

## MONOCLONAL ORIGIN OF MULTICENTRIC KAPOSI'S SARCOMA LESIONS

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**ABSTRACT**

**Background** Kaposi's sarcoma has features of both hyperplastic proliferation and neoplastic growth. Multiple lesions, in which spindle cells are prominent, often arise synchronously over widely dispersed areas. We tested the hypothesis that the spindle cells in these multicentric lesions originate from a single clone of precursor cells.

**Methods** To determine whether Kaposi's sarcoma is a monoclonal disorder, we assessed the methylation patterns of the androgen-receptor gene (*HUMARA*) in multiple lesions from women with the acquired immunodeficiency syndrome. In polyclonal tissues, about half the copies of each *HUMARA* allele are methylated, whereas in cells derived from a single clone all the copies of only one allele are methylated. To minimize contamination by normal DNA, we used microdissection to isolate areas composed primarily of spindle cells, the putative tumor cells.

**Results** Eight patients with a total of 32 tumors were studied. Of these tumors, 28 had highly unbalanced methylation patterns (i.e., predominant methylation of one *HUMARA* allele). In all the tumors that had unbalanced methylation from a given patient, the same allele predominated.

**Conclusions** These data indicate that Kaposi's sarcoma is a disseminated monoclonal cancer and that the changes that permit the clonal outgrowth of spindle cells occur before the disease spreads. (N Engl J Med 1997;336:988-93.)

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**K**APOSI'S sarcoma is the most common tumor in patients with the acquired immunodeficiency syndrome (AIDS).<sup>1,2</sup> The lesions associated with the disease have four characteristic components: thin-walled neovascular formations, extravasated red cells, inflammatory lymphocytes, and proliferating spindle cells. The spindle cells, which may be the primary abnormality, are more prominent in nodular tumors than in plaque or patch lesions. Although their origin is unknown, spindle cells have the characteristic immunohistochemical and ultrastructural features of endothelial cells.<sup>3,4</sup> The neoplastic or hyperplastic nature of spindle cells has long been debated,<sup>5,6</sup> but the demonstration that individual Kaposi's sarcoma lesions are clonal proliferations supports the idea that Kaposi's sarcoma is a neoplasm.<sup>7</sup> In affected patients multiple lesions can appear synchronously in widely dispersed areas, without evidence of a primary tumor as a source of metastasis. Therefore, a currently

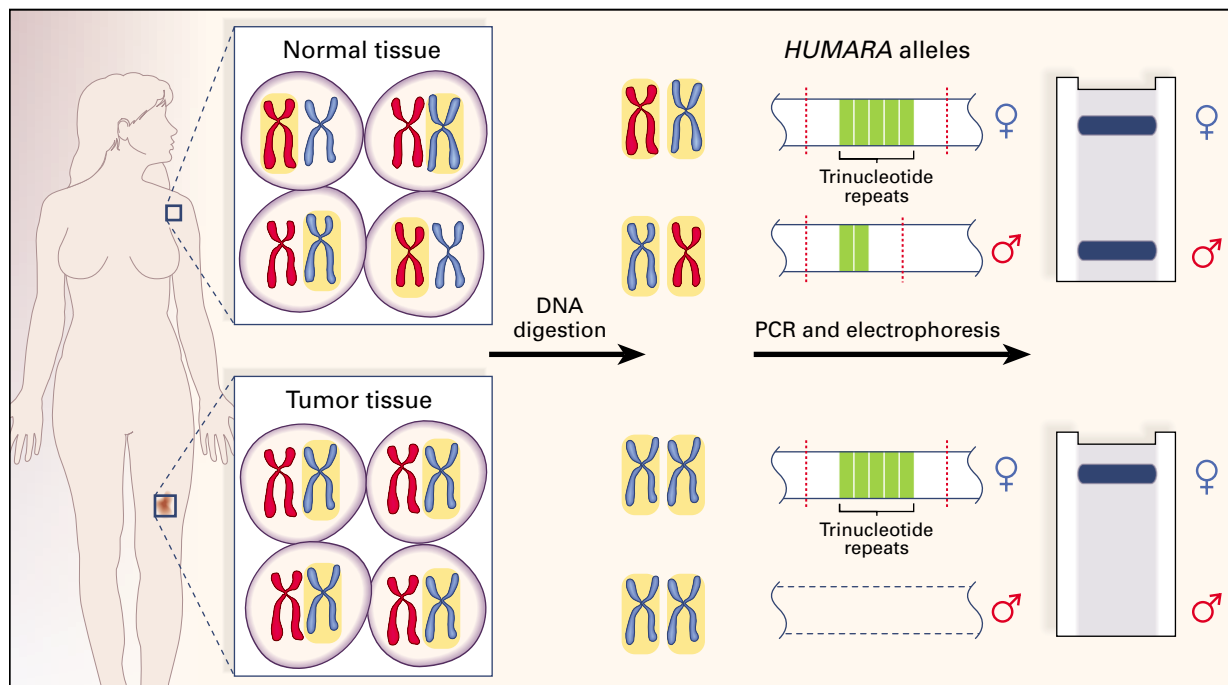
favored hypothesis is that the individual lesions of Kaposi's sarcoma arise in situ by neoplastic transformation of local precursors.<sup>8</sup> We tested this idea by analyzing multiple lesions from the same patient to determine their clonal relatedness.

