Kaposi's Sarcoma-Associated Herpesvirus/Human Herpesvirus Type 8-Positive Solid Lymphomas

A Tissue-Based Variant of Primary Effusion Lymphoma

Antonino Carbone,* Annunziata Gloghini,*
Emanuela Vaccher,[†] Michaela Cerri,[‡]
Gianluca Gaidano,[‡] Riccardo Dalla-Favera,[§] and
Umberto Tirelli[†]

From the Divisions of Pathology and Medical Oncology A,[†] Centro di Riferimento Oncologico, Istituto Nazionale Tumori, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Aviano, Italy; the Hematology Unit,[‡] Department of Medical Sciences, "Amedeo Avogadro" University of Eastern Piedmont, Novara, Italy; and the Institute of Cancer Genetics,[§] Columbia University, New York, New York*

Kaposi's sarcoma-associated herpesvirus (KSHV), also termed human herpesvirus type 8, is consistently identified in Kaposi's sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman's disease. Here we report four cases of KSHV-bearing solid lymphomas that occurred in AIDS patients (cases 1 to 3) and in a human immunodeficiency virus (HIV)-seronegative person (case 4). The patients presented extranodal masses in the abdomen (cases 1, 3, and 4) or skin (case 2), and nodal involvement, together with Kaposi's sarcoma (case 3). The gastrointestinal tract was involved in two patients (cases 1 and 3). The patients did not develop a lymphomatous effusion. KSHV was detected in the tumor cells of all cases by immunohistochemistry and by polymerase chain reaction. Epstein-Barr virus was detected in two of the HIV-related cases. All KSHV-positive solid lymphomas exhibited PEL-like cell morphology. To investigate the relationship of these disorders to PEL and to other AIDS-associated diffuse large cell lymphomas, KSHV-positive solid lymphomas were tested for the expression of a set of genes that were previously shown by gene profiling analysis to define PEL tumor cells. The results showed that expression of this set of genes in KSHV-positive lymphomas is similar to that of PEL but distinct from KSHV-negative AIDS-associated diffuse large cell lymphomas. Because pathobiological features of KSHV-positive solid lymphomas closely mimic those of PEL, our results suggest that KSHV-positive solid lymphomas should be considered as a tissue-based variant of classical PEL, irrespective of HIV status. (*J Mol Diagn* 2005, 7:17-27)

Kaposi sarcoma-associated herpesvirus (KSHV) was initially discovered in tissue biopsies of AIDS-related Kaposi's sarcoma (KS),¹ a tumor consistently infected by this virus. KSHV, also known as human herpesvirus 8 (HHV8),² is related to another human γ -herpesvirus, Epstein-Barr virus (EBV). Like EBV, KSHV is lymphotropic. Besides KS, KSHV has been shown to associate with three distinct lymphoproliferative disorders occurring most commonly in persons with human immunodeficiency virus (HIV) infection/AIDS:² primary effusion lymphoma (PEL),³ multicentric Castleman disease (MCD),⁴ and MCD-associated plasmablastic lymphoma.^{5,6} A new KSHV-associated lymphoproliferative disorder has recently been described in HIV-seronegative persons.⁷ This disease, termed "germinotropic lymphoproliferative disorder," is characterized by plasmablasts that are co-infected by KSHV and EBV and preferentially involve the germinal centers of lymph nodes.

Recently, KSHV has been detected by immunohistochemistry and/or polymerase chain reaction in lymphoma cases presenting as tissue masses in persons with MCD^{6,8-10} or AIDS-associated KS.¹⁰⁻¹⁴ These lymphomas were described histologically as diffuse large cell lymphoma (DLCL) or immunoblastic lymphoma with a PEL-like morphology.^{6,8,14} Previous studies have reported that similar to other AIDS-related non-Hodgkin's lymphomas (NHL), lymphomas containing KSHV can present as solid tumors in patients without other KSHV-associated disorders.^{2,12,15-17} All these KSHV-positive solid lymphomas were usually extranodal and extracavitary, although a lymphomatous effusion developed during follow-up in some cases.^{2,12} Thus, KSHV-positive solid lymphomas may represent a distinct category that is frequently, but not exclusively, associated with other KSHV-associated disorders.

Although most KSHV-positive solid lymphomas are morphologically reminiscent of PEL, it is unclear whether

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Address reprint requests to Antonino Carbone, M.D., Division of Pathology, Centro di Riferimento Oncologico, Istituto Nazionale Tumori, IRCCS, via Pedemontana Occidentale, Aviano I-33081, Italy. E-mail: acarbone@cro.it.

