

The Kaposi Sarcoma-associated Herpesvirus (KSHV) Is Present as an Intact Latent Genome in KS Tissue but Replicates in the Peripheral Blood Mononuclear Cells of KS Patients

By Lisa L. Decker,* Premalata Shankar,‡ Gulfaraz Khan,* Richard B. Freeman,§ Bruce J. Dezube,¶ Judy Lieberman,‡ and David A. Thorley-Lawson*

*From the *Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111; ‡Center for Blood Research, Harvard Medical School, Boston, Massachusetts 02115; §Department of Surgery, New England Medical Center, Boston, Massachusetts 02111; and ¶Division of Hematology/Oncology, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02215*

Summary

Short DNA sequences have been identified, originally in association with Kaposi's sarcoma (KS) biopsies, that are highly homologous to oncogenic, lymphotropic herpesviruses. Recently a virus, Kaposi sarcoma associated herpesvirus (KSHV) or human herpesvirus-8 (HHV-8), bearing these sequences has been identified in a cell line derived from a body cavity-based lymphoma. In this report, we show that the same sequences are present in KS biopsies as DNA molecules of a form and size characteristic of latent herpesviruses—large, covalently closed, circular episomes. The genomes migrate with an apparent size larger than the herpesvirus Epstein-Barr virus (172 kb). This form of the viral genome was found in four of four biopsies and three of five peripheral blood samples from KS patients. Linear forms of the viral genome, characteristic of viral replication, were not detected in the biopsies, but were present in the peripheral blood of three out of five patients. The sequences for KSHV/HHV-8 were also detected in the blood of four of five allograft patients and three of five healthy donors without KS suggesting that the virus is widespread throughout the human population.

Recently, short DNA sequences with homology to known herpesviruses were detected in the biopsies and blood of Kaposi sarcoma patients (1–3). These sequences were homologous to the oncogenic, lymphotropic herpesviruses Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS). This led to speculation that a novel oncogenic herpesvirus, KS-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8), had been discovered that might play a role in the pathogenesis of KS. Recently a herpesvirus bearing these sequences has been identified in cell lines derived from a body cavity B cell lymphoma (4–6). However, it remains to be demonstrated that the sequences present in KS patients are part of an intact herpesvirus genome.

Herpesviruses establish latent infections for the lifetime of the host. Their large DNA genomes, 100–250 kb (7), persist extrachromosomally in latently infected cells as covalently closed circles (CCC). The best studied example of this is Epstein-Barr virus (EBV) which can maintain a persistent, latent infection both in vivo and in vitro as a CCC episome (8–10). When the virus is reactivated, it begins to produce linear forms of the genome for packaging into vir-

ions. The circular and linear forms of herpesviral DNA, representing latent and replicative infection respectively, can be resolved based on differential migration in in situ lysis agarose gels (Gardella gels) (11, 12). The validity of this method has been established previously (11–13). In a large panel of cell lines which either lack or possess episomal DNA, as judged by restriction enzyme analysis and in situ fluorescence hybridization, the presence of the appropriately migrating DNA on a Gardella gel was consistently diagnostic for the presence or absence of episomal DNA (12). Furthermore, the conversion of the linear to the episomal form, in newly infected cells (13), and episomal to linear by double strand nicking (unpublished observations), can both be demonstrated with Gardella gels.

We show that the KSHV DNA sequences in KS biopsies migrated in Gardella gels at a position characteristic of the large circular DNA molecules detected in latent herpesvirus infections, whereas DNA characteristic of the linear form found during viral replication, was detected in peripheral blood mononuclear cells (PBMC). We have confirmed the presence of KSHV sequences in the biopsies and blood of

HIV-positive KS patients and extended these findings to demonstrate that the sequences are frequently present, at a lower level, in KS negative allograft and healthy donors.

Materials and Methods

Cell Lines and Cells. B95-8 (American Type Culture Collection [ATCC], Rockville, MD) is a marmoset B cell line, latently infected with EBV, in which ~5% of the cells are spontaneously replicating the virus. BJAB (ATCC) is an EBV-negative B cell tumor line.