We assessed clonal relatedness in Kaposi's sarcoma cells from women by studying patterns of X-chromosome methylation.<sup>9,10</sup> At an early stage of development, one of the two X chromosomes in each cell of a female embryo is inactivated by methylation, and that methylation status is retained in subsequent somatic-cell divisions. Thus, in polyclonal tissues, approximately half the cells have a methylated X chromosome from one parent, and half have a methylated X chromosome from the other parent. By contrast, in a clone derived from a common progenitor, the same X chromosome is methylated and the other is unmethylated in every cell. This principle is applicable only to tissues from female subjects who are heterozygous for an X-linked marker gene and whose normal tissue has an approximately balanced pattern of methylation. For the marker gene, we used the X-linked androgen-receptor gene (*HUMARA*), which has a highly polymorphic (with a greater than 90 percent prevalence of heterozygosity) trinucleotide-repeat sequence proximal to a methylation site and is thus suitable for study by an assay of clonality based on the polymerase chain reaction (PCR).<sup>11</sup> We used a methylation-sensitive restriction endonuclease (*HpaII*) to digest unmethylated *HUMARA* sequences before PCR amplification; both alleles of the gene are amplified in randomly methylated tissue, whereas in clonal tissue one allele is absent because all its copies are unmethylated (Fig. 1).

**METHODS**

Ten women with multiple nodular Kaposi's sarcoma lesions were recruited between April and June 1994 from among the patients of the Dermatovenereology Clinic of the University Teaching Hospital in Lusaka, Zambia. Human immunodeficiency virus (HIV) infection was confirmed serologically in eight women and diagnosed clinically in the other two, who declined serologic testing for HIV. Two women had received a single dose of intravenous vincristine two to five months before recruitment, and the remaining eight had not been treated for Kaposi's sarcoma. The median age of the women was 26 years (range, 20 to 35). All the women gave informed consent for their participation. The

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**Figure 1.** Clonality Assay of the *HUMARA* Gene.

In women, each somatic cell contains two X chromosomes, one derived from the father (red) and the other derived from the mother (blue). One of the X chromosomes is inactivated by methylation (yellow halo), but the other is active and unmethylated. Normal somatic tissue (upper rows) is a mosaic of cells. In some cells the maternally derived X chromosome is methylated, and in others the paternally derived chromosome is methylated. In tumor tissue (lower rows) the same X chromosome is methylated in all cells (the maternally derived chromosome, in this example). DNA from methylated chromosomes resists digestion by methyl-sensitive restriction endonucleases such as *HpaII*, which preferentially digest unmethylated DNA sequences. After *HpaII* digestion, DNA from normal tissue contains a mixture of maternal and paternal X-chromosome sequences, whereas DNA from clonal tissue contains one or the other, but not both.

Over 90 percent of people are heterozygous for the number of trinucleotide repeats in exon 1 of the X-linked androgen-receptor gene (*HUMARA*). After amplification by PCR of the region containing the repeats (delineated by red lines), the maternal and paternal *HUMARA* alleles can be separated by polyacrylamide-gel electrophoresis and visualized as bands on autoradiography. DNA from normal tissue, which contains a mixture of two methylated alleles, generates bands of approximately equal intensity for each (upper gel). DNA from a monoclonal population of cells generates a single band that corresponds to its one methylated allele (lower gel). In practice, tumors usually contain some polyclonal stromal cells and generate a second band, of diminished intensity.