Table 1. Epidemiological and Clinical Features of Patients with KSHV-Associated Solid Lymphomas

	Case 1	Case 2	Case 3	Case 4
Age/sex	39/M	24/F	41/M	75/M
HIV-serology (risk group)	Positive (homosexual)	Positive (heterosexual)	Positive (IVDU)	Negative
HIV disease*	A3	A2	C3	—
KSHV-related disease	—	—	KS	—
CD4 cell count/ μ L	20	282	104	470
HIV-RNA cp/mL	N/E	125.155	<50	—
PS (ECOG)	4	4	3	3
Stage (A. Arbor)	IVB	IVB	IVB	IVB
B-symptoms	High fever	High fever	High fever, night sweats	Severe weight loss
Site of involvement	Ln, GI-tract (duodenum, colon), pancreas	Ln, skin	Ln, GI-tract (ileum), Waldeyer's ring	Ln, Waldeyer's ring, soft tissue, spleen
Bulky disease	Present	—	—	Present
Major laboratory abnormalities:				
Severe anemia (≤ 8.0 g%)	Present	Present	Present	—
Thrombocytopenia ($\leq 100,000/\mu$ L)	Present	Present	Present	—
Coomb's test	Positive	Positive	Positive	Negative
Abnormal LDH serum level (≥ 460 U/mL)	Present	Present	Present	Present
Hypoalbuminemia (≤ 3 g%)	Present	Present	Present	Present
Therapy	CHOP _{LD} [†]	VCR-BLM [†]	CHOP _{LD} [†]	CHOP [§]
Outcome (days)	Died (15)	Died (25)	Died (45)	Died (55)
Cause of death	NHL	NHL	NHL	NHL

IVDU, intravenous drug user; KS, Kaposi's sarcoma; N/E, not evaluable.

*According to CDC '92; [†]cyclophosphamide, doxorubicin, vincristine, predmistine (CHOP) 50% of dose; [‡]vincristine, bleomycin; [§]CHOP standard dose.

they are pathobiologically and clinically related to this entity. To clarify this issue, we performed a comparative analysis of the clinical and biological features of KSHV-positive solid lymphomas, PEL, and KSHV-negative AIDS-related DLCL. We report that KSHV-positive solid lymphomas express several cellular genes identified as PEL-specific by gene expression profiling, and therefore may be regarded as a solid variant of PEL.

Materials and Methods

Case History

Major epidemiological and clinical features are summarized in Table 1. HIV infection was present in three of four cases (two with asymptomatic HIV-infection and one with previous KS). Informed written consent was obtained from the patients, and tissue collection was approved by the institutional review board. All patients presented poor general conditions (performance status, 3 to 4) and severe systemic symptoms, including high fever (39 to 40°C), night sweats, and weight loss (15 kg in 1.5 months). Advanced stage disease (stage IV) occurred in all cases. Tumor localization included both generalized lymphadenopathy and multiple extranodal involvement. Gastrointestinal tract and Waldeyer's ring were the most common extranodal sites. No patient developed lymphomatous effusion. Abnormal lactate dehydrogenase serum level, hypoalbuminemia and autoimmune anemia, and thrombocytopenia were detected in all except case 4. The HIV-positive patients died after one course of low-dose chemotherapy for disease progression. The median

survival was only 22 days. Autopsy was performed in patient 1. The HIV-negative patient died 55 days after the onset of the disease for disease progression. He received one course of full-dose chemotherapy.

Neoplastic Samples

The three cases of KSHV-positive solid lymphomas developing in HIV-positive patients belonged to a single institution series of 56 cases of systemic AIDS-DLCL. All cases were reviewed (by A.C. and A.G.) for the purpose of this study and were classified according to the 2001 World Health Classification of Tumors of Hematopoietic and Lymphoid Tissues.¹⁸ The panel included 15 centroblastic AIDS-DLCL and 38 immunoblastic AIDS-DLCL. Ten AIDS-PEL were also included in the study for clinical purposes (see Clinical Comparison section). Detailed phenotypic characterization of these cases has been reported previously.¹⁹ AIDS-PEL was classified on the basis of the peculiar clinicopathological and virological (eg, KSHV positivity) characteristics of the lymphoma.^{2,3,20,21} Tissues were fixed in Bouin solution or neutral buffered formalin. In most cases, a portion of unfixed tissue was snap-frozen in liquid nitrogen and stored at -80°C .

Analysis of Viral Infection

The samples from all of the cases included in the study were subjected to determination of tumor infection by KSHV and EBV. The presence of KSHV was ascertained by immunohistochemistry using a rat monoclonal anti-

body against the KSHV latency-associated nuclear antigen encoded by viral open reading fragment (ORF) 73 (Advanced Biotechnologies, Columbia, MD). Positive cases were also tested for viral interleukin-6 (vIL-6), a KSHV cytokine homologue, by using a rabbit polyclonal antibody (Advanced Biotechnologies). Paraffin-embedded tissue sections were pretreated in a microwave oven at 250 W for 30 minutes in EGTA solution (1 mmol/L, pH 8), and then immunostained on an automated immunostainer (Nexes; Ventana Medical Systems, Inc., Tucson, AZ) according to a modified version of the company's protocols. Negative controls, which were invariably negative, consisted of omission of the primary antibody and substitution with phosphate-buffered saline. Positive controls for ORF73 consisted of KS biopsy samples, whereas positive controls for vIL-6 consisted of AIDS-associated MCD biopsy samples.

The presence of KSHV was confirmed by polymerase chain reaction (PCR) analysis of three KSHV regions (ie, K330₂₃₃, ORFK9-3, ORF72) using previously reported primer sequences.²² PCR analysis was performed on DNA isolated from Bouin, formalin, and frozen tissues by using the Nucleospin tissue system (Macherey-Nagel GmbH and Co., Düren, Germany) according to the manufacturer's protocol with minor modification. Each PCR reaction used ~0.2 µg of genomic DNA, 100 pmol of each primer, 2 U of Taq polymerase, 100 mmol/L of each deoxynucleotide triphosphate, 1.5 mmol/L Tris-hydrochloride (pH 9.0), and 0.1% Triton X-100 in a final volume of 50 µL. PCR amplification was performed at 95°C for 6 minutes (1 cycle); 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds (35 cycles); and 72°C for 5 minutes (1 cycle). Amplification was performed with a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT). PCR products were visualized on 2% agarose gel containing ethidium bromide.

