

Rearrangements of the *BCL-6* Gene in Acquired Immunodeficiency Syndrome-Associated Non-Hodgkin's Lymphoma: Association With Diffuse Large-Cell Subtype

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Acquired immunodeficiency syndrome (AIDS)-associated non-Hodgkin's lymphomas (AIDS-NHL), a major source of morbidity and mortality among AIDS patients, are derived from B cells and can be classified into two main histologic categories, small noncleaved cell lymphoma (SNCL) and diffuse large-cell lymphoma (DLCL). DLCL includes two histologic subsets, ie, large noncleaved cell lymphoma (LNCCL) and large cell-immunoblastic plasmacytoid lymphoma (LC-IBPL). Several studies have shown that AIDS-SNCL is associated with the clonal accumulation of multiple genetic lesions, including Epstein-Barr virus (EBV) infection, activation of the *c-MYC* and *RAS* oncogenes, as well as inactivation of the *p53* tumor suppressor gene at variable frequencies. On the contrary, the molecular pathogenesis of AIDS-DLCL is largely obscure, because no genetic lesion other than EBV infection has been specifically identified in this group. In this

study, we have tested a panel of 40 AIDS-NHL for structural alterations of *BCL-6*, a putative proto-oncogene that is frequently altered in DLCL in the immunocompetent host. Our results show that rearrangements of *BCL-6* are present in 20% of AIDS-DLCL (5 of 24), including 2 of 8 LNCCL and 3 of 16 LC-IBPL, but in no case of AIDS-SNCL. *BCL-6* rearrangements were detected both in the presence and in the absence of EBV infection of the tumor clone, but in no case were associated with activation of *c-MYC* or mutations of *p53*. These data identify a novel genetic lesion in AIDS-DLCL and corroborate the notion that lymphomagenesis in AIDS follows two distinct molecular pathways that are associated with the development of histologically distinct types of AIDS-NHL.

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NON-HODGKIN'S lymphomas (NHL) represent one of the most common malignancies associated with human immunodeficiency virus (HIV) infection, and are recognized as an acquired immunodeficiency syndrome (AIDS)-defining condition.¹⁻³ Since their initial observation in 1982,⁴ the incidence of AIDS-associated NHL (AIDS-NHL) has been consistently increasing,^{1,2} and they now represent the most frequent HIV-associated malignancy in some AIDS risk groups, namely, the hemophiliacs.⁵ Indeed, some estimates project that 10% to 20% of all new NHL cases in the United States may eventually be related to AIDS.⁶

AIDS-NHL are almost invariably B-cell-derived NHL.^{1,2,7-12} When compared with NHL of similar histology arising in the immunocompetent host, AIDS-NHL display distinctive clinical features, including late stage at presentation, poor prognosis, and the frequent involvement of extranodal sites.^{1,2,7-12} Systemic AIDS-NHL are histologically heterogeneous, and have been initially classified into three distinct categories, including small noncleaved cell lymphoma (SNCL), large noncleaved cell lymphoma (LNCCL), and large cell-immunoblastic plasmacytoid lymphoma (LC-IBPL).^{7,9} Subsequently, most investigators have agreed to classify LNCCL and LC-IBPL as a single category under the term of diffuse large-cell lymphoma (DLCL).¹³

Some progress has been made in elucidating the molecular pathogenesis of AIDS-SNCL.¹⁻³ We and others have shown that AIDS-SNCL is associated at variable frequency with multiple genetic lesions, including Epstein-Barr virus (EBV) infection, *c-MYC* translocation, *RAS* gene family mutation, and *p53* inactivation by point mutation and allelic loss.^{1,3,14-26} On the other hand, the pathogenesis of AIDS-DLCL is relatively less defined. EBV infection appears to be the only genetic lesion associated with a significant fraction of these tumors, particularly with the subset displaying plasmacytoid features; *p53* lesions have not been found

and *c-MYC* activation is restricted to a small minority of cases.^{1-3,14-26}

Recently, rearrangements of the *BCL-6* gene, located on 3q27 and coding for a putative zinc finger transcription factor, have been identified as a recurrent genetic lesion of DLCL in the immunocompetent host.²⁷⁻³⁰ The same gene has also been called *LAZ-3*^{31,32} or *BCL-5*,³³ although the name *BCL-5* had already been assigned to a locus on 17q22³⁴ and the name *BCL-6* has been officially adopted.³⁴ Rearrangements of *BCL-6* result in truncation within 5' noncoding regulatory sequences and occur in 30% to 40% of DLCL.²⁸ Cytogenetic analysis has suggested that breakpoints at 3q27, the site of *BCL-6*, may occur also in AIDS-DLCL,³⁵ although the exact frequency has not been determined and the involvement of *BCL-6* has not been documented. These observations have prompted our analysis of *BCL-6* re-

