Kaposi sarcoma herpesvirus viral load as a biomarker for leptomeningeal involvement by primary effusion lymphoma

Kaposi sarcoma herpesvirus (KSHV, also known as human herpesvirus 8), is the etiologic agent of primary effusion lymphoma (PEL), a rare, aggressive B-cell lymphoma with a worse prognosis compared to other human immunodeficiency virus (HIV)-associated lymphomas.[1,2] Eighty percent of tumors are co-infected with Epstein Barr virus (EBV). KSHV also causes Kaposi sarcoma (KS), a form of multi-centric Castleman disease (MCD), and KSHV inflammatory cytokine syndrome (KICS).[3-5] PEL involvement of the cerebrospinal fluid (CSF) and leptomeninges (CSF-PEL) has been rarely reported, reflecting either true rarity or underdiagnosis.[6-8] A diagnosis of CSF-PEL is essential in both asymptomatic and symptomatic patients to guide therapy as CSF-PEL requires specific therapy directed to the leptomeningeal space.[7]

The gold standard for diagnosis of leptomeningeal involvement by lymphoma is cytologic examination of the CSF. Flow cytometry and molecular pathology to determine clonality of immunoglobulin gene rearrangements can also aid in diagnosis. However, these tests require experienced pathologists and specialized laboratory techniques generally only available in high-resource settings. Little is known about whether KSHV or EBV DNA viral loads (VL) are elevated in the CSF of patients with PEL with or without CSF involvement, or in other KSHV-associated diseases. The KSHV VL is markedly elevated in peripheral blood mononuclear cells (PBMC) and blood plasma in PEL, MCD, and KICS as well as PEL effusions.[1,9-13] We hypothesized the CSF KSHV VL would be elevated in CSF-PEL and could be utilized to diagnose and monitor disease activity. We also hypothesized the EBV VL could be used in a similar way in EBV$^+$ CSF-PEL.

We retrospectively identified patients with KSHV-associated diseases who underwent at least one CSF evaluation in the HIV and AIDS Malignancy Branch at the National Cancer Institute (NCI) between June 2007 and October 2020. All cases of PEL, MCD, and KS were pathologically confirmed in the NCI Laboratory of Pathology. KSHV and EBV tumor status were confirmed by latency-associated nuclear antigen staining or *in situ* hybridization against EBV-encoded small RNA, respectively. CSF-PEL was diagnosed via cytopathology supported by immunohistochemistry (Figure 1). KICS was diagnosed via published criteria after the exclusion of MCD and PEL.[5,13] Those with PEL and concurrent MCD or KS were classified as having PEL and not included in the MCD or KS groups. Patients were enrolled on an NCI Institutional Review Board-approved protocol to collect and analyze tissue with clinical correlations (*clinicaltrails gov. Identifier: NCT00006518*) via written informed consent in accordance with the Declaration of Helsinki.

We compared KSHV and EBV VL from fresh PBMC or stored plasma drawn at the same time points as CSF sampling. We measured CSF, plasma, and PBMC-associated KSHV and EBV VL via real-time quantitative polymerase chain reaction (PCR) with primers for KSHV *K6* and EBV *pol*. PBMC-associated KSHV and EBV VL were reported as viral DNA copies/10^6 PBMC with cellular equivalents quantified using human endogenous retrovirus 3 primers.[14] Plasma and CSF KSHV and EBV VL were reported as viral DNA copies/mL. We considered VL >1 copy/mL elevated. We assessed the sensitivity, specificity, and likelihood ratios of CSF KSHV VL at different cut points to predict positive cytology for PEL, performed receiver operator characteristic curve analyses, and evaluated the correlation between CSF, plasma, and PBMC-associated KSHV and EBV VL using Spearman correlation. Statistically significant differences in KSHV VL positivity were determined by Fisher's exact test.

We assessed 116 paired CSF and PBMC samples for which KSHV VL measurement and cytology were performed on the CSF from 38 HIV+ patients (26 PEL, 4 MCD, 3 KICS, and 5 KS) (Table 1; *Online Supplementary Table S1*). Of the 26 patients with PEL, four had positive cytology for CSF-PEL at baseline, and an additional patient had positive cytology at a later time point. None of the patients with CSF-PEL had neurologic symptoms to suggest CSF involvement. Overall, we analyzed 99 CSF samples from 26 patients with PEL and 17 from patients with other KSHV-associated diseases.

