda Cancer

Table 1
Comparison of polymorphic nucleotide patterns that identify several distinctive subgroups of HHV-8 genomes within the ORF 26 gene locus. Dashes indicate identities to the prototype A1 class sequence (BCBL-R) in the top line.

Sample Name	ORF26 POLYMORPHISMS																																				ORF26 CLASS	TMP (K14.1/ K15)
	64 67	66 63	68 83	68 86	70 43	73 73	98 81	10 32	10 55	10 57	10 86	11 22	11 32	11 39	11 41	11 58	11 77	12 06	12 94	13 97	14 90	15 45	ins	ins	ins	15 68	15 69	15 94	16 31	16 76	16 84	17 17	17 23	17 35	17 54	17 95	18 02	18 16	18 25		
BCBLR	G	T	T	T	G	G	T	C	G	A	C	G	A	A	A	C	C	G	G	C	G	C	^	^	^	G	G	G	T	A	C	C	C	C	C	G	G	T	G		
SAKS21							–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-161	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
IT-164	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-165	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-166	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-169	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G			–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-170	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-171	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-172	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
IT-173	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
IT-176	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-181	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	G	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-E40	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-218	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G			–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-219	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
IT-221	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-222	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G			–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-224	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-225	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-228	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-235	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
IT-268	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
IT-272	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
IT-276	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G			–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-281	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
IT-285	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
GR-03	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	G	G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
GR-77	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G			–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
GR-93	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
US-114	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G			–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
US-123	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
US-148	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
US-150	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
US-151	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
US-183	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	A/C	P
US-187	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
US-216	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C	P
TKS11							C	–	–	–	T	––	–	C	–	–	–	–	–	–	T	–	G			–	–	T	–	–	A	T	–	–	–	–	–	–	K	M
GR-100	–	–	–	–	–	–	C	–	C	–	T	–	–	C	–	–	–	–	–	–	T	–	G	G	G	–	–	T	–	–	A	T	–	–	–	–	–	–	–	K	P
GR-101	–	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	C	T	T	–	G			–	–	T	–	–	A	T	–	A	–	–	–	–	–	K	P
SAKS24							C	-	–	–	T	T	–	C	–	–	–	–	–	–	T	–				–	–	T	–	–	A	T	–	–	–	T	–	–	–	Q	P
UG-110	–	–	–	–	–	–	C	–	–	–	T	T	–	C	–	–	–	–	–	–	T	–	G	G	G	–	–	T	–	–	A	T	–	–	–	T	–	–	–	Q	P
KE-229	–	–	–	–	–	–	C	–	–	–	T	T	–	C	–	–	–	–	–	–	T	–	G	G	G	–	–	T	–	–	A	T	–	–	–	T	–	–	–	Q	P
HKS15							C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
CM-90	–	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
CM-95	–	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
KE-234	–	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–	G			–	–	T	–	–	–	T	–	–	–	–	–	–	–	R	P
US-217	–	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
CM-116	A	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
KE-231	A	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
UG-85	A	–	–	–	–	–	C	–	–	G	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	T	–	–	–	–	R	P
UG-65	A	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
UG-107	A	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	A	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
UG-124	A	–	–	–	–	–	C	–	–	–	T	–	–	C	–	T	–	–	–	–	T	–				δ	δ	T	G	–	–	T	–	–	–	–	–	–	–	R	P
UG-206	A	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	P
UG-208	A	–	–	–	–	–	C	–	–	–	T	–	–	C	–	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
UG-915	A	–	–	–	–	–	C	–	–	–	T	–	–	C	G	–	–	–	–	–	T	–				δ	δ	T	–	–	–	T	–	–	–	–	–	–	–	R	P
HKS21							C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	J	P
GR-92	–	C	C	C	–	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–	G			–	–	–	–	–	–	–	–	–	–	–	–	–	–	J	P
GR-94	–	C	C	C	T	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–	G	G	G	–	–	–	–	C	–	–	–	–	–	–	–	–	–	J	P
IT-220	–	C	C	C	–	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	J	P
KE-230	–	C	C	C	–	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	J	P
KE-232	–	C	C	C	–	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	J	P
KE-233	–	C	C	C	–	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	J	P
UG-064	–	C	C	C	–	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	J	P
US-133	–	C	C	C	–	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	J	P
US-215	–	C	C	C	–	–	C	A	T	–	–	–	G	C	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	J	P
431K							C	–	–	–	–	–	G	C	–	–	–	–	–	–	–	–				T	A	–	–	–	–	T	–	–	C	–	C	A	B1	P
UG-111	–	–	–	–	–	–	C	–	–	–	–	–	G	C	–	–	–	–	–	–	–	–				T	A	–	–	–	–	T	–	–	C	–	C	A	B1	P
021K							C	A	–	–	–	–	G	C	–	–	–	–	–	–	–	–				T	A	–	–	–	–	T	–	–	C	–	C	A	B2	P
IT-226	–	–	–	–	–	–	C	A	–	–	–	–	G	C	–	–	–	–	–	–	–	–				T	A	–	–	–	–	T	–	–	C	–	C	A	B2	P
UG-120	–	–	–	–	–	–	C	A	–	–	–	–	G	C	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	B2	P
UG-15	–	C	C	C	–	–	C	A	–	–	–	–	G	C	–	–	–	–	–	–	–	–				.	.	.	.	.	.	.	.	.	.	.	.	.	.	B2	P
UG-18	–	C	C	C	–	–	C	A	–	–	–	–	G	C	–	–	–	–	–	–	–	–				T	A	–	–	–	–	T	–	–	C	–	C	A	B2	P
UG-80	–	C	C	C	–	–	C	A	–	–	–	–	G	C	–	–	–	–	–	–	–	–				T	A	–	–	–	–	T	–	–	C	–	C	A	B2	P