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arrangements in a panel of AIDS-NHL representative of both the SNCL and DLCL subtypes. We wished to define the frequency of *BCL-6* involvement in AIDS-NHL and to correlate its presence with other genetic lesions that have been described in the pathogenesis of AIDS-NHL.

MATERIALS AND METHODS

Pathologic samples. Biopsy samples of lymph node, bone marrow, peripheral blood, or other involved organs from 40 patients with AIDS were collected during the course of standard diagnostic procedures. Thirty-two samples were derived from patients referred to the Department of Pathology, New York University (New York, NY) or to the Department of Pathology, Columbia University (New York, NY) and their histology was reviewed by D.M.K. Eight samples were derived from patients referred to the Departments of Hematology and Pathology, University of Southern California School of Medicine (Los Angeles, CA) and their histology was reviewed by D.S. Diagnosis was based on analysis of histopathology, immunophenotypic analysis of cell surface markers, and immunogenotypic analysis of Ig gene rearrangement.³⁶ In most cases, the fraction of malignant cells in the pathologic specimen was greater than 80%, as determined by cell suspension cytofluorometric or tissue section immunohistochemical analysis of cell surface markers and by Ig gene rearrangement analysis.

DNA extraction and Southern blot analysis. DNA was purified by digestion with proteinase K, "salting out" extraction, and precipitation by ethanol.³⁷ For Southern blot analysis,³⁸ 6 μ g of DNA was digested with the appropriate restriction endonuclease, electrophoresed in a 0.8% agarose gel, denatured, neutralized, transferred to Duralon filters (Stratagene, La Jolla, CA), and hybridized to probes that had been ³²P-labeled by the random primer extension method.³⁹ Filters were washed in 0.2 $\times$ SSC (NaCl/Na citrate)/0.5% sodium dodecyl sulphate (SDS) for 2 hours at 60°C and then autoradiographed using intensifying screens (Quanta III; Dupont, Boston, MA).

DNA probes. Ig gene rearrangement analysis was performed using a J_H probe⁴⁰ (a gift of Dr S.J. Korsmeyer) on *Hind*III, *Eco*RI, and *Bam*HI digests. The organization of the *BCL-6* locus was investigated by hybridization of *Xba*I, *Bam*HI-, and *Bgl*II-digested DNA to the human *BCL-6* probe Sac4.0.^{27,28} In selected cases, a second probe representative of the *BCL-6* locus, Sac0.8, was also used. The organization of the *c-MYC* locus was analyzed by hybridization of *Eco*RI- and *Hind*III-digested DNA to the human *c-MYC* probe MC413RC, representative of the third exon of the *c-MYC* gene.⁴¹ The presence of the EBV genome was investigated with a probe specific for the EBV termini (5.2-kb *Bam*HI-*Eco*RI fragment isolated from the fused *Bam*HI terminal fragment NJ-het).⁴²

RESULTS

Forty cases of systemic AIDS-NHL were studied, including 13 SNCL and 24 DLCL (8 LNCL and 16 LC-IBPL). In addition, 3 cases of CD30⁺ lymphomas, which have been sporadically reported in AIDS,⁴³ were also included. All cases displayed a predominant monoclonal B-cell population as determined by Ig gene rearrangement analysis (data not shown).