Generally, patients with PEL had well-controlled HIV at the time of their first CSF sampling (median HIV VL 10 copies/mL, interquartile range [IQR]: 0-491) with median CD4$^+$ T-cell count of 194 cells/μL (IQR: 75-309) (Table 1). All four patients with CSF-PEL at first sampling had an elevated KSHV VL (391-360,000 copies/mL) (Table 1; *Online Supplementary Table S1*). Only two patients of 22 with negative baseline cytology had an elevated CSF KSHV VL (*Online Supplementary Table S2*). Patient 5 had a KSHV VL of 192 copies/mL with negative cytology, but 4 weeks later, the CSF KSHV VL had increased to 16,000 copies/mL and cytology became positive. Patient 18 with multiply relapsed PEL had a KSHV VL of 960 copies/mL with negative cytology but we were unable to perform subsequent CSF

sampling as he was treated only briefly at our center. In patients with PEL at first CSF sampling, when using a cut-off of 391 copies/mL, the CSF KSHV VL had a sensitivity of 100% and specificity of 96% to detect CSF-PEL at that time point, with a positive likelihood ratio of 22 to predict positive cytology, and a negative likelihood ratio of <0.00001 to predict negative cytology.

Among the 12 patients with other KSHV-associated diseases, only one CSF sample had an elevated KSHV VL at 440 copies/mL with negative cytology and no known CSF