Absence of genetic variations relative to the reference is marked with dashes, whereas presence of variant nucleotides is indicated by the nucleotide corresponding letter. A dot indicates sequence not available. δ indicates nucleotide insertion; δ indicates nucleotide deletion. The numbering system conforms to that used by Zong et al. (2007).

Table 2
Comparison of polymorphic nucleotide patterns that identify distinctive subgroups of HHV-8 genomes within the T0.7 gene.

Sample name	ORF T0.7 POLYMORPHISMS																																	
	3 0	3 6	7 8	1 1 3	1 1 4	1 1 5	1 1 6	1 4 1	1 5 8	1 5 9	1 6 3	1 6 5	1 9 1	2 1 6	2 1 8	2 3 8	2 4 5	2 7 5	2 9 8	3 0 2	3 0 8	3 1 7	3 4 4	4 7 9	4 9 7	5 2 0	5 2 2	5 2 5	5 4 4	5 4 7	5 5 0	5 5 4	5 7 9	5 8 7	6 1 3	T0.7 class
BCBLR	A	G	G	G	T	C	T	G	C	C	G	A	C	A	A	7	G	T	C	C	G	C	C	C	C	A	G	T	C	T	G	G	T	C	G	A/C
GR-77	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A/C
GR-92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C
GR-94	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C
GR-101	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C
IT-220	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C
IT-224	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	A/C
US-114	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C
US-133	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C
US-215	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A/C
BCBL1	–	–	T	–	–	–	–	–		–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	C	–	–	C	–	–	–	–	A3
GR-93	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	C	–	–	C	–	–	–	–	A3
SKS1	–	–	–	–	–	–	–	–	–	–	–	–	–	G	–	–	A	C	–	–	–	–	–	–	–	G	–	C	–	C	C	–	–	–	–	C4
CM-95	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	G	–	C	–	C	C	–	–	–	–	C4
431K	–	–	–	–	–	G	–	–	–	–	C	–	–	G	G	–	A	C	–	–	–	–	–	–	T	–	–	C	–	C	C	–	G	–	–	B1
UG-107	–	–	–	–	–	G	–	–	–	–	C	–	–	G	G	–	A	C	–	–	–	–	–	–	T	–	–	C	–	C	C	–	G	T	–	B1
UG-15	–	–	–	–	–	G	–	–	–	–	C	–	–	G	G	–	A	C	–	–	–	–	–	–	T	–	–	C	–	C	C	–	G	–	–	B1
CM-116	–	–	–	–	–	G	–	A	–	–	C	–	–	G	G	–	A	C	–	–	–	–	–	–	T	–	–	C	–	C	C	–	G	–	–	B1
UG-64	–	–	–	–	–	G	–	A	–	–	C	–	–	G	G	–	A	C	–	–	–	–	–	–	T	–	–	C	–	C	C	–	G	–	–	B1
UG-65	–	–	–	–	–	G	–	A	–	–	C	–	–	G	G	–	A	C	–	–	–	–	–	–	T	–	–	C	–	C	C	–	G	–	–	B1
KE-230	–	–	–	–	–	G	–	A	–	–	C	–	–	G	G	–	A	C	–	–	–	–	–	–	T	–	–	C	–	C	C	–	G	–	–	B1
RKS3	G	–	–	–	–	–	–	–	–	T	C	–	–	G	–	–	A	C	T	A	–	T	–	T	T	–	–	C	–	C	C	–	–	–	–	B2
UG-18	G	–	–	–	–	–	–	A	–	–	C	–	–	G	–	–	A	C	T	A	–	T	–	T	T	–	–	C	–	C	C	–	–	–	–	B2
UG-80	G	–	–	–	–	–	–	A	–	–	C	–	–	G	–	–	A	C	T	A	–	T	–	T	T	–	–	C	–	C	C	–	–	–	–	B2
KE-232	G	–	–	–	–	–	–	A	–	–	C	–	–	G	–	–	A	C	T	A	–	T	–	T	T	–	–	C	–	C	C	–	–	–	–	B2
KE-233	G	–	–	δ	δ	δ	δ	–	–	–	C	–	–	G	–	–	A	C	T	A	–	T	–	T	T	–	–	C	–	C	C	–	–	–	–	B2
OKS7	G	A	–	–	–	–	–	–	–	–	C	G	A	G	–	–	A	C	–	A	A	–	–	–	T	–	–	–	–	C	C	–	–	–	–	B3
UG-110	G	A	–	–	–	–	–	–	T	–	C	G	–	G	–	–	A	C	T	A	–	–	T	–	T	–	–	–	T	C	C	T	–	–	–	B3

Absence of genetic variations relative to the reference is marked with dashes, whereas presence of variant nucleotides is indicated by the nucleotide corresponding letter; δ indicates nucleotide deletion. The numbering system and class designation conforms to that used by Poole et al. (1999), Kakoola et al. (2001) and Zong et al. (2002).