4-mm punch biopsies of cutaneous KS lesions were obtained with informed consent from HIV-positive KS patients, attending the Oncology Unit at Beth Israel Hospital, following human experimental guidelines of the U.S. Department of Health and Human Services and of Beth Israel Hospital. Biopsy specimens were dissected free of surrounding connective tissue, minced into small pieces and digested with 2.5 mg/ml of trypsin (Difco) and 10 µg/ml of collagenase type IV (Sigma Chem. Co., St. Louis, MO) in RPMI1640. The aliquots were pooled and subjected to Ficoll-Hypaque discontinuous gradient centrifugation at 600 g for 10 min. The interface cells were washed, counted and analyzed by Gardella gel (11). For the induction of replication, the biopsy cells were incubated overnight at 37°C in RPMI 1640 and 10% fetal calf serum.

PBMC were prepared from heparinized peripheral blood by Ficoll-Hypaque discontinuous gradient centrifugation. The cells were then washed and prepared for Gardella gel analysis as described (13).

In Situ Lysis Gardella Gels. Gardella gels were performed as described previously (11) with modifications (8, 12).

DNA PCR Analysis. Lanes of the gel were excised, cut into slices, and analyzed for EBV by PCR as described previously (8, 14).

Gels containing biopsy material or peripheral blood samples from KS patients were soaked in 1% SDS for 15 min and TBE for ~45 min before further manipulation. After melting the gel slices, 25–100 ng carrier tRNA was added to each. DNA was then obtained from the slices by extracting with phenol three times and chloroform/iso-amyl alcohol (24:1) one time. 2.5 M ammonium acetate and 100% ethanol were used to precipitate the DNA. After washing in 70% ethanol and air drying, the DNA was solubilized in 50 µl HPLC H₂O at 4°C overnight. PCR amplification of the 233-bp KS330₂₃₃ KSHV sequence was performed with 25 µl of the sample, essentially as described (2), with the addition of a hot start and 1 unit of Taq extender (Stratagene Inc., La Jolla, CA) per reaction. As a positive control, cloned PCR product, kindly supplied by Dr P. Moore (Cornell Medical School), was used. EBV DNA was used as a negative specificity control. As negative controls, five samples of 10⁶ BJAB cells and/or five no DNA controls were performed with every set of PCR reactions and a complete Gardella gel analysis was performed on BJAB cells and PBMC from an HIV-negative donor who was also KSHV negative by PCR (Table 1 donor 3). No specific KSHV PCR product was ever detected in the specificity or negative control samples.

The PCR products of DNA from sequential slices of a Gardella gel lane were analyzed in two rows of the same gel. In every case where photographs are spliced the samples are continuous, from the same gel, just different rows from the gel, and from the same film and photograph with the same exposure.

Cloning and Sequencing of the PCR Product. 5 µl of DNA from slices positive for the putative KSHV were subjected to an addi-

tional 30 cycles of PCR amplification. The resultant 233 bp product was purified using the Qiaex DNA gel extraction kit (Qiagen, Inc., Chatsworth, CA) or through electro-elution onto DEAE SSNA 45 paper (Schleicher & Schuell, Inc., Keene, NH). The TA Cloning Kit (Invitrogen, San Diego, CA) was used to subclone the purified PCR products according to manufacturer's instructions except the XL-1 blue strain of *E. coli* was used for transformation. The presence of an appropriately sized insert was verified by digestion of the plasmid DNA with EcoRI (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Double-stranded DNA sequencing by the dideoxy method was performed with the Sequenase Version 2.0 kit (United States Biochem. Corp., Cleveland, OH) using M13 reverse and T7 promoter 23mer as primers (New England Biolabs, Beverly, MA). Comparison to the published sequence of KSHV confirmed that we had identified and amplified the correct sequence.

Results

Analysis by DNA PCR of biopsies and PBMC from HIV-positive KS patients confirmed previous results that the sequences could be consistently detected in the biopsies (Table 1). However, we detected the sequences at a much higher frequency (90%) in the PBMC than reported previously (56%) (3). The sequences were also detected in the PBMC from immunosuppressed allograft patients and healthy donors. To detect the sequences in these individuals it was necessary to test multiple samples of 1 × 10⁶ PBMC (Table 2), whereas 10⁶ PBMC was always sufficient for KS patients. Negative control PCR reactions using the BJAB cell line were always tested in parallel and were never positive, indicating that the results were not due to contamination.