Infection by EBV was investigated by multiple approaches, including *in situ* hybridization and PCR. EBER *in situ* hybridization was performed on Bouin- or formalin-fixed tissue sections, as previously described.^{23,24} PCR analysis of EBV was performed with primers SL-1 and SL-3 representative of the EBV nuclear antigen-1 gene, as previously reported.²⁵ In the case of EBV-positive samples, immunostaining for LMP1 was performed with a LMP1-specific antibody (Dakopatts A/S, Glostrup, Denmark) and the alkaline phosphatase anti-alkaline phosphatase method.²⁶

Molecular Analysis of Clonality and of BCL-6 Mutations

PCR analysis of immunoglobulin heavy chain (IgV_H) and light chain (IgV_L) and TCR-γ chain gene rearrangements was performed according to standard protocols. For IgV_H gene analysis, samples were amplified with a set of six V_H gene family-specific primers that hybridize to sequences in framework region (FR) 1 and/or FR2 in conjunction with a J_H degenerated primer.^{27,28} The sequences of FR1 V_H primers have been reported previously.^{27,28} The sequences of FR2 V_H primers are: 5'-GGA CAA RGG CTT

GAG TGG AT-3' (V_H1.1), 5'-GGA MAA SGS CTT GAG TGG AT-3' (V_H1.2), 5'-GGG AAR GGV CTG GAG TGG AT-3' (V_H4-5), 5'-GDT CCG CCA GGC TCC AG-3' (V_H3.11), 5'-GGT CCG SCA AGC TCC AG-3' (V_H3.12), 5'-GAT CCG TCA GCC CCC AG-3' (V_H2), 5'-GGA AAA GGT CTG GAG TGG GT-3' (V_H3.21), 5'-GGG AAG GGT CTG GAG TGG GT-3' (V_H3.22), 5'-GGG AAA GGG CTG GAG TGG GT-3' (V_H3.22a), and 5'-TCG AGA GGC CTT GAG TGG-3' (V_H6). Sequence of the degenerated J_H primer is: 5'-CTY ACC TGA RGA GAC RGT GAC C-3'. PCR was performed for 35 cycles (40 for paraffin-embedded biopsy specimens) with an annealing temperature of 60°C. For IgVL analysis, Samples were amplified with a set of V_k and V_λ gene family-specific primers hybridizing to sequences in FR1 in conjunction with the appropriate J_k or J_λ degenerated primers.^{27,28} PCR was performed for 35 cycles (40 for paraffin-embedded biopsy specimens) with an annealing temperature of 60°C.

Analysis of TCR-γ chain gene rearrangement was performed by amplification of V_γJ rearrangement with a set of three V_γ forward primers (V_γI, V_γII, and V_γIII/IV) and a set of reverse primers (J_γ1/2, JP1/2, and JP) complementary to J_γ 3' region. Four distinct PCR reactions were performed as follows: 1) V_γI/J_γ1/2; 2) V_γI/JP1/2 + JP; 3) V_γII/J_γ1/2 + JP1/2 + JP; 4) V_γIII/IV/J_γ1/2 + JP1/2 + JP. The sequence of V_γI is: 5'-TAC ATC CAC TGG TAC CTA CAC CA-3', the sequence of V_γII is: 5'-TCA TAC AGT TCC TGG TGT CC-3', and the sequence of V_γIII/IV is: 5'-TCA TTC ACT GGT ACC GGC AGA AAC CAA A-3'. The sequence of J_γ1/2 is: 5'-CCC GTC GAC TAC CTT GGA AAT GTT GTA TTC TTC-3', the sequence of JP1/2 is: 5'-CCA GGT GAA GTT ACT ATG AG-3' and the sequence of JP is: 5'-AAG CTT TGT TCC GGG ACC AA-3'. PCR was performed for 35 cycles (40 for paraffin-embedded biopsy specimens) with an annealing temperature of 58°C.

Southern blot studies for Ig gene rearrangement analysis and for TCR β/γ gene rearrangements could not be performed because of lack of sufficient material from these retrospectively collected cases. Rather, we applied multiple PCR assays for detection of Ig and TCR-γ gene rearrangements, because this technique requires lesser amounts of DNA.

Analysis of BCL-6 mutations was performed as previously reported.²⁸ *In situ* hybridization technique was used to detect immunoglobulin light chain messenger RNA (mRNA). κ and λ light chain mRNA was detected in Bouin-fixed, paraffin-embedded sections, by using a commercial kit (DAKO A/S, Glostrup, Denmark) under conditions recommended by the supplier.