Institute in Kampala in 2001 (Kajumbula et al., 2006). Fig. 1 shows the neighbor-joining tree of all sequences including the already known reference subtypes (A, B, C, D, E and F), clearly identified on the basis of consistent topological associations and bootstrap values above 90%. Among the African K1 sequences, 11 were identified as A5 (52.4%), four were identified as B3 (19%), three as B1 (14.3%), two as C (9.5%) and one sequence as subtype F (4.8%). Among the European and North American K1 sequences, 14 were identified as A (66.7%) and seven as C (33.3%) subtypes. Other K1 variants (D, E and Z) were not detected.

The GenBank accession numbers of the 42 new ORF K1 sequences are FJ884606 to FJ884626 and GU097416 to GU097435.

K14.1/15 P and M genotypes

K14.1/15 genomic region at the right-hand side of HHV-8 genomes has been amplified in all 68 samples using a triple primer PCR set, covering the divergent junction of the two subtypes of K15 gene, designed to distinguish the P (362 bp) from the M (450 bp) allele on the basis of the amplified product size; a further specific primer set has been used to amplify K15 M (370 bp) (Poole et al., 1999). All samples except one had the P allele. The single K14.1/15 M allele was identified in a sample obtained from a Cameroonian patient. These results are in agreement with previous studies showing a predominance of P allele in most African, European and North American geographical regions and the segregation of M allele mainly in Western Africa countries. The 362-bp K14.1/15 amplimers, obtained from 11 samples including four classic and two epidemic KS from European, three endemic and two epidemic KS from African patients, as well as the 450-bp M allele amplimer of a single Cameroonians sample, were subjected to sequence analysis. All P allele sequences, except one, were 100% identical to the reference strain U85269 (P) in the analyzed sequence. A single Ugandan sequence (UG-65) showed two synonymous nucleotide changes at nucleotide positions 238 (T to C) and 246 (G to C) and one non-synonymous nucleotide substitution at position 273 (T to C). The M allele of CM-95 sample was identical to the reference sequence U75698 (M).

HHV-8 variant distribution in endemic, classic, iatrogenic and epidemic KS

Among the African cases 14 samples (11 from Uganda and 3 from Cameroon) were endemic KS obtained from skin biopsies during 1971–1975 period before the AIDS epidemic (Levy et al., 1986) and 9 (3 from Uganda and 6 from Kenya) were HIV-associated KS obtained during 1990–1991 period (Table 3). Using prevalently the ORF 26 subtype distribution, there was not a significant difference in the distribution of R, J and Q genotypes between endemic (50%, 7.1% and 7.1% respectively) and epidemic KS (55.5%, 33.3% and 11.1%, respectively). Subtype B was identified only in endemic KS (35.8%) and was not identified in the few KS epidemic samples obtained from Uganda, although B2 subtype was present in more than 50% of samples from Ugandan patients enrolled at the University of Kampala in 2001 (Kajumbula et al., 2006).

Twenty-eight classic KS from Italy and USA contained, as expected, A/C subtype, only the samples IT-226 and US-133 contained B2 and J subtype, respectively. A major divergence was observed among the seven Greek samples which were 42.8% within A/C, 28.6% within K and 28.6% within J subtype. A more heterogeneous distribution was observed among epidemic KS: 7 out of 10 (70%) belonged to A/C, 2 to J and 1 USA sample belonged to R subtype.