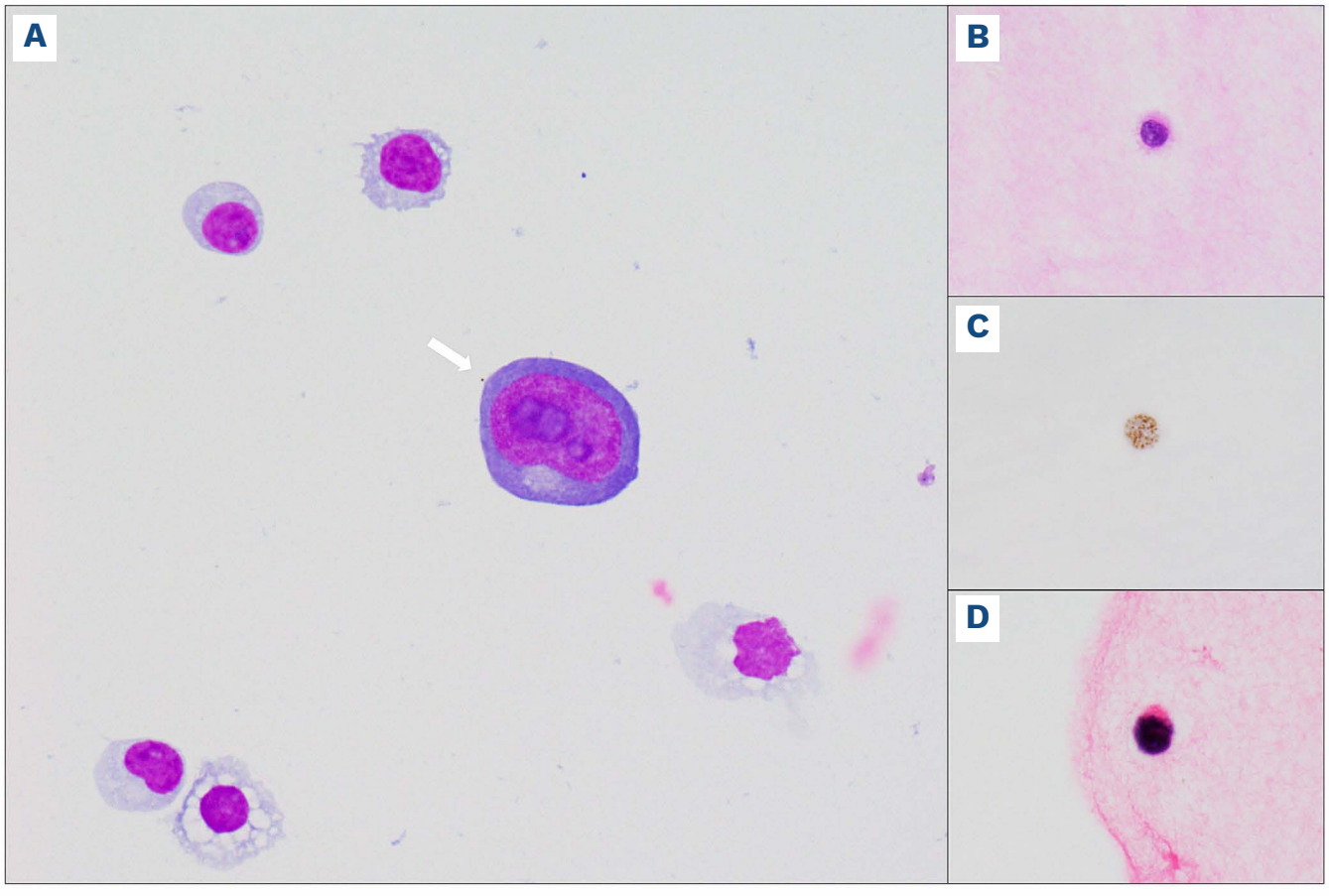

Figure 1. Cerebrospinal fluid of patient with leptomeningeal primary effusion lymphoma. Panel (A) shows cytology cerebrospinal fluid (CSF). The white arrow indicates a malignant lymphocyte. Panel (B) shows a paraffin-embedded malignant cell from the same patient's CSF. The malignant cells are positive for both Kaposi sarcoma herpesvirus by (C) latency nuclear antigen staining (LANA) in brown and for Epstein Barr virus by (D) EBV-encoded small RNA (EBER) staining in dark blue.

Table 1. Characteristics of patient samples evaluated for Kaposi sarcoma herpesvirus viral load.

	PEL	MCD	KICS	KS
Number of patients	26§	4	3	5
Number of samples	99	8	4	5
CD4+ T cells/μL*, median (IQR)	194 (75-309)	76 (55-99)	56 (41-75)	511 (256-2,571)
HIV copies/mL*, median (IQR)	10 (0-491)	909 (349-24,285)	38 (14-82)	5,233 (1,271-150,999)
Number of CSF baseline samples with elevated KSHV VL**	6	1	0	0
CSF KSHV copies/mL in all samples at all time points, median (IQR)	1 (0-12,400)	0 (0-1)	0 (0-0)	1 (0-1)
CSF KSHV copies/mL in patients with PEL at baseline, median (IQR)	0 (0-1)	-	-	-
CSF KSHV copies/mL in patients with PEL with CSF-PEL all time points†, median (IQR)	4,9750 (7,000-1525,000)	-	-	-
CSF KSHV copies/mL in patients with PEL without CSF-PEL all time points, median (IQR)	0 (0-1)	-	-	-
PBMC KSHV copies/10^6 cells all time points, median (IQR)	1 (0-835)	1 (0,-7,722)	1 (0-618,183)	1 (1-1,538)
Plasma KSHV copies/mL all time points, median (IQR)	225 (0-1,850)	8,000 (1,450-20,000)	23,825 (112-76,875)	1050 (0-6,000)

*As measured at the time of first cerebrospinal fluid (CSF) sampling. **Elevated Kaposi sarcoma herpesvirus (KSHV) defined as >1 copy/mL. Four patients had cytology positive for CSF-PEL, 1 patient developed positive cytology on subsequent CSF sampling, and 1 patient had elevated CSF-KSHV with negative cytology and no further follow-up to determine significance of the elevated KSHV in the CSF. †From 5 patients with CSF-PEL with multiple CSF samplings. §Includes 7 patients who had both PEL and MCD. CSF-PEL: leptomeningeal primary effusion lymphoma confirmed by cytology; IQR: interquartile range; KICS: KSHV inflammatory cytokine syndrome; KS: Kaposi sarcoma; MCD: multicentric Castleman disease; PBMC: peripheral blood mononuclear cells; PEL: primary effusion lymphoma; VL: viral load; HIV: human immunodeficiency virus.