Immunohistological Studies

All 56 cases included in the study had been immunophenotyped as previously described.¹⁹ To further investigate the phenotype of KSHV-positive solid lymphomas and to determine their relatedness to PEL, we tested the expression of an additional panel of proteins that are specifically expressed by PEL tumor cell, as previously documented by gene expression profiling analysis.²⁹ Immunohisto-

chemistry was done for antigens for which antibodies suitable for paraffin-embedded tissue sections were available. The KSHV-positive solid lymphomas along with a representative subset of KSHV-negative AIDS-DLCL of the centroblastic and immunoblastic type were tested for the expression of the following proteins: granzyme A (GRA), aquaporin-3 (AQP3), mucin-1 (MUC1), selectin P ligand (SELPLG), and vascular endothelial growth factor (VEGF). They were investigated by commercially available antibodies [GRA, AQP3, SELPLG, VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), Muc-1 (Chemicon Int., Temecula, CA)]. They were tested on paraffin-embedded sections with a previous step of antigen retrieval (30 minutes in EGTA-solution in microwave oven at 250 W) and immunostained on an automated immunostainer (Nexes). Negative controls were performed as described above.

RNA Isolation and Real-Time Reverse Transcriptase (RT)-PCR Relative Quantification Experiments

The four cases of KSHV-positive solid lymphomas were tested by real time RT-PCR for the expression of a subset of genes selected among the genes specifically up-regulated in PEL tumor cells (in hierarchic order). These genes are known to characterize PEL relative to other normal and neoplastic B cells.²⁹ Total RNA was isolated from frozen (cases 1, 3, 4) or formalin-fixed (case 2) samples using the Nucleospin RNA II system (Macherey-Nagel) according to a modified version of the company's protocol.³⁰ In particular: tissues were digested with Proteinase K (10 mg/ml) at 56°C for 2 to 3 hours with gentle agitation until complete digestion; to ensure complete removal of genomic DNA, the sample was incubated for 1 hour at 37°C with DNase I on the column. RNA was then eluted with 60 μ l of RNase-free water. All RNA were quantitated by spectrophotometer and OD 260/280 ratios >1.9 were obtained for all samples, indicating high purity. The conversion of total RNA to single-stranded cDNA was performed using the High-Capacity cDNA Archive kit (PE Applied Biosystems, Inc., Foster City, CA).

Real-time RT-PCR relative quantification analyses were performed using the ABI Prism 7700 sequence detection system instrument and software (PE Applied Biosystems). Assays-on-demand, consisting of a mix of unlabeled PCR primers and TaqMan MGB probe (FAM dye-labeled) for the target genes: GRA (assay ID, Hs00196206), IL-2 receptor β -chain (IL-2RB) (assay ID, Hs00386697), AQP3 (assay ID, Hs00185020), IL-10 (assay ID, Hs00174086), DAZLA (assay ID, Hs00154706), MUC1 (assay ID, Hs00410317), phosphatidic acid phosphatase type 2B (PAP2B) (assay ID, Hs00170359), IRF4 (assay ID, Hs00180031), and predeveloped TaqMan assay reagent (FAM-MGB) for β 2-microglobulin (product number, 4333766T) were purchased from PE Applied Biosystems. Assays-on-demand gene expression product have been designed by the producer for the detection and quantitation of specific human genetic sequences in RNA samples converted to cDNA.

Table 2. R^2 Values and Standard Curve Slopes Calculated for Each Target by Real-Time RT-PCR

Target	R^2 value	Slope
β 2-microglobulin	1	-3.260
Granzyme A	0.993	-3.438
Interleukin-2 receptor β chain	0.985	-3.253
Aquaporin 3	0.995	-3.739
Interleukin 10	0.990	-3.347
DAZLA	0.993	-3.438
Mucin 1	0.985	-3.238
Phosphatidic acid phosphatase type 2B	0.995	-3.418
IRF4	0.992	-3.344

The R^2 (correlation coefficient) value is an indication of the quality of the fit of the standard curve to the standard data points plotted, with values closer to 1 indicating a better fit of the data to the line. The slope of the standard curve is directly related to the average efficiency of amplification and may be used to calculate the PCR efficiency. A reaction with 100% efficiency will produce a slope of -3.32.

For evaluation of PCR efficiencies, serial dilutions of cDNA from CRO-AP/5 PEL cell line were used to construct a standard curve. PCR was performed with 25 μ l of TaqMan Universal PCR Master Mix (PE Applied Biosystems), 2.5 μ l probe/primer mix, and serial dilutions of cDNA (12.5 ng, 25 ng, 50 ng, 100 ng) in a 50- μ l final reaction mixture. After a 2-minute incubation at 50°C to allow for UNG cleavage, AmpliTaq Gold was activated by incubation for 10 minute at 95°C. Each of the 50 PCR cycles consisted of 15 seconds of denaturation at 95°C and hybridization of probe and primers for 1 minute at 60°C. All reactions were done in triplicate and the threshold cycle (C_T) values obtained were plotted against the base 10 log of the ng cDNA. C_T is defined as the cycle at which the fluorescence is determined to be statistically significant above background and is inversely proportional to the log of the initial copy number. The higher the value, the lower the mRNA content.³¹ As shown in Table 2 in which the R^2 values and standard curve slopes calculated by the instrument for each target are reported, all assays resulted in a good correlation coefficient and high PCR efficiency.