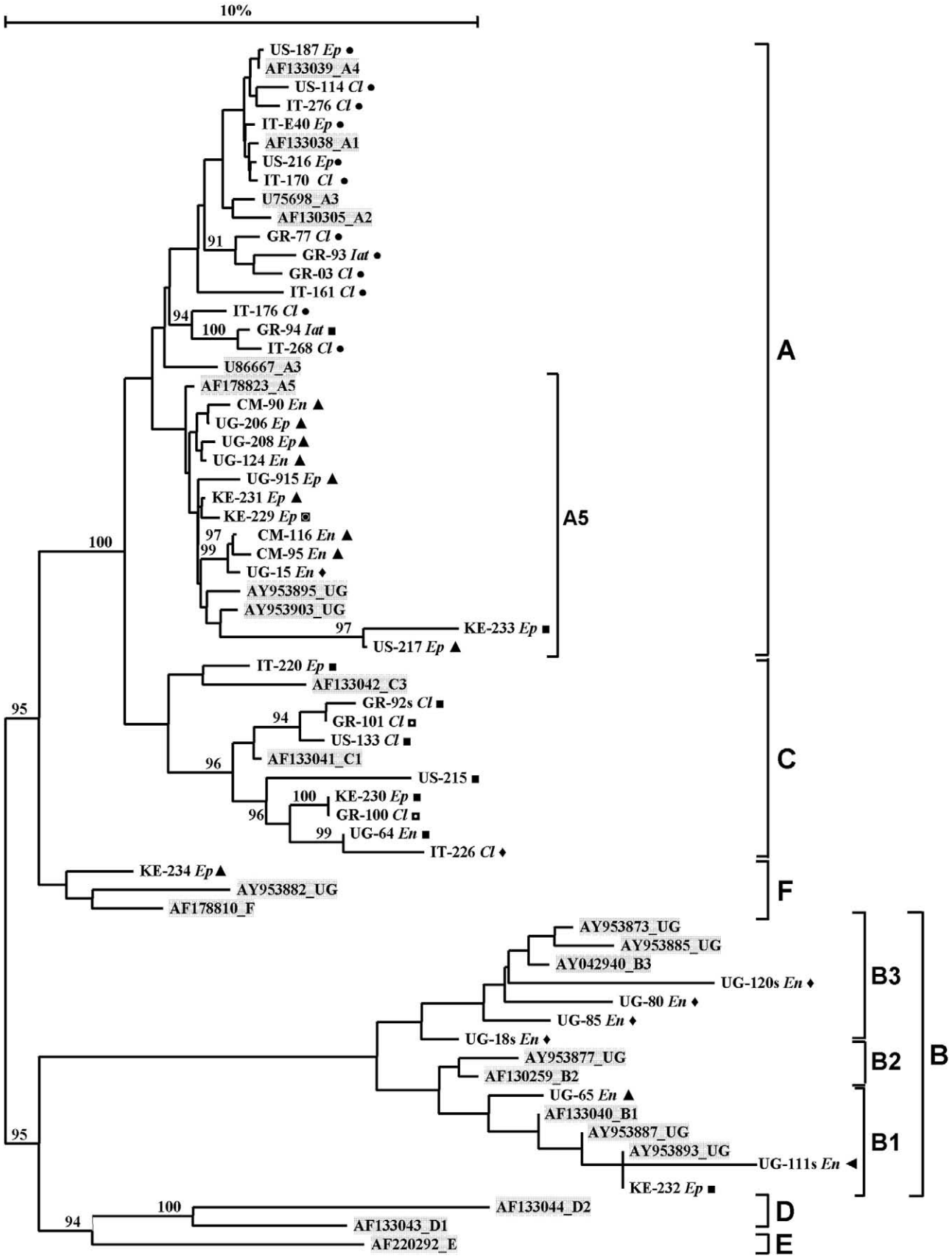

Fig. 1. Phylogenetic tree based on K1 sequences constructed with the TREECON software. Samples designated CM, GR, IT, KE, UG and US are from Cameroon, Greece, Italy, Kenya, Uganda and Unites States, respectively (present study). Samples designated with a GenBank numbers followed by UG are from Uganda (Kajumbula et al., 2006). AF1133038, AF130305, U86667, AF133039, AF178823, AF133040, AF130259, AY042940, AF133041, AF133042, AF133043, AF133044, AF220292 and AF178810 have been reported in the GenBank as reference sequences of A1, A2, A3, A4, A5, B1, B2, B3, C1, C3, D1, D2, E and F subtypes, respectively. Sample designation *Cl*, *En*, *Ep* and *Iat* indicates classic, endemic, epidemic and iatrogenic KS, respectively. Corresponding subtypes of the ORF 26 has been also reported near the sequence name: ●, A/C; ■, J; , K; , Q; ▲, R; ♦, B2; ◄, B1. Numbers above the branches indicate the bootstrap values that are grater than 90%.

Table 3
Clinical and epidemiological features of the patients included in the study and designation of HHV-8 variants of ORF 26, T0.7, K1 and K14.1/K15 amplimers.