Detection of KSHV-associated Sequences in Large Circular DNA Molecules Present in KS Biopsies. The in situ lysis/Gardella gel technique can resolve the circular and linear forms of herpesvirus genomes. This is demonstrated for EBV in Fig. 1, A and B. Fig. 1 A shows a Gardella gel of 10⁶ B95-8 cells analyzed by Southern blotting for EBV sequences. Approximately 5% of the cells are known to be replicating the virus at any one time while the remainder of the cells are latently infected. The resolution of the circular and linear forms of the viral DNA can be clearly seen. We have modified the Gardella gel technique to detect small numbers of EBV infected cells or genomes by performing DNA PCR on sequential slices from the gel (8). Fig. 1 B shows the results obtained from analysis of 10 cells from the B95-8 cell line. A signal is detected at the migration point for both

Table 1. Summary of Tissues and Donors Positive for the KSHV Sequences

Tissue	Origin	No. Donors positive/ no. tested
Biopsy	KS	5/5
PBMC	KS	8/9
PBMC	Allograft	4/5
PBMC	Healthy	3/5

Table 2. Detailed Analysis of PBMC from Allograft and Healthy Individuals

Origin	Donor	No. samples positive*/no. tested
Allograft	1	6/30
	2	0/8
	3	9/10
	4	1/12
	5	1/12
Healthy	1	1/17
	2	1/15
	3	0/10
	4	2/10
	5	0/10
BJAB	KSHV negative cell line	0/40

*Each replica contained DNA extracted from 10⁶ PBMC's

circular and linear forms of EBV DNA. The signal intensity for linear DNA is much stronger than for circular DNA, because lytically infected cells have much higher genome copy numbers than latently infected cells (15).

We have applied this type of analysis to cells from four KS biopsies, but with PCR amplification for the KSHV-associated sequences. The results are summarized in Table 3 and shown in detail for one biopsy in Fig. 1 C. A PCR signal was seen for DNA migrating in the region characteristic of circular DNA, but no signal was detected in the region expected for a linear form of a herpesvirus genome. The KS DNA appeared to migrate slower than we have observed for EBV. This difference could be due to variation between gels or represent a genuine size difference. To assess this, we measured the relative mobility of circular EBV and KS DNA, by loading a KS biopsy sample and B95-8 cells in parallel lanes of the same gel (Fig. 2, A and B). This experiment confirmed that the KSHV DNA does migrate slower than EBV suggesting a significantly larger genome size (11).

To confirm that the DNA in the biopsies was circular, it was necessary to demonstrate that it would migrate faster when in the linear form. Incubation of Burkitt lymphoma biopsies at 37°C results in reactivation of the endogenous EBV genome to produce infectious virus (16). To generate the linear form of the KSHV DNA we attempted to reactivate viral replication by incubating biopsy specimens at 37°C for ~8 h. The DNA from the cells was then analyzed in parallel with freshly isolated biopsy cells and B95-8 cells. Prior incubation of the biopsy cells resulted in a PCR signal being detected at a faster mobility than the circular KS or EBV control DNA, consistent with the migration expected with a linear form of a herpesvirus genome (Fig. 2, C and D). These experiments demonstrate that the herpes-