Quantitative RT-PCR of the studied samples was performed in duplicate using 20 ng of cDNA (or 120 ng of cDNA for paraffin-extracted RNA) in 50- μ l reaction volumes as above described. C_T values for β 2-microglobulin were used for normalization purposes. The averages of the normalized C_T values from a sample of centroblastic AIDS-DLCL,¹⁸ already studied by gene expression profiling analysis,²⁹ were used to determine the relative changes in gene expression of the KSHV-positive samples by the comparative C_T method ($2^{-\Delta\Delta CT}$).³¹

Clinical Evaluation

The clinical features and outcome of the three HIV-positive patients with KSHV-positive solid lymphomas were compared with those of 53 HIV-positive patients affected by KSHV-negative DLCL (38 immunoblastic, 15 centroblastic). Ten patients with a diagnosis of PEL were also included in the comparison study. All patients were diagnosed and treated at the CRO Aviano National Cancer

Figure 1. KSHV/HHV8-positive solid lymphomas. **A:** H&E stain. **B:** Immunohistochemistry for KSHV ORF73 protein. **C:** PCR for KSHV sequences (K330₂₃₃). In cases 3 and 4 DNA was extracted from both formalin and frozen tissue samples. C+, positive control (CRO-AP/6 PEL cell line); M, DNA molecular weight marker. Original magnifications, $\times 40$.

Institute, from April 1987 to June 2002. At diagnosis, all patients were evaluated for extent of HIV disease and NHL by physical examination, chest radiography, computed tomography of the thorax and abdomen, bone marrow aspiration and biopsy, lumbar puncture for spinal fluid, blood chemistry, CD4 cell count, and HIV viral load (since 1997). Stages for lymphomas were assigned according to Ann Arbor staging system.³² The revised classification system for HIV infection by the Centers for Disease Control was used for the AIDS diagnosis; according to World Health Organization only Centers for Disease Control clinical criteria were adopted.³³ Patients were treated mainly with doxorubicin-based combination chemotherapy, as part of phase II or phase III study, as previously reported.³⁴

Results

Demonstration of KSHV by Immunohistochemistry and PCR

Among the 56 cases of systemic AIDS-DLCL included in the study, KSHV was detected only in three cases. A

fourth case of KSHV-positive solid lymphoma that occurred in an HIV-negative person was identified in the course of the present study. The case was detected at the time of pathological diagnosis of lymphoma. Immunohistochemistry for KSHV ORF73 protein revealed that this antigen was detected in the nucleus of almost all of the tumor cells of all four cases (Figure 1). On the contrary, staining for vIL-6 was cytoplasmic and was restricted to a limited number of tumor cells (not shown). These data suggest that in the lymphomas infected with KSHV most tumor cells were in latent phase. On PCR analysis, bands compatible with KSHV genomic sequences were detected in all four cases (Figure 1).

Histopathological Analysis of the KSHV-Positive Solid Lymphomas

Abdominal tumors from two patients (cases 1 and 3) were extranodal lymphomas. In both cases, multiple nodular lesions of up to 5 cm in size were observed in the gastrointestinal tract. In case 3, a neck lymph node and Waldeyer's ring were also involved by the lymphoma. In case 2, tumors were observed in the dermis of the upper

Table 3. Molecular Characteristics of KSHV-Positive Solid Lymphomas

Case	<i>In situ</i> hybridization, kappa/lambda	IgV _H rearrangement	IgV _L rearrangement		BCL-6 mutations, frequency $\times 10^{-3}$	TCR- γ rearrangement
			Kappa chain	Lambda chain		
1	ND	+	+	+	3	—
2	ND	+	+	—	0	—
3	+	—	—	—	0	—
4	ND	—	—	—	6.5	—

ND, not done; +, clonal rearrangement identified; —, no clonal rearrangement identified.

left limb and chest wall, measuring ~2 cm in diameter. In case 4, superficial multiple lymphadenopathies were noticed at presentation.

Histologically, the lymphomas were composed of a proliferation of transformed lymphoid cells with large nuclei having prominent central nucleoli and abundant cytoplasm (immunoblastic-like cells) (Figure 1). Binucleation and multinucleation were not frequent. Two cases (cases 1 and 4) showed greater pleomorphism and a broader range of cell size and anaplastic features (large anaplastic cells), when compared with the others (Figure 1).

Demonstration of EBV by in Situ Hybridization, Immunohistochemistry, and PCR

By EBER *in situ* hybridization, two of four cases resulted positive for EBV (cases 2 and 3). These results were confirmed by PCR analysis of EBV nuclear antigen-1 region of EBV. EBV infection was not associated with LMP1 expression.

Molecular Analysis of Clonality and of BCL-6 Mutations

Results concerning clonality are shown in Table 3. A clonal IgV_H and IgV_L rearrangement was identified in two cases of KSHV-positive solid lymphomas (cases 1 and 2). Conversely, cases 3 and 4 yielded no IgV_H or IgV_L rearrangement; these same cases were readily amplified with a control gene fragment of similar size. Analysis of TCR- γ rearrangement scored negative in all cases. Mutations of BCL-6 were identified in two cases (cases 1 and 4). By *in situ* hybridization technique, monotypic λ mRNA was detected in case 3, establishing its B-cell clonality.

Immunophenotypic Analysis

Immunohistological analysis revealed that three of four cases (cases 1, 2, and 3) were positive for CD45. All four cases expressed CD3, CD43, CD138, EMA, and MUM1/IRF4 (Table 4). Usually, the cases showed CD3 positivity only in a small fraction of cells. Case 2 showed a subset of cells positive for CD20 and CD79a, whereas the other cases were negative for these markers. Three of four cases (cases 2, 3, and 4) were positive for CD30. The

expression of CD30 was variable, a small population of cells being stained in cases 3 and 4.