Analysis of *BCL-6* rearrangements. The *BCL-6* gene contains at least 9 exons spanning approximately 26 kb of genomic DNA.²⁸ Sequence analysis has shown that the first exon is noncoding and that the translation initiation codon is located within the third exon.²⁸ Rearrangements of *BCL-6* can be detected by Southern blot analysis using a probe

(Sac4.0) and restriction enzymes (*Bam*HI and *Xba*I) that, in combination, explore a region of 15.2 kb containing the 5' portion of the *BCL-6* gene^{28,30} (Fig 1). This same region was previously shown to contain the cluster of chromosomal breakpoints detected in NHL of the immunocompetent host.^{28,31} Cases showing an abnormally migrating band in only one digest were further studied by hybridizing the Sac4.0 probe to additional digests (*Bgl*II) or, alternatively, by hybridizing *Bam*HI and *Xba*I digests to a probe (Sac0.8) derived from the *BCL-6* first intron, which, being located 3' of the breakpoint cluster, explores the reciprocal chromosome 3 (Fig 1). Only cases showing abnormally migrating bands with two restriction enzymes and/or two probes were scored as rearranged.

Rearrangements of *BCL-6* were detected in 5 of 24 AIDS-DLCL (20.8%), both in the LNCL (2 of 8; 25%) and in the LC-IBPL (3 of 16; 18.7%) variants (Table 1 and Fig 1). All cases of AIDS-SNCL and CD30⁺ lymphomas displayed a germline *BCL-6* locus (Table 1 and Fig 1). The location of the breakpoints detected in AIDS-NHL corresponds to the pattern most commonly observed in DLCL of the immunocompetent host^{28,31} (Fig 1).

Other genetic lesions. The other genetic lesions investigated in the panel of AIDS-NHL included infection by EBV of the tumor clone, activation of the *c-MYC* and *RAS* proto-oncogenes, and inactivation of the *p53* tumor suppressor gene. The experimental strategies used to investigate these lesions have been described in detail elsewhere.^{14,15,44} For some of the cases, the molecular characterization of these genetic lesions had been previously reported^{14,15,45}; for the other cases, it has been assessed in the course of this study.

EBV infection was assessed by Southern blot hybridization using a probe representative of the EBV termini⁴² that allows us to analyze clonality in EBV-infected tissues²⁴ (Fig 2). A monoclonal infection was detected in 5 of 13 (38%) SNCL, 17 of 24 DLCL (71%) (3 of 8 [37.5%] LNCL and 14 of 16 [87.5%] LC-IBPL), and 3 of 3 (100%) CD30⁺ cases.

Rearrangements of *c-MYC* were tested by hybridizing *Hind*III- and *Eco*RI-digested DNAs with a probe representative of *c-MYC* exon 3⁴¹ (Fig 2). Rearrangements were present in 13 of 13 SNCL (100%), 5 of 24 (20.8%) DLCL (2 of 8 [25%] LNCL and 3 of 16 [18.7%] LC-IBPL), and 2 of 3 CD30⁺ cases.

Mutations of *p53* and *RAS* were analyzed by a two-step strategy. Single-strand conformation polymorphism (SSCP) analysis was applied to *p53* exons 5 through 9 (in 29 cases) or *p53* exons 5 through 8 (in 6 cases) (Fig 2) and to N-, K-, and H-*RAS* exons 1 and 2 (in 29 cases); cases displaying an altered electrophoretic pattern by SSCP were further studied by DNA direct sequencing of the PCR product. *p53* mutations were scored in 8 of 13 (61.5%) SNCL, but in none of the DLCL tested (0 of 22). Finally, *RAS* activation by point mutation was positive in 3 of 13 (23%) SNCL and in 1 of 16 (6%) DLCL tested.

The molecular features of the cases displaying *BCL-6* rearrangements are listed in Table 2. Overall, *BCL-6* rearrangements were detected both in the presence and in the absence of clonal EBV infection of the tumor, whereas c-