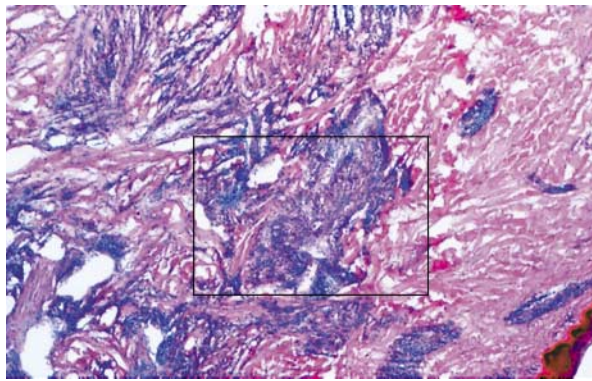
study protocol was reviewed and approved by the institutional review boards of the University Teaching Hospital and the National Cancer Institute.

Biopsy specimens were obtained from five widely separated superficial cutaneous nodular tumors in each patient, as well as from nonadjacent normal skin. The specimens were immediately snap-frozen in liquid nitrogen and kept frozen until they were sectioned. At that time, the specimens were mounted in Optimal Cutting Temperature compound (Miles, Elkhart, Ind.), and frozen sections 10  $\mu\text{m}$  thick were cut on a cryostat. The sections used for microdissection were fixed on slides and stained with hematoxylin and eosin.

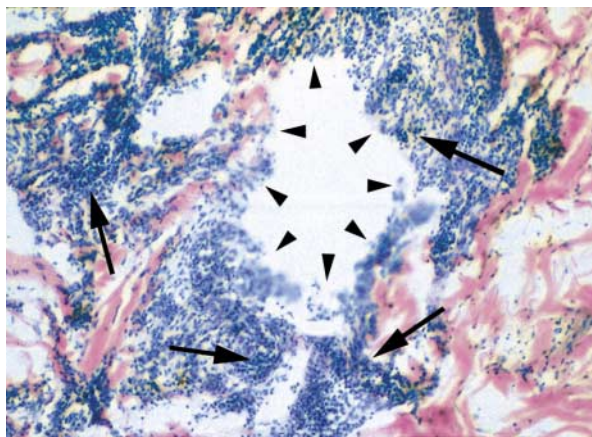
The number of CAG repeats in exon 1 of the *HUMARA* gene was determined for each allele. DNA for these assays was obtained from unstained sections of whole tissue, which were first washed with distilled water to remove the Optimal Cutting Temperature compound. The tissues were digested with proteinase K buffer, and the DNA was extracted with phenol and chloroform. Regions containing microsatellite repeats were amplified by direct PCR with *Taq* polymerase and one of the following primer pairs (designed from GenBank entry HUMARA01): 5'GAAGGGGA-

GGCGGGGTAAGGGAAGT3' and 5'CGACTGCGGCTGTGAAGGTGCTGT3'; 5'TCCAGAATCTGTCCAGAGCGTGC3' and 5'GCTGTGAAGGTGCTGTTCCAT3'; or 5'GCGCGAAGTGATCCAGAAC3' and 5'CCAGGACCAGGTAGCCTG3'. Allele-specific PCR fragments were separated on vertical 8 to 12 percent denaturing polyacrylamide gels, stained with ethidium bromide, purified with GeneClean (Bio 101, La Jolla, Calif.), ligated into pCRII vectors, and cloned in *Escherichia coli* (Invitrogen, San Diego, Calif.). Plasmid DNA was prepared and used as template to determine the number of microsatellite-repeat units in each allele by the dideoxy chain-termination method with the Sequenase kit (USB, Cleveland) or by cycle sequencing with the Femtomole kit (Promega, Madison, Wis.).

To obtain samples of tumor DNA and normal DNA, the tissue sections were subjected to microdissection with a 30-gauge needle under magnification by a power of 40, as previously described.<sup>12</sup> Tumor material was dissected from areas with a high proportion of spindle cells and a low proportion of lymphocytes (Fig. 2). Normal tissue was obtained from areas of dermis, including glands, lymphocytes, or both, in the biopsy specimens of nonadjacent normal skin. However, in two cases adjacent normal skin was used