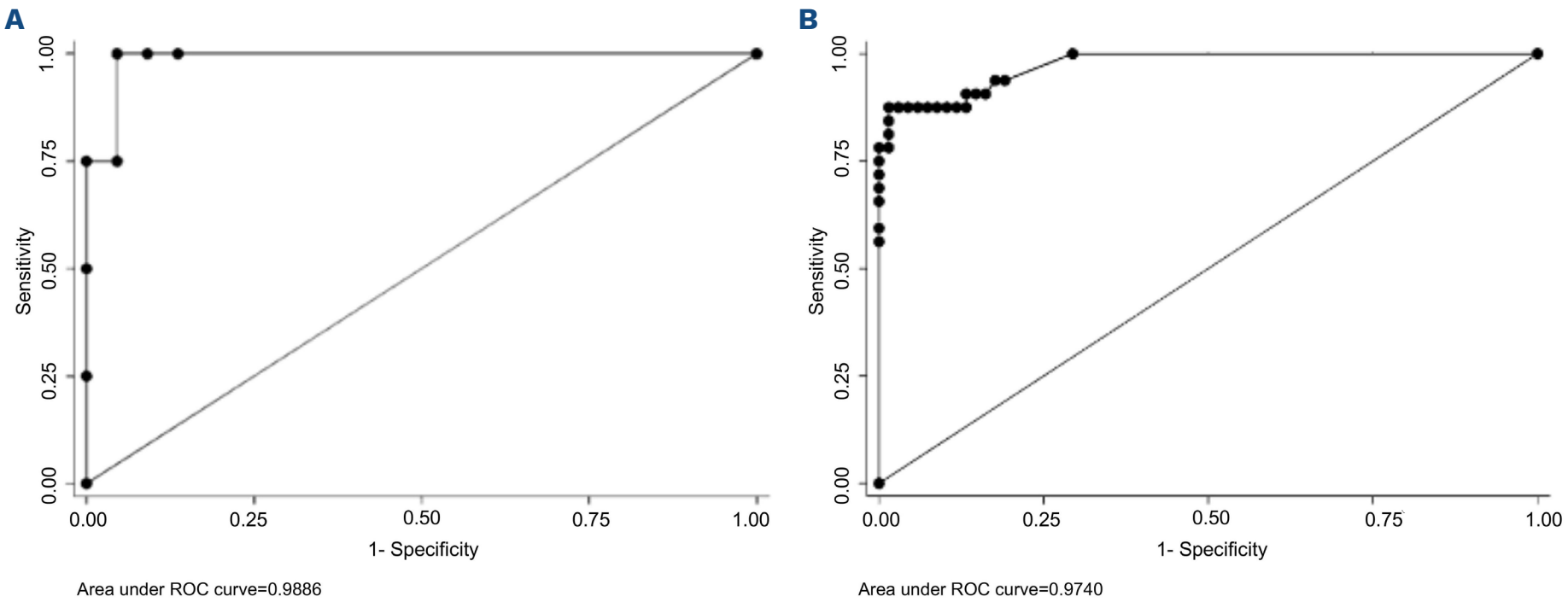

Figure 2. Receiver operator characteristic curves of the Kaposi sarcoma herpesvirus viral load in the cerebrospinal fluid to detect leptomeningeal involvement in patients with primary effusion lymphoma. (A) First cerebrospinal fluid (CSF) sampling (area under the curve=0.989) and (B) at any cerebrospinal fluid sampling time point (area under the curve=0.974). ROC: receiver operator characteristics.

pathology (Table 1). This patient had active MCD and a PBMC-associated KSHV VL of 91,000 copies/10^6 cells. He was successfully treated for MCD and was followed for several years without development of PEL. At first CSF sampling, the CSF KSHV VL in all patients with any KSHV-associated disease revealed that when using a cut-off of 391 copies/mL, the KSHV VL had a sensitivity of 100% and specificity of 94% to detect CSF-PEL with a positive likelihood ratio of 17 and a negative likelihood ratio of <0.00001.