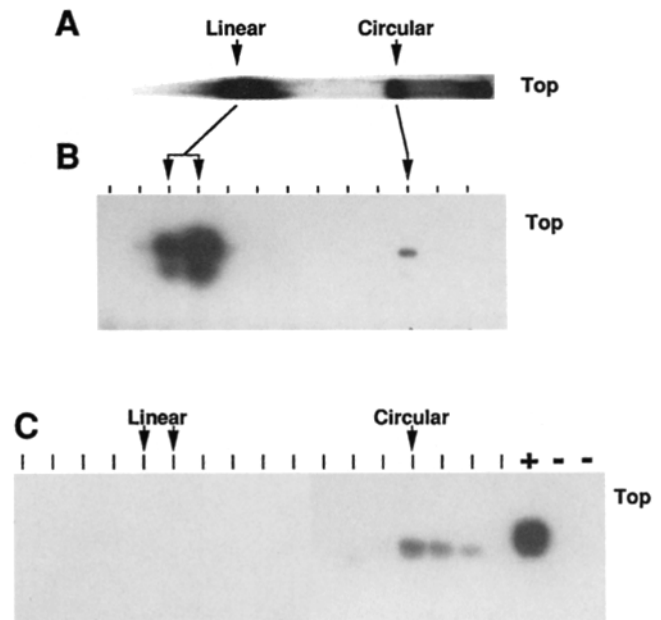


Figure 1. Gardella gel analysis for EBV and KSHV. (A) Southern blot analysis of a Gardella gel on which 10⁶ B95-8 cells were fractionated. Cells were lysed in situ, fractionated and analyzed by Southern blotting as described previously (12). The gel is shown sideways for comparison to B (below). The top of the gel is indicated. B95-8 is a marmoset cell line that contains a mixture of cells latently or lytically infected with EBV. Note the resolution obtained between the circular form of the viral genome, characteristic of latently infected cells, and the linear form characteristic of lytically infected cells. (B) As A but only 10 B95-8 cells, mixed with 10⁶ EBV-negative BJAB cells as carrier, were loaded on the gel and instead of Southern blotting directly, the gel was sliced, DNA extracted from each slice, the DNA subjected to PCR and the PCR products analyzed by Southern blotting. Vertical lines (/) denote slots of the gel loaded with DNA PCR product from each sequential gel slice. The slice closest to the top and the approximate correlation with A are indicated. The first slice of the Gardella gel was not analyzed by PCR because it was not made from low melting agarose to maintain the integrity of the sample well. (C) As B except the DNA PCR analysis was for the putative KSHV sequence KS330₂₃₃ and the cells were from the KS biopsy of patient 1. PCR analysis was as described (1). Vertical lines (/) denote analysis for the PCR products from each gel slice. (+) is a positive control of cloned PCR product and (-) are two negative control lanes (no DNA or 10⁶ BJAB cells).

virus-like sequences in KS can also migrate in the Gardella gel system at mobilities diagnostic for the linear form of a herpesvirus genome and suggest that viral reactivation can occur in culture although we cannot unequivocally exclude the possibility that the linear forms of the viral genome were generated by double stranded nicking rather than replication of the circular genomes.

Circular and Linear Forms of the KSHV DNA Are Found in the Peripheral Blood of KS Patients. DNA sequences associated with the putative KSHV have been detected by PCR in the peripheral blood of KS patients (3). We have analyzed the peripheral blood of five KS patients for the presence of circular and linear forms of the viral DNA using Gardella gels and the results are summarized in Table 3. Examples of the analysis from two KS patients and an HIV-

Table 3. Summary of Results Obtained from Gardella Analysis of Biopsies and PBMC from KS Patients

Patient#	KS Biopsy		PBMC	
	Episomes	Linear	Episomes	Linear
KS1	+	-		
KS2*	+	-	+	+
KS3	+	-		
KS4	+	-	-	+
KS5†			-	-
KS6			+	-
KS7			+	+
PBMC‡			-	-

*The PCR products from this donor were sequenced. See Fig. 4.

†No KSHV DNA was detected in the unfractionated PBMC from this patient.

‡PBMC from a healthy donor.

negative donor, who was negative for KSHV by DNA PCR, are presented in Fig. 3. We detected KSHV sequences in Gardella gel analysis of PBMC from four of the five donors tested. Of these four, circular DNA was detected in three (for example Fig. 3, A and B) and linear DNA was detected in three (for example Fig. 3 A). The presence of linear KSHV DNA raised the possibility that active viral replication is ongoing in the peripheral blood of at least some KS patients. The alternative explanation, that the linear sequences were derived by double stranded nicking of circular molecules during preparation of the samples, was unlikely because the biopsy materials were treated more harshly than the peripheral blood yet linear DNA was not detected in biopsy material.

It is possible to distinguish replication from nicking of the circular genome by comparing the sequences of the linear and circular forms. If the linear DNA was derived through nicking then the sequence of the linear and circular PCR products should be the same. If, on the other hand, they are derived through DNA replication, the sequences may vary due to the incorporation of random errors during this process. We therefore subcloned and sequenced the PCR products of the linear and circular DNA from the peripheral blood and biopsy of the same donor (Table 3, Fig. 4). As expected, the sequences were very similar and only the regions containing variations are shown. The sequences of one isolate, derived from the circular DNA of the biopsy, and two independent clones of the circular DNA from the peripheral blood were identical to each other and showed only a single base change (C > T at position 1033) compared to the published sequence. By comparison, the sequences of two independently derived clones from the linear DNA from peripheral blood showed three nucleotide changes (C > T at position 1033, A > T at

