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Advances in Brief

Identification of a Common Clonal Human Immunodeficiency Virus Integration Site in Human Immunodeficiency Virus-associated Lymphomas[1]

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Abstract

Infection with human immunodeficiency virus type 1 (HIV-1) is associated with a high incidence of lymphoma. Typically, the lymphomas are B-cell in origin, and although they occur in the setting of HIV-1 infection, historical studies have found no evidence for the presence of HIV-1 within the transformed B-cells. We describe a new class of large cell lymphoma wherein HIV p24 expression within the tumor specimens was found to be extremely high. In the first case, HIV was expressed in the tumor-associated transformed T-cells. In three other cases, HIV was found to be highly expressed in tumor-associated macrophages. These tumors exhibited a mixed immunophenotype histologically. Analysis by inverse polymerase chain reaction, using HIV long terminal repeat primers, demonstrated monoclonal HIV integration sites for all four tumors. Direct sequencing of the T-cell lymphoma inverse polymerase chain reaction products identified the HIV integration site within the *fur* gene, just upstream from the c-*fes/fps* protooncogene. Using segments of the *fur* gene as a probe, the other three monoclonal integration sites mapped to the same region. Although the integration and up-regulation of c-*fes/fps* was localized to the tumor cells within the T-cell lymphoma, the cells containing the monoclonal HIV in the other mixed immunophenotype lymphomas are currently unknown. These observations suggest that HIV may contribute directly to lymphomagenesis and identify a common site of HIV integration within a subset of acquired immunodeficiency syndrome lymphoma.

Introduction

Early in the acquired immunodeficiency syndrome epidemic, B-cell lymphomas began to appear in individuals infected with HIV-1[3] (1). Although these B-cell lymphomas develop in patients infected with HIV-1 (1, 2), the tumor cells from these individuals show no evidence for the presence of HIV-1. This result has been substantiated by other studies which also failed to detect HIV within the tumor clone (3). Therefore the role that HIV plays in the pathogenesis of B-cell lymphomas in HIV-infected individuals is thought to be indirect as a result of the effect of HIV on immunoregulation. The mechanisms by which HIV-associated lymphomas develop appear to be multifactorial but there does not appear to be any direct association with retrovirus infection such as seen in animal models (4). Indeed, certain animal retroviruses have been shown to induce cellular transformation through integration near cellular oncogenes, such as that seen in ALV-associated B-cell lymphomas, which occur in ALV-infected chickens. Molecular studies performed on the avian tumors demonstrated that both ALV and truncated forms of *ALV* genes integrated near c-*myc* causing constitutive c-*myc* expression (5). Unlike ALV-associated B-cell lymphomas, the HTLV-associated lymphomas do not arise through an obvious insertional mutagenic process. Rather, HTLV-1 is found randomly integrated in a large number of HTLV-1-associated T-cell lymphomas. B-cell lymphomas are not the only malignancies seen in the setting of HIV infection. Additionally, non-B-cell (6–8) and mixed immunophenotype lymphomas (9, 10) have been described in the literature. In a recent molecular and immunophenotypic study, almost one-half of the large cell lymphomas were of the mixed immunophenotype category (9). Over the past 3 years, an increasing number of non-B-cell lymphomas have been diagnosed at San Francisco General Hospital. These include T-cell, mixed immunophenotype, and angioimmunoblastic lymphadenopathy-like lymphomas. In the process of characterizing these lymphomas, some of them showed a high degree of HIV p24 antigen expression as analyzed by immunocytochemistry (7). In order to find out if this subset of HIV-associated lymphomas is different than the B-cell lymphomas, we used an IPCR to determine HIV clonality and to map HIV integration sites. In the current study, we found evidence for a common integration site in this class of non-B-cell lymphoma.

Materials and Methods

Lymphoma Specimens. Lymphoma samples were obtained at diagnosis and/or autopsy and were stored at −70°C. Specimens submitted for formaldehyde fixation/paraffin embedding, immunohistochemistry, and nucleic acid isolation were carefully aliquoted to ensure representative samples. All specimens were obtained after appropriate consent and in accordance with the University of California, San Francisco Committee on Human Research guidelines.

Immunocytochemistry. The frozen tissue was thin-sectioned and stained using a modified immunoperoxidase method (11). Antibodies used include: anti-human IgM; CD19; CD45; CD20; CD45RO; CD4; CD8; CD3; CD25; CD14; CD30; gp41 (Dako Patts, Carpinteria, CA); CD5 (Becton-Dickinson, San Jose, CA); and anti-HIV p24 (Dr. J. R. Carlson, University of California, Davis, Davis, CA).

Southern Analysis. Southern analysis was performed to verify the presence of HIV in the lymphomas using the same HIV probe as previously described (7).

IPCR. DNA was extracted from the tissue samples and IPCR was performed with modifications to a previously described method (12). DNA (0.1 μg) was digested with *Sau*3A and ligated in a 200-μl reaction (1× ligase buffer-40 units of T4 DNA ligase) for 36 h at 15°C. The reaction was purified by ethanol precipitation and followed by IPCR (Fig. 1) [100 pM primers (CW1B and CW2H; Fig. 1)-1 μl ligated product-1× buffer-20 nM deoxynucleotide triphosphates, 2.5 units of *Taq*I DNA polymerase (Promega Biotech, Madison, WI)] in a Perkin Elmer/Cetus (New England Biolabs, Beverly, Massachussetts) thermocycler. Conditions were 94°C melting for 1.5 min, 50°C annealing for 1.5 min, and 72°C extension for 3 min, for a total of 60 cycles. Amplified products were separated on an ethidium-stained gel (1% agarose-1.5% NuSieve gel; FMC BioProducts, Rockland, ME).