A



B

**Figure 2.** Representative Samples of Kaposi's Sarcoma Tissue before and after Microdissection.

Panel A ( $\times 40$ ) shows a section of a Kaposi's sarcoma nodule from Patient 2, with prominent proliferating spindle cells. Panel B ( $\times 100$ ) shows the area within the rectangle in Panel A after a portion containing tumor (arrowheads) has been removed by microdissection. Microdissection is used to obtain a sample free of dermis and lymphocytes (arrows) that are presumed to contain normal DNA rather than tumor DNA. (Hematoxylin and eosin.)

because of the scant cellularity of the biopsy specimens of non-adjacent normal skin. The tissue scrapings were suspended in 15 to 30  $\mu\text{l}$  of proteinase K buffer (0.1 mg of proteinase K per milliliter, 0.1 mol of EDTA per liter, 1 percent polysorbate 20 [Tween 20], and TRIS-hydrochloric acid [pH 8.0]). The suspensions were incubated overnight at 42°C, then inactivated by heat at 95°C for five minutes before their subsequent manipulation.

Clonality was determined with an adaptation<sup>13</sup> of the *HUMARA* methylation assay.<sup>11</sup> In brief, 10 units of the methyl-sensitive restriction endonuclease *HpaII* was added to 5  $\mu\text{l}$  of the DNA-preparation mixture, incubated at 37°C for one to three hours, and then inactivated by heat at 95°C for five minutes. Half this solution was then added to a 10- $\mu\text{l}$  reaction mixture for PCR amplification of the *HUMARA* gene with the primer pair 5'-GCTGIGAAGGTTGCTGTTCCTCAT3' and 5'-TCCAGAATCTGTTCCAGAGCGTGC3'.<sup>14</sup> The reaction mixture contained 50 pmol of each primer, 20 nmol of each deoxynucleotide tri-

phosphate, 0.2  $\mu\text{l}$  of [ $\gamma$ -<sup>32</sup>P]deoxycytidine triphosphate (600 Ci per millimole), 1 mM TRIS-hydrochloric acid (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01 percent gelatin (wt/vol), and 0.1 unit of *Taq* polymerase. Thermal cycling was carried out at 94°C for 45 seconds, 63°C for 45 seconds, and 72°C for 40 seconds, for a total of 35 cycles. The reaction products were subjected to electrophoresis on 6 percent denaturing polyacrylamide sequencing gels and visualized by autoradiography on photographic film. A 75 percent diminution in allelic intensity (relative to the intensity of the remaining allele) was arbitrarily considered to be indicative of unbalanced methylation (i.e., predominant methylation of one *HUMARA* allele) and — assuming that the methylation pattern of the alleles was balanced in the corresponding normal DNA — clonal derivation of tumor DNA.

## RESULTS

Two of the 10 patients were excluded from further analysis because they were homozygous for the *HUMARA* gene. The eight heterozygous patients had from 7 to 20 CAG trinucleotide repeats at that locus, with a minimal difference of 3 repeats (i.e., 9 base pairs) between the two alleles (Table 1). In two cases, there was scant DNA from normal dermis that could not be amplified after digestion with a restriction endonuclease. Digested normal DNA from the other six patients contained two allelic bands of approximately equal intensity. Hence, balanced methylation of normal-dermis DNA was demonstrated in six of the eight patients.

Of the 40 tumors, 32 could be studied for clonality; in the rest, there was insufficient material for microdissection (2 tumors) or a failure of PCR amplification after endonuclease restriction (6 tumors). The six women who had balanced methylation patterns in normal dermis had a total of 27 tumors that could be evaluated. Unbalanced methylation (the predominance of one *HUMARA* allele) was found in 23 of these tumors (85 percent); the remaining 4 had balanced methylation (Fig. 3). The two patients whose samples of normal-dermis DNA could not be amplified by PCR had a total of five tumors that could be evaluated, all of which had unbalanced methylation.

In each patient, the same *HUMARA* allele was methylated in all the tumors with unbalanced methylation (Fig. 3). The larger allele was preferentially methylated in three patients, and the smaller allele predominated in the other five. No patient had tumors with discordant patterns of unbalanced methylation. When the two patients were excluded in whom balanced methylation of normal DNA could not be confirmed, the remaining six patients had a total of 23 tumors with concordant methylation of one of the two alleles: two patients with 5 tumors each, one with 4, and three with 3 each. Assuming that the chances of methylation of either allele were equal, the probability of concordant patterns was 50 percent for two tumors, 25 percent for three tumors, 12.5 percent for four tumors, and 6.25 percent for five tumors. Under the hypothesis that the alleles in