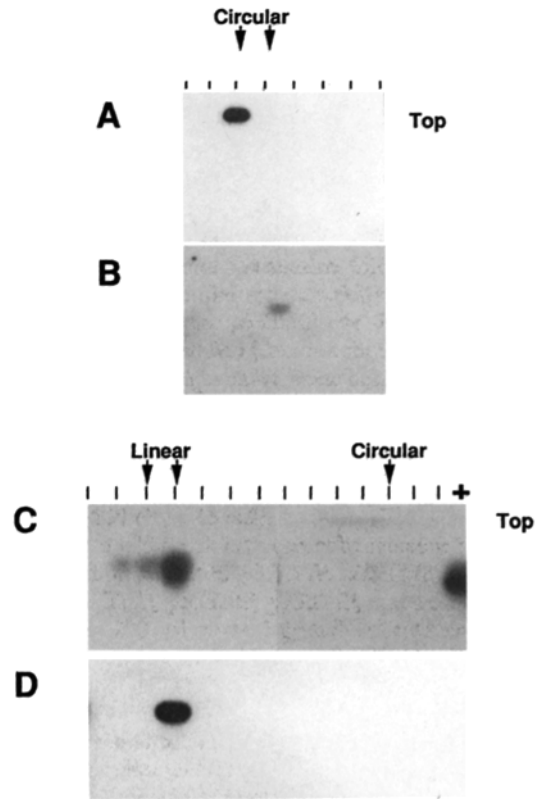


Figure 2. Relative mobility of the circular and linear forms of KSHV DNA compared to circular EBV DNA. (A) Cells from an EBV infected cell line analyzed for EBV DNA. Only the region containing circular DNA is shown. (B) Cells from the KS biopsy of patient no. 2 fractionated in a parallel lane of a Gardella gel and analyzed KSHV. Only the region containing circular DNA is shown. (C) As B but the cells were preincubated at 37°C for ~8 h to produce linear genomes. (D) As C but the cells were from patient no. 4.

position 1044 and T > C at position 1090) one of which was identical to the change seen in the sequences from the circular DNA. The observed differences in sequence between the linear and circular forms demonstrates that the linear DNA was not derived through nicking of the circular DNA. Furthermore, the presence of the same single nucleotide change in both circular and linear DNA is consistent with the linear DNA being derived by replication of the same genome that gave rise to the circular DNA.

Discussion

The results presented here suggest that KSHV, like other herpesviruses, is prevalent in the human population. The higher number of positive reactions found in two of the immunosuppressed individuals further raises the possibility that the virus may be reactivated by immunosuppression. It is also apparent that the amount of the DNA sequences detected by PCR is considerably higher in the HIV-positive KS patients than in the allograft or healthy donors. This may reflect higher levels of virus infection before the develop-

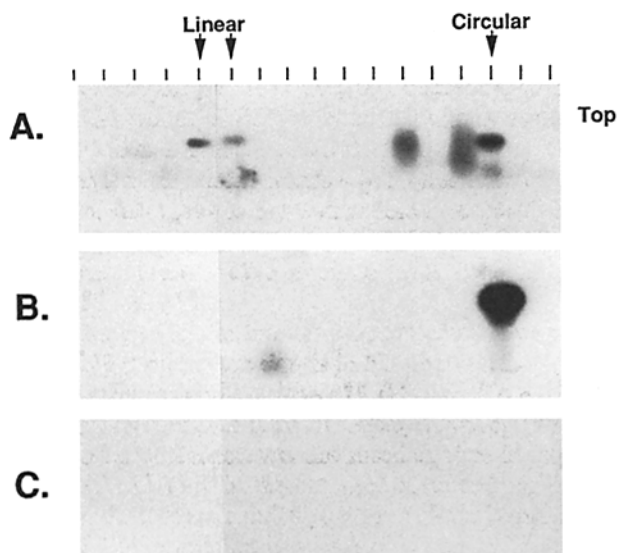


Figure 3. Detection of circular and linear forms of the KSHV DNA in the peripheral blood of KS patients. (A) PBMC from KS patient no. 2 demonstrating the presence of both linear and circular forms of the viral genome. (B) PBMC from KS patient no. 6 demonstrating the presence of circular DNA only. (C) PBMC from a control donor who was both KS negative and HIV negative.

ment of the tumor, due to HIV immunosuppression, or production of virus from tumor sites other than the skin since we have not detected viral replication in skin biopsies.