To determine the relationship of the KSHV-positive solid lymphomas with the KSHV-negative AIDS-DLCL and PEL, we analyzed the expression of a subset of genes identified by gene profiling that distinguish PEL tumor cells from other AIDS-related NHL.²⁹ All KSHV-positive solid lymphomas displayed a profile that is clearly indistinguishable from that of PEL. In fact, all four cases expressed GRA, AQP3, MUC1, SELPLG, and VEGF (Figure 2 and Table 4). Conversely, all these markers, with the exception of SELPLG, scored negative in the KSHV-negative AIDS-DLCL cases (not shown).

Table 4. Virological and Immunophenotypic Characteristics of KSHV-Positive Solid Lymphomas

	Case 1	Case 2	Case 3	Case 4
KSHV (IHC)	ORF73+ vIL-6-/+	ORF73+ vIL-6-/+	ORF73+ vIL-6-/+	ORF73+ vIL-6-*
KSHV (PCR)	KS ₃₃₀ + ORF72+ ORF9-3+	KS ₃₃₀ + ORF72+ ORF9-3+	KS ₃₃₀ + ORF72+ ORF9-3+	KS ₃₃₀ + — —
EBER (ISH)	—	+	+	—
EBNA1 (PCR)	—	ND	+	—
LMP1 (IHC)	—	—	—	—
CLA	+/-	-/+	+	—
EMA	+/-	-/+	-/+	+
MUM1/IRF4	+	+	+	+
CD138/syn-1	+/-	+	-/+	+
CD30	—	+/-	-/+	-/+
CD20	—	-/+	—	—
CD40	—	ND	—	—
CD79a	—	+/-	—	—
CD3	-/+	-/+	—*	-/+
CD5	—	—	—	—
CD43	+/-	+	-/+	-/+
CD45RO	-/+	ND	-/+	—
CD10	ND	—	—	—
Ki-67/MIB1	+	+	+	+
Granzyme A	+	+	+	-/+
AQP3	+	+	+	—*
MUC1	—*	-/+	-/+	NE
SELPLG	+	+	+	+
VEGF	+	-/+	+/-	+

IHC, immunohistochemistry; ISH, *in situ* hybridization; CLA, common leucocyte antigen; EMA, epithelial membrane antigen; syn-1, syndecan-1; AQP3, aquaporin 3; MUC1, mucin 1; SELPLG, selectin P ligand; VEGF, vascular endothelial growth factor; +, Almost all neoplastic cells positive; +/-, majority of neoplastic cells positive; -/+, minority of neoplastic cells positive; —*, few neoplastic cells positive; —, all neoplastic cells negative; ND, not done; NE, not evaluable.

Figure 2. KSHV-positive solid lymphomas positively immunostain for molecules that are specifically expressed by PEL tumor cells.²⁹ Original magnifications, $\times 40$.

Real-Time RT-PCR Relative Quantification

To further define the phenotype of KSHV-positive solid lymphomas in relation to that of PEL, we tested the four KSHV-positive solid lymphomas by real-time RT-PCR for the expression of the genes that are more specifically up-regulated in PEL (see Materials and Methods).²⁹ Of note, mRNAs encoded by KSHV were not represented in this set of genes. As shown in Figure 3, all of the tested genes appeared overexpressed in the KSHV-positive solid lymphomas. However, some of the genes were expressed at lower levels in some of the cases than in the control case (calibrator). According to a relative quantification assay, gene expression was variable among the cases. For example, patient 3 appeared to have rather divergent results compared to the other three cases and the PEL cell line. Patient 3 demonstrated relatively reduced mRNA expression of several of the PEL-related genes. However, this case was the only one in which a

large amount of normal lymphocytes were admixed to tumor cells. The samples from the other cases showed a tumor cell population $\geq 95\%$.

The expression of the molecules related to the tested genes on tumor cells was confirmed by immunohistochemical staining (Figure 2, Table 4). However, there was a lack of concordance between the immunohistochemistry data and the quantitative real-time RT-PCR data for MUC1. All cases showed high expression levels of MUC1 mRNA, but correspondingly low protein expression.

Clinical Comparison

Comparative clinical study was performed on three groups of HIV-positive patients with NHL: the KSHV-positive solid lymphoma group (3 patients), the KSHV-negative DLCL group (38 immunoblastic, 15 centroblastic patients), and the PEL group (10 patients). Age distribution,

Figure 3. KSHV-positive solid lymphomas display expression of a subset of genes selected among the genes specifically up-regulated in PEL tumor cells.²⁹ The results of a relative quantification assay is reported as a fold-difference relative to a calibrator sample. Relative quantification of target gene expression was calculated from ABI Prism 7700 Relative Quantification software that calculates relative levels of gene expression using the comparative C_T method of data analysis.³¹ A sample of centroblastic AIDS-DLCL,¹⁸ already studied by gene expression profiling analysis²⁹ is used as calibrator. The calibrator expressed all of the tested genes, but at lower levels than those reported for PELs. A PEL cell line termed CRO-AP/5, whose parental sample was previously tested by gene expression profiling analysis,²⁹ is included in the real-time RT-PCR relative quantification experiments to determine the expression of the same subset of genes. The x-axis of the graph lists all of the target involved in the analysis. Within each target group, the software displays the relative quantity of the target in each sample. In the y-axis are the relative quantities graphed on a logarithmic scale. The quantities are shown relative to the calibrator sample. Each increment corresponds to a 10-fold difference in gene expression. Because the calibrator sample is compared to itself, the level of cDNA expression in the calibrator always appears as 1 (1E + 00).

sex, HIV category, and previous anti-retroviral therapy were similar for the three groups. The HIV disease distribution differed significantly: previous AIDS diagnosis was found more frequently in the PEL patients (60%) than in the KSHV-positive solid lymphomas (33%) and in the DLCL patients (17%) ($P = 0.01$). Performance status, stage of NHL, incidence of B-symptoms, and bulky dis-

ease did not show substantial differences among the patients.