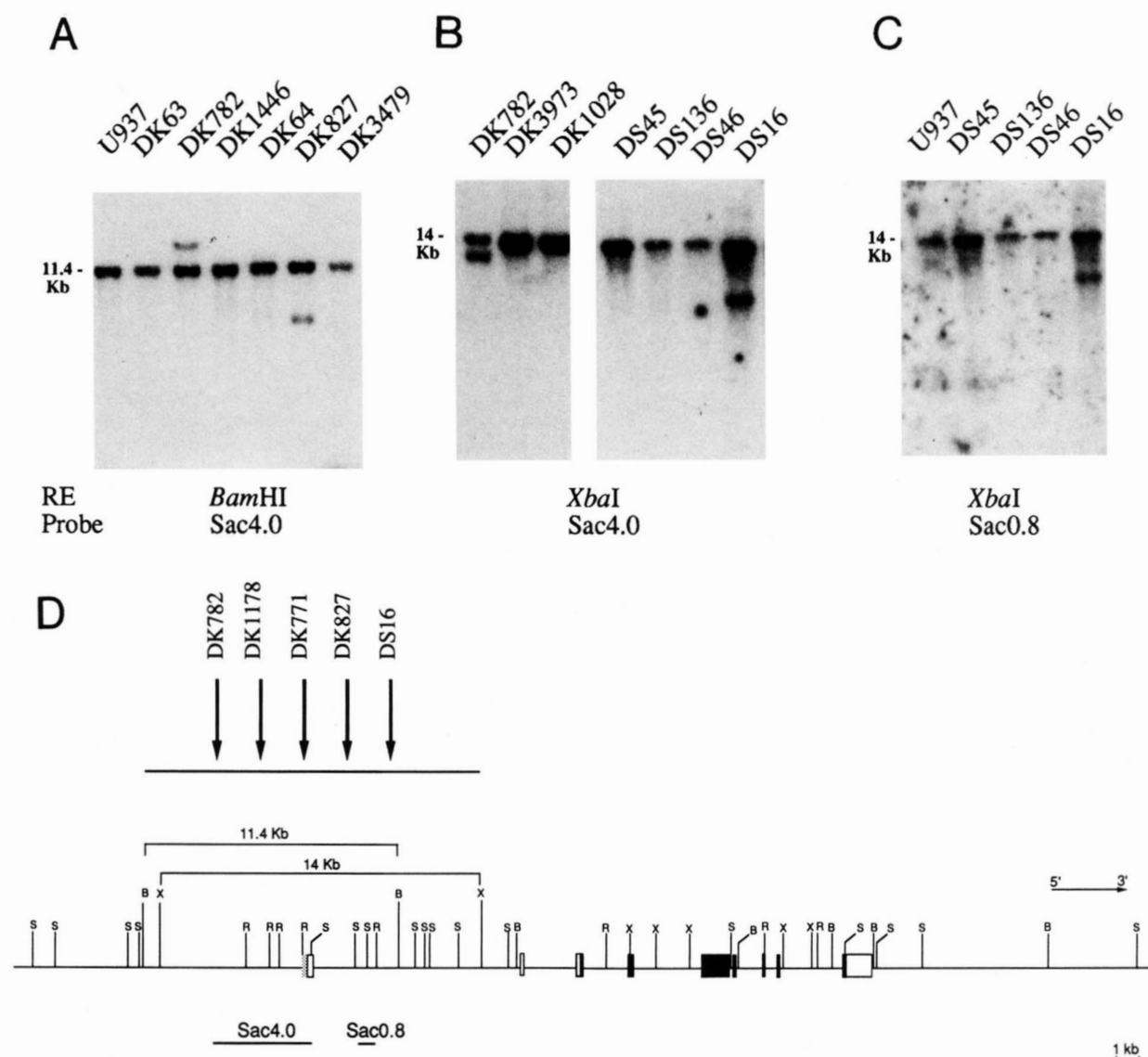


Fig 1. Analysis of *BCL-6* rearrangements in AIDS-NHL (A, B, and C) and restriction map of the germline *BCL-6* locus (D). (A, B, and C) DNAs were digested with *Bam*HI (A) or *Xba*I (B and C) and hybridized to probes Sac4.0 (A and B) or Sac0.8 (C). The *BCL-6* germline bands detected by *Bam*HI (11.4 kb) and *Xba*I (14 kb) are indicated. U937 was used as a *BCL-6* germline control. Among the cases shown, rearrangements were detected in cases DK782, DK827, and DS16, represented by AIDS-DLCL. (D) Exon-intron organization of the *BCL-6* gene. Coding and noncoding exons are represented by solid and open boxes, respectively. The transcription initiation site has not been mapped (shaded box on 5' side of first exon). The breakpoints detected in AIDS-NHL are indicated by arrows. Restriction enzyme symbols are S, *Sac*I; B, *Bam*HI; X, *Xba*I; R, *Eco*RI. RE, restriction enzyme.

MYC alterations and *p53* mutations were consistently absent in the cases displaying *BCL-6* rearrangements.