Seventeen patients with PEL, including the five with CSF-PEL, had more than one CSF analysis (range, 2-23). Of the 99 CSF samples from patients with PEL, 32 were positive by cytology, and 30 of these 32 samples (93.8%) had an elevated KSHV VL. The median CSF KSHV VL in patients with CSF-PEL at any time point was 49,750 copies/mL (IQR: 7,000-1525,000), and in patients with PEL without CSF-PEL, the median CSF KSHV VL was 0 (IQR: 0-1). Using the cut-off of 391 copies/mL, the KSHV VL had a sensitivity of 91% and specificity of 87% to detect CSF-PEL at any given time point, with a positive likelihood ratio of 6.8 and negative likelihood ratio of 0.11 (Figure 2).

In those with positive cytology undergoing CSF-directed therapy, the KSHV VL generally tracked with treatment response. There were 41 CSF samples taken from the five patients with CSF-PEL receiving CSF-directed therapy (*Online Supplementary Table S3*). Thirty-one were positive by cytology for CSF-PEL, and 29 had an elevated KSHV VL. In cytology-positive samples, the median CSF KSHV VL was 19,500 copies/mL (IQR: 4,825-1925,000). The KSHV VL remained elevated in patients whose cytology remained positive despite treatment, and it became negative in the one patient who received multiple lines of therapy and remarkably had eventual clearance of his CSF (*Online Supplementary Figure S1*).

Among all patients with PEL, the CSF EBV VL was not helpful to diagnose CSF-PEL, irrespective of EBV status of the PEL tumor. Fifteen percent of CSF samples from all patients with PEL and 17% from patients with EBV^+ CSF-PEL had an elevated EBV VL. At all time points, the median CSF EBV VL was 0 copies/mL when evaluating all patients with PEL and in those specifically with EBV^+ PEL. There was no statistically significant difference in the CSF EBV VL between patients with EBV^+ PEL with and without positive CSF cytology (P=0.72). In patients with PEL, there was no correlation between CSF and PBMC or plasma KSHV VL (r=-0.05, P=0.63; r=-0.15, P=0.12, respectively). There was a moderately strong correlation (r=0.54, P=<0.00001) between the KSHV VL in PBMC and plasma. The CSF EBV VL was weakly correlated with PBMC EBV VL (r=0.26, P=0.01) and moderately correlated with plasma EBV VL (r=0.36, P=0.004). EBV VL in PBMC and plasma had a moderately strong correlation (r=0.65, P=<0.00001).

To our knowledge this is the largest study of CSF-PEL and the first to report the use of the CSF KSHV VL in any KSHV-associated disease. Our finding of five cases of CSF-PEL among 26 patients with PEL suggests CSF-PEL is underdiagnosed and CSF sampling at baseline should be routine. None of our patients with CSF-PEL had neurologic symptoms suggestive of leptomeningeal disease, reinforcing the importance of specifically looking for CSF-PEL with sensitive diagnostics. In resource-limited settings where PCR-based testing for KSHV is increasingly available, the KSHV VL could be particularly useful to relieve strain on scarce pathologic services by ruling out CSF-PEL in samples with undetectable or very low KSHV VL, negating the need for

cytology review.[15] Moreover, an elevated KSHV VL with negative cytology should prompt additional CSF sampling and close patient follow-up for the development of CSF-PEL. In summary, an elevated KSHV-VL in the CSF in patients with PEL is a sensitive and specific test for detecting CSF involvement and can be used as a tumor biomarker for diagnosis and to monitor treatment response.

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Contributions

KL, RR, VM, DW, and RY designed the study; KL, RR, RM, RY, and TSU cared for patients; KL, VM, and RR collected and analyzed data; EJ, SP, MR, and ACF provided pathologic confirmation of cases; VM, EMCC, WM, KM, and RR performed KSHV viral load assays; KL and RY drafted and revised the initial manuscript. All authors contributed to writing and approving the manuscript.

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Data-sharing statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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