Region	Year of diagnosis	Sample ID	Age	Sex	HIV/Risk factors	Country	Diagnosis	ORF K1 class	ORF 26 class	T0.7	K14.1/K15
Africa	1971	UG-15	55	M	−	Uganda	Endemic	A5	B2	B1	P
	1971	UG-18	35	M	−	Uganda	Endemic	B3[a]	B2	B2	P
	1972	UG-64	70	M	−	Uganda	Endemic	C	J	B1	P
	1972	UG-65	67	M	−	Uganda	Endemic	B1	R	B1	P
	1973	UG-80	34	M	−	Uganda	Endemic	B3	B2	B2	P
	1973	UG-85	35	M	−	Uganda	Endemic	B3	R		P
	1974	CM-90	60	M	−	Cameroon	Endemic	A5	R		P
	1974	CM-95	58	M	−	Cameroon	Endemic	A5	R	C4	M
	1974	UG-107	49	M	−	Uganda	Endemic		R	B1	P
	1974	UG-110	42	M	−	Uganda	Endemic		Q	B3	P
	1974	UG-111	34	M	−	Uganda	Endemic	B1	B1		P
	1975	UG-120	66	M	−	Uganda	Endemic	B3	B2		P
	1975	UG-124	39	M	−	Uganda	Endemic	A5	R		P
	1975	CM-116	37	M	−	Cameroon	Endemic	A5	R	B1	P
	1990	UG-206	20	F	+	Uganda	Epidemic	A5	R		P
	1990	UG-208	36	M	+	Uganda	Epidemic	A5	R		P
	1990	UG-915	25	M	+	Uganda	Epidemic	A5	R		P
	1991	KE-229	21	F	+	Kenya	Epidemic	A5	Q		P
	1991	KE-230	34	M	+	Kenya	Epidemic	C	J	B1	P
	1991	KE-231	32	M	+	Kenya	Epidemic	A5	R		P
	1991	KE-232	45	M	+	Kenya	Epidemic	B1	J	B2	P
	1991	KE-233	27	F	+	Kenya	Epidemic	A5	J	B2	P
	1991	KE-234	23	M	+	Kenya	Epidemic	F	R		P
Europe	1983	IT-161	54	M	−	Italy	Classic	A3	A/C		P
	1983	IT-164	20	M	−	Italy	Classic		A/C		P
	1984	IT-165	75	M	−	Italy	Classic		A/C		P
	1984	IT-166	65	M	−	Italy	Classic		A/C		P
	1984	IT-169	83	M	−	Italy	Classic		A/C		P
	1984	IT-170	56	M	−	Italy	Classic	A1	A/C		P
	1984	IT-171	63	F	−	Italy	Classic		A/C		P
	1985	IT-172	75	F	−	Italy	Classic		A/C		P
	1985	IT-173	71	F	−	Italy	Classic		A/C		P
	1985	IT-176	68	M	−	Italy	Classic	A	A/C		P
	1990	IT-181	32	M	+	Italy	Epidemic		A/C		P
	1990	IT-E40	42	M	+/Hemoph	Italy	Epidemic	A4	A/C		P
	1991	IT-218	65	M	−	Italy	Classic		A/C		P
	1991	IT-219	37	M	+	Italy	Epidemic		A/C		P
	1991	IT-220	39	M	+	Italy	Epidemic	C3	J	A/C	P
	1991	IT-221	66	M	−	Italy	Classic		A/C		P
	1991	IT-222	74	M	−	Italy	Classic		A/C		P
	1991	IT-224	74	M	−	Italy	Classic		A/C	A/C	P
	1991	IT-225	78	M	−	Italy	Classic		A/C		P
	1991	IT-226	79	M	−	Italy	Classic	C	B2		P
	1991	IT-228	65	M	+	Italy	Epidemic		A/C		P
	1992	GR-03	77	M	−	Greece	Classic	A3	A/C		P
	1992	GR-77	88	M	−	Greece	Classic	A3	A/C	A/C	P
	1992	GR-92	81	M	−	Greece	Classic	C1	J	A/C	P
	1992	GR-93	47	M	Transplanted	Greece	Iatrog	A3	A/C	A3	P
	1992	GR-94	53	M	Transplanted	Greece	Iatrog	A3	J	A/C	P
	1992	GR-100	78	M	−	Greece	Classic	C	K		P
	1992	GR-101	65	F	−	Greece	Classic	C1	K	A/C	P
	1996	IT-235	80	F	−	Italy	Classic		A/C		P
	2002	IT-268	80	M	−	Italy	Classic	A3	A/C		P
	2002	IT-272	75	F	−	Italy	Classic		A/C		P
	2003	IT-276	76	M	−	Italy	Classic	A4	A/C		P
	2004	IT-281	80	M	−	Italy	Classic		A/C		P
	2008	IT-285	70	F	−	Italy	Classic		A/C		P
North America	1975	US-114	76	M	−	USA	Classic	A4	A/C	A/C	P
	1975	US-123	87	M	−	USA	Classic		A/C		P
	1976	US-133	81	M	−	USA	Classic	C1	J	A/C	P
	1977	US-148	49	M	−	USA	Classic		A/C		P
	1977	US-150	79	M	−	USA	Classic		A/C		P
	1990	US-151	76	M	−	USA	Classic		A/C		P
	1990	US-183	52	M	+	USA	Epidemic		A/C		P
	1990	US-187	59	M	+	USA	Epidemic	A4	A/C		P
	1990	US-215	43	M	+	USA	Epidemic	C	J	A/C	P
	1990	US-216	29	M	+	USA	Epidemic	A1	A/C		P
	1990	US-217	32	M	+	USA	Epidemic	A5	R		P

[a] Phylogeny determined on K1 partial sequence.

The phylogenetic analysis of three regions (ORF 26, T0.7 and K1) allowed the identification of 9 genetic patterns all present within the 12 genotype patterns defined by Hayward and Zong (2007). Thus, viral gene chimerism does not seem to be a currently frequent phenomenon in strain reassortment. Moreover, based on the analysis of ORF 26, T0.7 and K1 variability, there was no clear molecular aggregate reflecting the period of tumor collection or clinical-epidemiological type of KS.

Table 4
PCR primer sequences used to amplify HHV-8 genome regions.