The Gardella technique is an in situ lysis gel that allows the detection of linear, episomal and integrated forms of herpesvirus DNA based on their characteristic migration. This technique can therefore be used to distinguish cells that are latently infected, containing episomes, from cells that are replicating the virus, containing linear forms of the genome (8). Based on this analysis we have found that the herpesvirus like sequences in the biopsies and peripheral blood of KS patients are contained within large, episomal, covalently closed, circular DNA structures, a genomic form characteristic of latent herpesviruses. This is direct evidence that KSHV has an intact herpesvirus genome and rules out the possibility that the viral DNA in the biopsies is all integrated, as has been speculated recently (17).

In fresh KS biopsy specimens we detected only the circular form of KSHV DNA associated with latent infection. We cannot exclude the possibility that an undetectably small fraction of cells is replicating the virus at a low level. However, by analogy with other herpesviruses, the viral genome copy number in a cell replicating the virus should be much higher, therefore easier to detect, than a latently infected cell. For example, B95-8 cells replicating EBV in vitro have several thousand copies of the viral DNA (15) whereas latently infected cells in vivo only have 2-5 copies (14) (see for example Fig. 1 B). The fact that we detect circular not linear DNA, therefore allows us to safely conclude that most of the infected cells are latently infected. The lack of viral replication explains why viral particles have not been readily detected in KS biopsies. The predominance of latently infected cells with low genome copy numbers also explains why PCR amplification is required to detect KSHV sequences by both in situ PCR (18) and Gardella gels.

One alternative interpretation of our results is that the circular episomes in biopsy material are present in infiltrating PBMCs. However, this explanation is unlikely because we have demonstrated that PBMC contain linear genomes, but have not detected linear genomes in biopsy material and Boshoff et al. have used in situ PCR to demonstrate that the KSHV-associated sequences are in the KS tumor cells (18).

We have found evidence for viral replication occurring in the peripheral blood mononuclear cells of KS patients. This conclusion is based upon the finding that linear genomes were detected and that they had different sequences from the circular forms found in the blood. Retrospective analysis of the patients we had studied revealed that the one individual with plentiful circular DNA, but no linear DNA, in the PBMC was the only patient to be currently treated with the drug ganciclovir which acts by inhibiting the replication of herpesvirus DNA. This coincidence raises the possibility that the KSHV may be susceptible to ganciclovir.

Our experiments do not address the question of whether KSHV-HHV8 plays a causal role in the pathogenesis of KS itself. The KS-associated herpesvirus shows highest levels of DNA sequence homology to lymphotropic herpesviruses and is found in B lymphocytes (19). EBV is highly B lymphotropic yet it is present in and causally associated with

	1031						1091	
	GA	CCCCGTGTTT	CCCATGGTTCG	TGCCCGACGA	ACTGGGGCAC	GCTATTCTGC	AGCAGCTGTT	GGTG
Bio. Circ.	--	--t-----	-----	-----	-----	-----	-----	----
PBL Circ.	--	--t-----	-----	-----	-----	-----	-----	----
	--	--t-----	-----	-----	-----	-----	-----	----
PBL Lin.	--	--t-----	--t-----	-----	-----	-----	-----c	----
	--	--t-----	--t-----	-----	-----	-----	-----c	----

Figure 4. Sequence analysis of KSHV from the PBMC and tumor biopsy. Each sequence was derived from an independently isolated clone of PCR product from the same donor (see legend to Table 2). The top line is the published sequence for KS330₂₃₃ (1) and the numbers refer to the nucleotide numbering used in that reference. The sequences

below are our aligned sequences showing only the region that differed from the published sequence. (Bio. Circ.) Circular DNA from the KS biopsy. (PBL Circ.) Circular DNA from the PBMC.

the development of non-lymphoid tumors especially the epithelial cell tumor nasopharyngeal carcinoma. By analogy, the KS herpesvirus may also be lymphotropic and

normally benign in these cells, but becomes oncogenic when it inappropriately gains access to the precursor cells for KS.

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Address correspondence to David A. Thorley-Lawson, Department of Pathology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111.

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