DISCUSSION

DLCL represents the most frequent type of AIDS-NHL in the HIV-infected adult.⁸ Despite its epidemiologic relevance, the molecular pathogenesis of these tumors is largely unclarified.³ In this study, we report a novel genetic lesion in AIDS-NHL that appears to be restricted to the AIDS-DLCL histologic type. Our analysis of the genomic configuration of *BCL-6* in a panel of AIDS-NHL indicates that

BCL-6 rearrangements are involved in approximately 20% of AIDS-DLCL, whereas they are consistently negative in AIDS-SNCL. In this respect, *BCL-6* rearrangements may be considered the first identified genetic lesion specific for the DLCL type among AIDS-NHL. *BCL-6* rearrangements are present in both subgroups of DLCL, ie, LNCCL and LC-IBPL, and occur both in the absence and in the presence of EBV infection of the tumor clone (Table 2). On the other hand, *BCL-6* rearrangements were never detected in AIDS-DLCL carrying *c-MYC* alterations (Table 2). Future studies of larger series of cases will clarify whether these two genetic

Table 1. Frequency of *BCL-6* Rearrangements in AIDS-NHL

SNCCCL	DLCL*		CD30 ⁺ NHL†
	LNCCCL	LC-IBPL	
0/13	2/8	3/16	0/3

*The DLCL included in the panel can be further distinguished into two subgroups (LNCCCL and LC-IBPL), as previously reported.^{7,9}

† NHL expressing the CD30 cell surface antigen.⁴³

lesions represent mutually exclusive events in the pathogenesis of AIDS-DLCL.

These data are consistent with a model of AIDS-lymphomagenesis that suggests that genetically distinct pathways are specifically associated with different histologic types of AIDS-NHL.^{3,14} The molecular pathway leading to AIDS-SNCCCL involves *c-MYC* rearrangements, *p53* mutations, and EBV infection in 100%, 60%, and 40% of the cases, respectively.^{3,14-26} The presence of somatic hypermutation in the Ig variable regions used by AIDS-SNCCCL points to chronic antigen stimulation as an additional mechanism in the development of these tumors.⁴⁶ The second genetic pathway is associated with AIDS-DLCL and involves EBV in the large majority of cases, as well as *c-MYC* and/or *BCL-6* rearrangements in a fraction of cases.¹⁴⁻²⁶ These distinct pathogenetic mechanisms correlate with a number of clinical features that distinguish AIDS-SNCCCL from AIDS-DLCL, including different age at onset and different CD4 counts at the time of lymphoma development.^{1,2,8}

Although the number of AIDS-DLCL studied is presently

Table 2. Molecular Features of AIDS-DLCL

Patient No.	Histology	Clonality	<i>BCL-6</i>	EBV	<i>c-MYC</i>	<i>p53</i>	<i>RAS</i>
DK782	LNCCCL	+	+	-	-	-	-
DK1178	LNCCCL	+	+	-	-	-	-
DK1028	LNCCCL	+	-	-	-	-	-
DK3973	LNCCCL	+	-	+	-	-	-
DK773	LNCCCL	+	-	-	+	-	-
RDF834	LNCCCL	+	-	+	+	-	-
DK1452	LNCCCL	+	-	-	-	-	-
DK64	LNCCCL	+	-	+	-	-	+
DK771	LC-IBPL	+	+	+	-	-	-
DK827	LC-IBPL	+	+	+	-	-	-
DS16	LC-IBPL	+	+	+	-	-	ND
DK3537	LC-IBPL	+	-	+	+	-	-
DK3357	LC-IBPL	+	-	+	-	-	-
DK63	LC-IBPL	+	-	+	-	-	-
DK1446	LC-IBPL	+	-	+	+	-	-
DK3479	LC-IBPL	+	-	-	-	-	-
DK2092	LC-IBPL	+	-	+	-	-	-
DS17	LC-IBPL	+	-	-	-	-	ND
DS45	LC-IBPL	ND	-	+	-	-	ND
DS46	LC-IBPL	+	-	+	+	-	ND
DS93	LC-IBPL	+	-	+	-	-	ND
DS136	LC-IBPL	+	-	+	-	-	ND
DS155	LC-IBPL	+	-	+	-	-	ND
DS165	LC-IBPL	+	-	+	-	-	ND

The results of the analysis of EBV, *c-MYC*, *p53*, and *RAS* of some of these cases have been previously reported.^{14,15,45}

Abbreviation: ND, not done.