Locus	Primer name	Sequences (5′–3′)	Nucleotide position	Amplicon size (bp)	References
ORF 26_{233}	$KS26_{233}A$	AGCCGAAAGGATTCCACCAT	47,386–47,405	233 bp	Chang et al. (1994)
	$KS26_{233}B$	TCCGTGTTGTCTACGTCCAG	47,599–47,618		
ORF 26	KS26/D	GCTAACCCTTCTAGCGTTGGC	47,006–47,026	999 bp	This study
	KS26/E	CTTGCAACCGACAGAATATCAG	47,983–48,004		
ORF 26	LGH2575-R	GTGCTTGACGATCTGTCC	47,638–47,655	620 bp	Zong et al. (2007)
	LGH2574-L	CAGAAACAGGGCTAGGTAC	48,239–48,257		
K1	LGH2089	GTTCTGCCAGGCATAGTC	21–38	1035 bp	Poole et al. (1999)
	LGH2088	AATAAGTATCCGACCTCAT	1,037–1,055		
K1-5′	K1 A	TTTGTGGCGGCCCTTGTGTA	43–62	522 bp	This study
	K1 B	ACCCCTCAGTTTGGCTCATCAG	543–564		
K1-3′	K1 C	GTGTGCACAGCCATCAA	401–417	689 bp	This study
	K1 D	TTTTAATAAGTATCCGACCTCA	1,068–1,089		
T0.7/K12-5′	LGH2076	GCTGCAATGTACTGCCATG	117,288–117,306	485 bp	Zong et al. (2002)
	LGH2017	CATTCAGGACACGAGTTGC	117,754–117,772		
T0.7/K12-3′	LGH2018	TGCTTTAATGCGGAGAGG	117,538–117,555	398 bp	Zong et al. (2002)
	LGH2075	CTCCAATCCCAATGCATGGA	117,916–117,935		
K14.1 (P)	LGH2079	GAGATCACTCTCCAACCAC	134,548–134,566	363 bp	Poole et al. (1999)
	LGH2033	GGAGTGCCTTCCGTATAG	134,892–134,909		
K14.1 (M)	LGH2079	GAGATCACTCTCCAACCAC	134,400–134,418	450 bp	Poole et al. (1999)
	LGH2506	CACAGTCACCTATGCTAG	134,832–134,849		
K15 (M)	LGH2473	CATGCAGCGAGCTTGAGA	136,045–134,062	370 bp	Poole et al. (1999)
	LGH2474	CTTTGAGTACTGTTTGTG	136,397–136,414		

Nucleotide positions of HHV-8 ORF 26, K1 and K14.1/15 (P) primers are given on the GenBank sequence number NC_009333 and K14.1/15 (M) on the GenBank sequence number NC_003409. Nucleotide positions of HHV-8 T0.7 are given on the GenBank sequence number GQ_994935.1.

Discussion

Before the onset of the AIDS epidemic in the early 1980 s, KS showed extreme geographical variation in incidence even within the African continent, with particularly high rates in Zaire, Uganda and Tanzania (Cook-Mozaffari et al., 1998). Seroepidemiological studies, however, conducted on samples collected before AIDS epidemic showed that several populations in sub-Saharan African have had also in the past high HHV-8 seroprevalence but endemic KS was confined primarily to East and Central Africa and variably in West Africa (Dedicoat and Newton, 2003). Several studies have analyzed the genetic variation of HHV-8 and have mapped the ethno-geographic distribution of major clades (Hayward and Zong, 2007), but few reports are available on HHV-8 variant distribution before the AIDS epidemic, particularly in geographical regions where major changes in KS incidence have occurred in association with HIV infection.

In this study, the genetic variations of three different genomic loci of HHV-8, the conserved ORF 26 and T0.7 and the hypervariable K1 region as well as the P or M alleles of the K14.1/K15 junction segment have been analyzed in endemic, classic, iatrogenic and epidemic KS in patients from sub-Saharan Africa, Italy, Greece and USA. The endemic African KS were diagnosed during 1971–1975, in a period preceding AIDS epidemic (Levy et al., 1986), and AIDS-associated KS diagnosed during 1990–1991. Based on the complete sequence of ORF 26 and according to the classification of Zong et al. (2007), subtype B2 was mainly detected in endemic KS, while R subtype, former C3, followed by J where similarly represented in both tumor types. The absence of ORF 26 subtype B2 in our epidemic KS, however, may be merely due to the underrepresentation of Ugandan epidemic KS versus the higher number of Kenyan cases. In a recent study performed on HHV-8-positive DNA samples isolated from peripheral blood of 31 Ugandan patients with epidemic KS, indeed the B2 and the R subtype of the ORF 26 predominated across 11 Ugandan ethnic group with no evidence of distinct ethnic or geographic distribution (Zong et al., 2007; Kajumbula et al., 2006). Moreover, the ORF 26 J subtype, previously identified among two subjects of Ganda tribe (Zong et al., 2007; Kajumbula et al., 2006), was also found among one endemic Ugandan KS and three epidemic Kenyan KS samples in the present study. The majority of African samples, including the four ORF 26 J variants, were found to belong to ORF T0.7 B1 and B2 subtypes, which have been shown to predominate in sub-Saharan Africa (Zong et al., 2002).