**TABLE 1.** SELECTED CLINICAL AND GENOTYPIC CHARACTERISTICS OF 10 WOMEN WITH AIDS-RELATED KAPOSI'S SARCOMA.

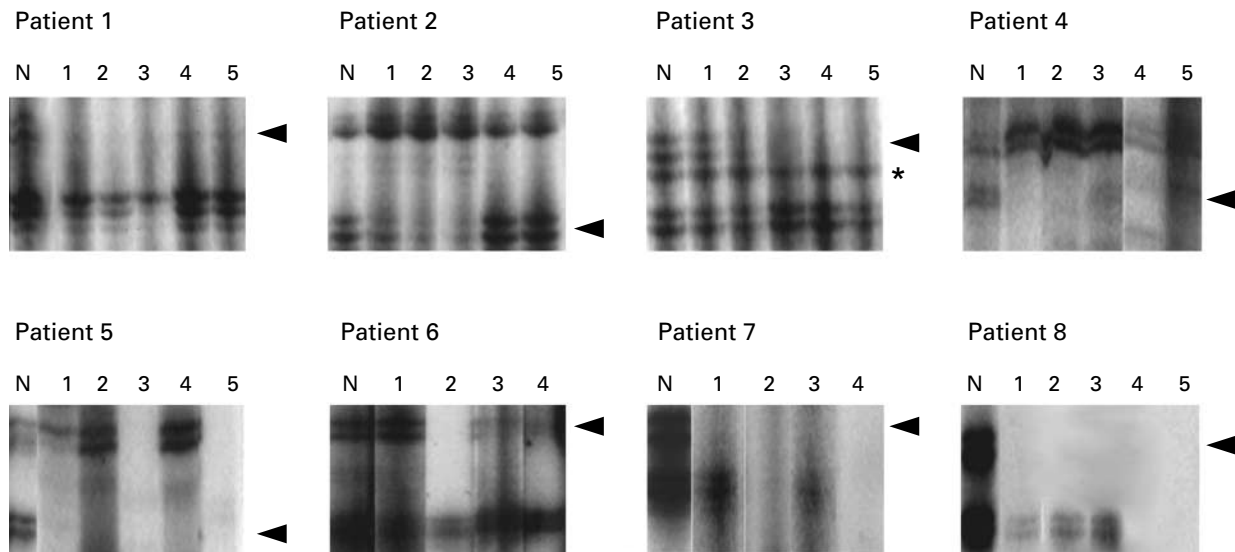
PATIENT No.	AGE (YR)	HIV SEROLOGIC STATUS	TIME FROM KAPOSI'S SARCOMA DIAGNOSIS TO BIOPSY (MO)	PREVIOUS TREATMENT	No. OF CAG REPEATS IN HUMARA*		No. OF TUMORS WITH UNBALANCED METHYLATION†
					ALLELE 1	ALLELE 2	
1	30	Positive	0	None	11	15	5
2	—	Not tested‡	—	None	13	20	3
3	35	Positive	5	Vincristine§	7	12	4
4	26	Positive	2	Vincristine§	13	19	5
5	25	Positive	0	None	8	19	3
6	—	Not tested‡	—	None	12	18	3
7	25	Positive	3	None	12	15	2
8	23	Positive	0	None	10	18	3
9	20	Positive	0	None	10	10	Not tested
10	35	Positive	0	None	13	13	Not tested

\*The numbers shown are the numbers of microsatellite repeats within exon 1 of the androgen-receptor gene, as described in the text.

†Five tumors from each patient were studied at biopsy.

‡HIV infection was diagnosed clinically in this patient.

§One intravenous dose was given at the time of the diagnosis of Kaposi's sarcoma.



**Figure 3.** Assessment of the Clonality of Kaposi's Sarcoma Lesions from Patients Heterozygous for the *HUMARA* Allele.

DNA from four or five distinct tumors (lanes 1 through 5) and normal skin (N) from each of eight patients was digested with *Hpa*II and amplified by PCR. The dark bands are due to the presence of methylated *HUMARA* alleles, with larger alleles causing the upper bands and smaller alleles the lower ones. The presence of multiple bands is attributable to the slippage of DNA polymerase during amplification. The arrowheads designate diminished or absent parental alleles owing to the unbalanced methylation pattern in clonal populations of cells. The asterisk indicates a constant band not specifically detected by the *HUMARA* assay; the blank lanes represent samples containing DNA that could not be amplified by PCR after digestion by a restriction endonuclease.