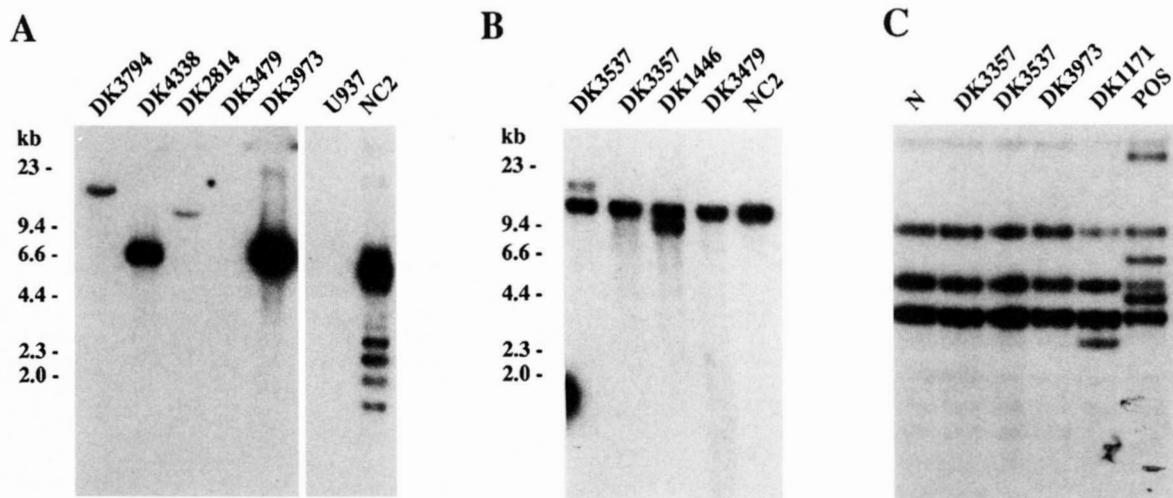


Fig 2. Analysis of EBV infection (A), *c-MYC* rearrangements (B), and *p53* mutations (C) in AIDS-NHL. (A) Analysis of EBV termini heterogeneity in AIDS-NHL. DNAs were digested with *Bam*HI and subjected to Southern hybridization using a DNA probe specific for the fused termini of the EBV genome.⁴² U937, a monocytic leukemia cell line, is used as a negative control. A lymphoblastoid cell line derived by EBV infection of normal polyclonal B cells (NC2) is used as control for polymorphic EBV termini. Representative samples of AIDS-NHL, both positive (DK3794, DK4338, DK2814, and DK3973) and negative (DK3479), are shown. (B) Southern blot analysis of *c-MYC* rearrangements in AIDS-NHL. Genomic DNAs from the cases shown was digested with *Hind*III and probed with clone MC413RC,⁴¹ representative of *c-MYC* exon 3. A lymphoblastoid cell line (NC2) was used as control for *c-MYC* germline configuration. Among the cases shown, two cases of AIDS-DLCL (DK3537 and DK1446) display a *c-MYC* rearrangement. (C) Analysis by PCR-SSCP of the *p53* gene in AIDS-NHL. Representative examples are shown for *p53* exon 5. Samples were scored as abnormal when differing from the normal control (N). A sample known to harbor a *p53* mutation was used as positive control (POS). Among the cases shown, DK1171, a case of AIDS-SNCCCL, shows a *p53* mutation that was further characterized by direct sequencing of the PCR product.

limited, our results suggest that the frequency of *BCL-6* rearrangements in AIDS-DLCL is significantly lower than that in DLCL in the immunocompetent host, where *BCL-6* rearrangements occur in more than 40% of the cases.³⁰ It is possible that the genetic pathogenesis of these two groups of tumors is different, and that the molecular mechanisms active in AIDS-DLCL are characterized by a higher degree of heterogeneity. Among DLCL in the immunocompetent host, *BCL-6* rearrangements are associated with distinct clinical features, including the extranodal origin of the lymphoma and the lack of bone marrow involvement.⁴⁷ In addition, the presence of this rearrangement appears to represent a favorable prognostic marker.⁴⁷ Future studies are warranted to determine whether *BCL-6* rearrangements are associated with distinctive clinical features in AIDS-NHL as well.

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