Generalized lymphadenopathy was detected in 100% of patients with KSHV-positive solid lymphomas, in 57% of patients with DLCL and in only 20% of patients with PEL ($P = 0.03$). However, when the KSHV-positive solid lymphoma was compared with the DLCL the differences

Table 5. Significant Clinical Comparisons among HIV-Infected Patients with KSHV-Positive Solid Lymphomas (KSHV-SL), Primary Effusion Lymphomas (PEL), and Diffuse Large Cell Lymphomas (DLCL)

Variables	KSHV-SL n (%) (n = 3)	PEL n (%) (n = 10)	DLCL n (%) (n = 53)	P value
Prior AIDS	1	6 (60)	9 (17)	0.01*
Site of involvement				
Lymph nodes	3 (100) [†]	2 (20)	30 (57) [†]	0.03*
Pleura	—	9 (90)	—	≤0.0001*
Pericardium	—	6 (60)	—	≤0.0001*
Peritoneum	—	3 (30)	—	≤0.0001*
Major laboratory findings				
Hb concentration (g%)	7.80 [‡]	9.99 ^{‡§}	11.40 [§]	0.004 [¶]
Mean (±SD)	(±0.34)	(±1.48)	(±2.25)	
Hypoalbuminemia (≤3 g%)	3 (100)	8 (80)	15 (28)	≤0.0001*
Outcome				
OS probability (%)				
6 Months	—	50	68	
12 Months	—	40	43	0.001 [¶]

* χ^2 test; [†]KSHV versus DLCL $P = 0.1$ (Fisher's exact test); [‡]KSHV-SL versus PEL $P = 0.008$ (Fisher's exact test); [§]PEL versus DLCL $P = 0.03$ (Fisher's exact test); [¶]Kruskal Wallis test; ^{||}overall survival.

of incidence of adenopathy disappeared ($P = 0.1$). The overall incidence of extranodal disease was similar for the three groups ranging from 100% in both the KSHV-positive solid lymphomas and the PEL, to 91% in the DLCL group ($P = 0.05$). The number of patients with two or more extranodal sites were similar in the three groups ($P = 0.7$). However, the incidence of malignant serous involvement was significantly ($P \leq 0.0001$) higher in patients with PEL than in patients with KSHV-positive solid lymphomas and in patients with DLCL, in which serous involvement was absent. Nonsignificant differences in the distribution of other extranodal localization (ie, gastrointestinal tract, Waldeyer's ring, bone marrow, liver, spleen, central nervous system, and skin) were detected among the three groups.

The mean (±SD) CD4 cell count was similar among the patient groups, being 137.67/ μ l (±137.62) in the KSHV-positive solid lymphoma group, 144.65/ μ l (±122.80) in the DLCL group, and 95.80/ μ l (±88.95) in the PEL group. The mean (±SD) leukocyte and platelet count did not show significant differences in the groups ($P = 0.7$ and $P = 0.3$, respectively). The mean (±SD) hemoglobin concentration was significantly lower in KSHV-positive solid lymphoma patients (7.80 g/% ± 0.34) compared to the PEL patient (9.99 g/% ± 1.48), and the DLCL patients (11.40 g/% ± 2.25) ($P = 0.004$). However, when the KSHV-positive solid lymphoma patients were compared with the PEL patients, this difference disappeared ($P = 0.08$).

Abnormal lactate dehydrogenase serum level was detected in 100% of the KSHV solid lymphoma patients, in 70% of PEL patients, and in 58% of DLCL patients, but the difference was not significant ($P = 0.2$). The distribution of hypoalbuminemia differed significantly among the three groups ($P \leq 0.0001$). However, no significant difference in the incidence of the hypoalbuminemia was found when the KSHV-positive solid lymphoma patients were compared with PEL patients ($P = 0.4$). Therapy use was similar in the three groups ($P = 0.7$), but response rate distribution was not evaluated because of the heterogeneity of treatment used.

Mortality rates were similar among the three groups ($P = 0.5$) and NHL progression was the most common cause of death for all patients. However, the outcome was significantly poorer in the KSHV-positive solid lymphoma group compared to the other two groups. The probability of survival at 6 and 12 months was 0 in the KSHV-positive solid lymphoma group, 50% and 40% in the PEL group, and 68% and 43% in the DLCL group ($P \leq 0.001$) (Table 5).