In Patients 1 and 4, all five tumors studied showed unbalanced methylation patterns; in Patient 5, three tumors showed such patterns and DNA from the other two tumors could not be amplified. Patients 2, 3, and 6 had balanced methylation patterns in one or two tumors each, but there was unbalanced methylation in the remaining three or four tumors. For Patients 7 and 8, the samples of DNA from normal skin could not be amplified after endonuclease digestion; for these patients, the lanes denoted N represent undigested DNA. Patients 7 and 8 each had two or three samples of tumor DNA that showed unbalanced methylation, and the remaining samples from these patients could not be amplified. In each of the eight patients, all the tumors with unbalanced methylation had the same methylated allele.

different tumors from the same patient were independent of each other with respect to their methylation patterns, the combined probability of the observed results was less than  $0.00001$  ( $0.0625^2 \times 0.125 \times 0.25^3$ ).

## DISCUSSION

We have demonstrated concordance among the patterns of methylation of the X-linked *HUMARA* alleles in different Kaposi's sarcoma tumors from a given female patient. This finding indicates that multiple Kaposi's sarcoma lesions in the same patient arise from a single clone of cells. This evidence argues that Kaposi's sarcoma is a disseminated monoclonal cancer.

The specificity of the tumor DNA we analyzed was enhanced by increasing the spindle-cell content of the samples by microdissection. Nevertheless, some tumors with balanced methylation may have contained excessive normal stromal DNA. We estimate that the tumor DNA content of a sample must be at least 80 percent for unbalanced methylation to be detected consistently by autoradiography. An additional consideration is the limited recovery of DNA after microdissection, a likely cause of the failure of PCR amplification after endonuclease restriction in several samples.

Previous tests of clonality in spindle cells from Kaposi's sarcoma lesions have yielded variable results. Immunostaining of spindle cells has been reported to be heterogeneous, which would be inconsistent with monoclonality.<sup>5</sup> However, opposite findings have also been reported with this technique.<sup>4</sup> Spindle cells typically have a diploid DNA content,<sup>15,16</sup> but some high-grade lesions appear to be aneuploid, which would indicate the clonal proliferation of abnormal cells.<sup>17,18</sup>

Our approach assumes that multiple stem cells form the normal tissue from which spindle cells arise.<sup>19</sup> We used unaffected dermis as a polyclonal control because this normal tissue has not been identified. But if this tissue arises from a single cell after the embryologic phase of X-chromosome inactivation, it would have an unbalanced methylation pattern and would invalidate our assay. Although we cannot rule out this possibility, our findings cannot be explained by the presence of local monoclonality in patches of such normal tissue, because we found concordant allelic methylation in widely separated tumor nodules in the same patient. Validation of our results may require that the cell of origin of spindle cells and its distribution in normal tissues be identified.

The role of human herpesvirus 8 (HHV-8) in Kaposi's sarcoma is uncertain. This virus, which is found nearly universally in Kaposi's sarcoma tissues, may be necessary for the development of the disease.<sup>20</sup> Plausibly, HHV-8 infection may transform a circulating precursor cell, which in tissues develops into spindle

cells. In lymphocytes transformed by the related Epstein-Barr virus (EBV), the linear EBV genome becomes circular by joining its ends in a way that produces episomes of variable size. Clonally related cells contain episomes in one or several specific sizes, indicating that the entry and circularization of EBV precede clonal outgrowth.<sup>21</sup> If intracellular HHV-8 becomes circular in a similar way, then the structure of the fused termini could be an independent indicator of clonality in Kaposi's sarcoma.

Our data imply that each lesion of Kaposi's sarcoma arises from a monoclonal population of circulating progenitor cells that home to multiple local sites and proliferate. The circulating cells are potentially related to the spindle-shaped cells that can be cultured from peripheral blood, whose concentration is increased in HIV-infected patients who have Kaposi's sarcoma or are at high risk for it.<sup>22</sup> We do not know whether this process occurs in the endemic or transplantation-associated cases of Kaposi's sarcoma. In addition, it remains to be demonstrated whether the neoplastic clone persists over time in recurrent Kaposi's sarcoma, as has been demonstrated with regard to B-lymphocyte neoplasms.<sup>23</sup> These data also warrant the examination of Kaposi's sarcoma lesions for other genetic changes, such as mutations, rearrangements, amplifications, deletions, and allelic losses, to further our understanding of this enigmatic disorder.

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