Phylogenetic studies based on the complete K1 gene indicate that A5, B3 and B1 variants were present among epidemic KS and, in agreement with previous results by Kakoola et al. (2001), the A5 and B3 were predominating. One sample from a Kenyan epidemic KS (KE-234) clustered with samples K1–43/Berr (France) (Lacoste et al., 2000), San2 (Botswana) (Whitby et al., 2004) and HKS22 (Uganda) (Kajumbula et al., 2006) within the new subtype F, contributing to the designation of this subtype as a new African variant. Comparison of the subtype assignments at the conserved ORF 26 and hyper variable K1 region resulted in a strong linkage between the two loci: all K1 B3 and all A5 sequences, except one, were linked to B2 ORF 26 subtype. All African viral genomes but one had the P allele at the K14.1/15 loci and, as expected, the single M allele was present in a tumor sample from a Cameroonian patient. These results are in agreement with previous studies reporting a high prevalence of P allele in Central-Eastern Africa and an equal representation of M and P alleles in Central-Western-African regions (Lacoste et al., 2000;Poole et al., 1999;Kakoola et al., 2001;Kasolo et al., 2007;Zong et al., 2002). Thus, HHV-8 subtype distribution in Sub-Saharan Africa, mainly Uganda, is suggestive of a conserved pattern of HHV-8 variants which has not changed during AIDS epidemic.

Moreover, HHV-8 variants ORF 26 A/C, T0.7 A/C and K1 A broadly predominated in classic and epidemic KS from Italian and USA patients diagnosed during 1975–2008. Only one classic (US-133) and one epidemic KS (IT-220) showed ORF 26 J, linked to T0.7 A/C and K1 C subtype. Moreover, among Greek cases 57% contained ORF 26 A/C and 43% J variants and all but one showed ORF T0.7 A/C subtype. All the samples were positive for P allele at K14.1/15 locus in concordance with the low prevalence of M alleles among French KS patients (Lacoste et al., 2000). Higher prevalence of M alleles has been reported in patients from Scandinavia (2 out of 5 classic KS cases with K15 M allele virus, 40%), from Israel (1 out of 4 classic KS with K15 M allele virus, 25%) and from Saudi Arabia (2 out of 7 iatrogenic KS with K15 M allele virus, 28.6%) (Poole et al., 1999; Zong et al., 2002). Moreover, among Russian KS patients, a frequency of 57% (4 out of 7 cases) and 28% (9 out of 32 cases) of K15 M allele virus was reported by Lacoste et al. (2000) and Kadyrova et al. (2003), respectively.

In a previous study including classic and AIDS-associated KS primarily in patients from Tennessee and Maryland (USA), Zong et al. (2002) reported the identification of a considerable number of M alleles. This observation could have been due to different ethnic background of patients, indeed Zhang et al. (2001), who reported a prevalence of 56% K15 M and 44% K15 P genotypes among patients with KS in South Texas, presumably linked to the large predominance of Hispanic population in this region.

Several studies have reported that HHV-8 subtype K1 A display more aggressive characteristics of pathogenicity in HIV-associated KS (Boralevi et al., 1998). In a recent studies on classic KS, it has been reported that subtype A was significantly more frequently isolated in patients affected by classic KS with high viral load and fast progression than in individuals with low viral load and slow progression in which subtype C was prevalent (Mancuso et al., 2008). Conversely HHV-8 subtype E, identified among Amerindians tribes with a 53% HHV-8 seroprevalence, seems to be not associated with KS development given the very low incidence of the tumor in this population (Biggar et al., 2000). However, in other studies, the correlations between genotype and HHV-8-related pathologies with a different degree of aggressiveness were not confirmed (Cook et al., 1999;Lacoste et al., 2000). Paradigmatic is the observation that K1 A5 and B subtypes, prevalent in Uganda, were also hyperendemic in Botswana (Whitby et al., 2004), a country with high prevalence of HHV-8 infection and low incidence of KS, suggesting that besides HHV-8 infection, a still unknown cofactor is necessary for KS development.

HHV-8 infection and KS development have also been linked to a predisposing genetic background. Some studies have shown that variability in alleles of the human leukocyte antigen (HLA) complex, particularly those encoding HLA-DR, may influence response to infection with several human pathogens and have been suggested as markers of susceptibility to KS development (Gaya et al., 2004). This observation suggests the need for evaluating the risk of progression associated with HHV-8 variants in a larger study population along with determination of HLA haplotypes and HLA-DR polymorphisms.

In conclusion, this study adds new insight to the current knowledge of worldwide HHV-8 molecular epidemiology by providing the first in depth analysis of the viral genetic variability in African patients diagnosed with epidemic KS and with endemic KS prior of AIDS epidemic and in North America and European patients with classic and with epidemic KS. Although the virus has genetic regions of high variability, approaching that of HIV-1 *env* gene, the HHV-8 subtypes remained stably distributed before and after the AIDS epidemic. These results suggest that the increased incidence of epidemic KS in low-incidence countries was not related to the spreading of high pathogenic HHV-8 variants and furthermore suggest the presence of other cofactors in high-risk KS countries pre-existing in the pre-AIDS era.