Discussion

This study reports on four cases of solid lymphomas, all carrying KSHV/HHV8 and exhibiting PEL-like morphology. Three cases were AIDS-related solid lymphomas, whereas one case occurred in a HIV-seronegative person. Because our data unequivocally demonstrate the tumor cell localization of KSHV genome and protein, we believe that these cases, irrespective of HIV status, belong to the entity of HHV-8-associated lymphoma. Furthermore, our findings clearly show that KSHV can associate with solid lymphomas that, in the AIDS setting, should be distinguished from the other KSHV-negative AIDS-DLCL. The KSHV-positive solid lymphomas occurred both in extranodal and nodal sites, without secondary lymphomatous effusions. Among KSHV-associated malignancies, only KS was found in one case (case 3).

Based on the immunophenotypic results, there were difficulties in assigning lymphoid lineage in these KSHV-positive solid lymphomas using usual phenotypic markers. These lymphomas with infrequent or no definite immunophenotypes after immunomorphological analysis, showed clonally rearranged Ig genes in two cases (cases 1 and 2), and a monotypic λ mRNA expression in case 3. Although an Ig rearrangement could not be identified in case 4, this lymphoma showed the presence of BCL-6 mutations that are characteristically associated with B-cell malignancies deriving from B cells that have experienced the germinal center reaction. Conversely, a clonal rearrangement involving TCR- γ chain gene was never

found in any of the four KSHV-positive solid lymphomas. These data allow us to speculate that, despite their phenotypes, all these lymphomas were consistently represented by a monoclonal B-cell population, as documented by genotypic studies. It is interesting that the reported PELs were of B- or null-cell immunophenotypes, whereas genetic analysis revealed B-cell genicity in most of the cases.²¹

To the best of our knowledge less than 20 cases of KSHV-positive solid lymphomas have been reported so far.⁸⁻¹⁷ In those reports, the term PEL-like was used in a broad sense, based mostly on morphology. The results of our comparative gene expression analysis of KSHV-positive solid lymphomas versus PEL reveal previously unrecognized phenotypic relationships of these tumor entities, and greatly extend the characterization of KSHV-positive lymphomas through the identification of a large number of genes specifically expressed by the tumor cells. Extending the previous observations, the profile shown in Figures 2 and 3 demonstrates that all of the tested genes appeared overexpressed in the KSHV-positive solid lymphomas. Consistent with these results, KSHV-positive solid lymphomas show relatedness to the PEL-specific profile in the gene expression levels of a significant number of genes, confirming the relatedness of these lymphomas to PEL. Therefore, it is evident that molecular diagnostic techniques, including analysis of viral infection, molecular analysis of clonality, and immunohistological studies, are of primary importance to define this apparently distinct type of lymphoma. In the diagnostics practice, early diagnosis of KSHV-positive solid lymphomas and the possibility to inhibit tumor growth might be prognostically useful.

From the clinical standpoint, KSHV-positive solid lymphomas presented with severe systemic symptoms, generalized lymphadenopathy, and multiple extranodal involvement, particularly gastrointestinal tract and Waldeyer's ring. The most consistent laboratory findings at diagnosis are anemia and hypoalbuminemia, the last because of nephrotic syndrome. Two additional features are notable in HIV-infected patients: autoimmune thrombocytopenia and Coomb's test positivity, markers of HIV as well as HHV-8 immune dysregulation.^{35,36}

The lymphoma shows aggressive behavior and rapidly fatal outcome in all patients, with a median survival of only 22 days. However, the features of KSHV-positive solid lymphomas in HIV setting do not appear to be unique. In this study, a clinicopathological comparison has been made in a single institution between three different groups of HIV-related lymphomas: KSHV-positive solid lymphomas, PEL, and the DLCL group. This kind of comparison is significant in that all patients were staged, pathologically evaluated, and clinically treated by the same group of physicians, rendering the evaluation more feasible and reliable.

In HIV-infected patients, the spectrum of clinical presentation of KSHV-positive solid lymphomas is comparable with that of DLCL. KSHV-positive solid lymphomas and DLCL are frequently confined to nodal and extranodal sites, on the other hand, PEL is mostly confined to body cavity surfaces. Moreover, PEL occurs more fre-

quently in the late stage of HIV infection and immunodepletion appears to be more severe in this group (median CD4 cell count, 95.80/ μ l) in comparison with the other two groups. The differences in CD4 cell count distribution are not statistically significant, but the data need to be confirmed in a larger series.

Two major laboratory abnormalities, anemia and hypoalbuminemia, were found more frequently in both KSHV-positive solid lymphomas and PEL than in DLCL. In this retrospective series we could not determine the primary cause of anemia and hypoalbuminemia. However, it is noteworthy that these laboratory features are common in other KSHV-related diseases, ie, MCD.

Mortality was similar among the three lymphoma groups, but the KSHV-positive solid lymphoma exhibited a more aggressive clinical course and a more unfavorable prognosis compared to PEL and DLCL. Prospective studies are needed to recognize prognostic factors and to define the best treatment strategy for these patients.

In conclusion, although the outcome was significantly poorer in KSHV-positive solid lymphomas compared to the other two groups of malignancies, no significant differences in clinical features were found comparing these lymphomas with AIDS-DLCL group. On the other hand, laboratory abnormalities of the KSHV-positive solid lymphoma patients were similar to those of PEL patients. Because pathobiological features of KSHV-positive solid lymphomas mimic those of PEL, they should be considered in the spectrum of KSHV-associated lymphomas. In this sense, KSHV-associated lymphoma can take two forms: effusion lymphoma type and solid lymphoma type. Previous reports of cases of PEL relapsing as a solid gastrointestinal tumor³⁷ would support this conclusion.

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