Materials and methods

Kaposi's sarcoma patients and DNA sample isolation

All tissue specimens were obtained from KS skin lesions and preserved in liquid nitrogen at −192 °C. African samples were from the University Centre for Health Sciences, Yaounde, Cameroon ($n=3$ endemic), from the Pathology Department of Nairobi University in Kenya ($n=6$ epidemic) and from the Mulago Hospital and Makerere University in Kampala, Uganda ($n=11$ endemic and $n=3$ epidemic). European cases were from Pathology Department of Athens University Medical School, Greece ($n=5$ classic and $n=2$ iatrogenic), from the Institutes of Dermatological Sciences, University and IRCCS Ospedale Maggiore of Milan in North Italy ($n=7$ classic, and $n=3$ epidemic) and from the Dermatology Department of Second University of Naples, in South Italy ($n=16$ classic and $n=2$ epidemic); North American cases ($n=6$ classic and $n=5$ epidemic) were from New York's Sloan-Kettering/Memorial Hospital New York City, USA. DNA from frozen biopsies was extracted by proteinase K treatment (150 μg per ml at 60 °C for 30 min) in lysis buffer (10 mM Tris–HCl pH 7.6, 5 mM EDTA, 150 mM NaCl, 1% SDS), followed by phenol and phenol–chloroform–isoamyl alcohol (25:24:1) DNA extraction and ethanol precipitation in 0.3 M sodium acetate (pH 4.6). Genomic DNA from 61 cases was previously extracted and subjected to PCR amplification with oligonucleotides amplifying a 233-bp DNA fragment within the HHV-8 ORF 26 (Buonaguro et al., 1996;Chang et al., 1994). Six previously uncharacterized tumors from patients undergoing surgical resection from 1996 to 2008 were extracted and included in this study. DNA quality test was carried out on all DNA samples by amplification with specific oligoprimers targeting a fragment within the exon 7 of *TP*53 gene (Buonaguro et al., 1996), and DNA quantity analysis, evaluated by spectrophotometric measurements, provided data that all samples were suitable for the viral DNA amplifications.

PCR amplification of ORF 26, K1 and K14.1/15 region

Five segments of the HHV-8 genomes were amplified by PCR: (1) ORF 26 (999 bp); (2) K1 (1068 bp); (3) ORF T0.7 (648 bp) ; (4) K14.1/K15 (P) (363 bp) and K14.1/K15 (M) (450 bp), covering the divergent K14.1/K15 junction of the two alleles; and (5) K15 M allele (370 bp). Primers and nucleotide positions are listed in Table 4. PCR amplification reactions were performed in 50 L reaction mixture containing 100 to 300 ng of target DNA, 20 pmol of each primer, 50 mM KCl, 2.5 mM $MgCl_2$, 100 mM Tris–HCl pH 8.3, 0.1% Triton X-100, 50 mM of each dNTP and 1.8 U of thermostable AmpliTaq DNA polymerase (Applied Biosystems, Foster City CA). DNA was amplified in a Perkin-Elmer GeneAmp PCR System 9700 thermal cycler with the following steps: an initial 1-min denaturation at 94 °C, followed by 40 cycles of 55 °C for 1 min, 72 °C for 2 min, 94 °C for 30 s and a final annealing at 55 °C for 1 min with 5-min elongation at 72 °C. A reaction mixture containing genomic DNA, extracted from NIH 3T3 murine cell line, was used as negative control and was included in every set of 5 clinical specimens.

Nucleotide sequencing and phylogenetic analysis

PCR amplification products were extracted with phenol and chloroform-isoamyl alcohol and purified by precipitation at 37 °C for 15 min in 1.25 M NaCl with 10% polyethylene glycol (PEG 6000) (Tornesello et al., 2000). Purified DNA samples were subjected to direct nucleotide sequencing (Winship, 1989). Briefly DNA samples (30–100 ng) were denatured at 95 °C, in presence of 10% DMSO, immediately cooled in liquid nitrogen and subsequently sequenced with the Sequenase 2.0 kit according to manufacturer's instructions (GE Healthcare) modified in the labeling step (3 min on ice). Aliquots of PCR amplified products were further sequenced on both strands by Primm Srl Laboratorie (Milan, Italy) using the fluorescent dye terminator technology and ABI 3730 DNA sequencers (Applied BioSystems, Foster City, CA). Nucleotide sequences were edited with Chromas Lite 2.01 (http://www.technelysium.com.au/chromas.html) and converted to FASTA format.

Multiple sequence alignments of HHV-8 sequences from the present study and reference strains reported in the GenBank were performed with clustal W tool of MegAlign program of the Lasergene software (DNASTAR Inc., V7.0.0). Phylogenetic trees were constructed transforming the aligned sequence data into a distance matrix by the Kimura's two parameter model (Kimura, 1980), followed by the neighbor-joining bootstrap analysis (Saitou and Nei, 1987), which was executed with the TREECON software package (Van de and De, 1994). Boot strapping, with 1000 replicates confirmed the robustness of the three major branches with bootstrap values above 90%.

Statistical analysis

The data were analyzed with Epi Info 6 Statistical Analysis System Software (Version 6.04b, 1997, Centers for Disease Control and Prevention, USA). Unpaired t test was used for comparisons of continuous variables (i.e. age); Yates-corrected χ^2 test and, where appropriate, two-sided Fisher's exact test were used for comparison of categorical data. Differences were considered to be statistically significant when p values were less than 0.05.

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