In order to verify the amplified products, the DNA from the agarose gel was transferred to a nylon membrane and prepared for hybridization. The probes used for hybridization (LTRP, Fur1, and Fur2) are listed in Fig. 1. The probes were labeled with digoxigenin and detected by chemiluminescence (Boehringer-Mannheim, Mannheim, Germany).

Individual amplified products were isolated from a separate gel and purified. Sequencing of the products was performed as previously described (13).

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[3] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; ALV, avian leukosis virus; HTLV, human T-cell lymphotrophic virus; IPCR, inverse polymerase chain reaction; LTR, long terminal repeat.

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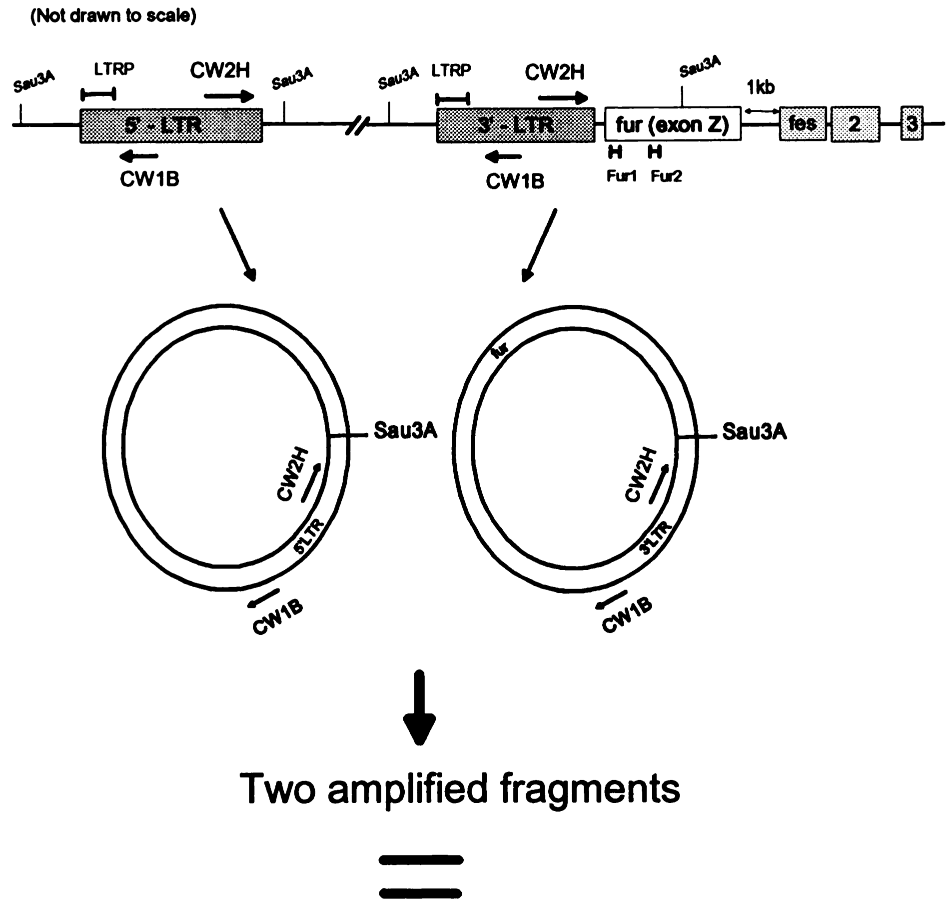

Fig. 1. Schema of IPCR to detect integration sites. Primers CW1B and CW2H flank the LTR region in the opposite direction. Sequence and location of primers and probes. *LTRP*, long terminal repeat probe; *kb*, kilobase.

Primers / Probes	Sequence
CW1B	5'-GGATCCGGATCCCCTNGNTAGCCAGAGAGCTC-3'
CW2H	5'-AAGCTTAAGCTTAGCTTGCCTTGAGTGCNTCA-3'
LTRP	5'-GGTCTCTCTNGTTAGACCAG-3'
FUR1	5'-TGAGGAATAGTTGAGCCCCAAGTCCTGAAG-3'
FUR2	5'-CAGCACCCCTTCCATGTGGAGAAAGGAGTG-3'

N: G,A,T,C

Results and Discussion

The histology of all four lymphomas was that of a diffuse aggressive lymphoma consisting mainly of a predominance of what surgical pathologists call "large cells" or "large immunoblastic cells" as described in the Working Formulation for lymphoma classification (14). The tumors in Cases 1, 3, and 4 were heterogeneous consisting of a predominant lymphocyte background admixed with a distinct population of macrophages (histiocytes). The presence of the macrophages was verified by immunohistochemistry (CD14 and/or CD68). The tumor in Case 2 was almost exclusively composed of large atypical lymphocytes. The complete immunophenotypes are: Case 1, B-cell (CD20) on an angioimmunoblastic lymphadenopathy background admixed with reactive T-cells (CD45RO) and CD68-reactive macrophages (histiocytes); Case 2, T-cell (CD4, CD5, CD45RO, CD30, p24, gp41); Case 3, Ki-1-positive T-cell lymphoma (CD30, CD45RO, CD3) admixed with CD14/CD68-reactive macrophages (histiocytes); and Case 4, Ki-1 positive lymphoma (CD30) admixed with CD14/CD68-reactive macrophages/histiocytes. Immunocytochemical staining with anti-HIV p24 antibody demonstrated that the virus was localized to cells phenotypically similar to macrophages in Cases 1, 3, and 4 and localized to T-cells in Case 2.

Fig. 2 shows the histology from Cases 2 and 3. The tumor from Case 2 has been described previously in more detail (7). The histology of Case 2 demonstrates the aggressive nature of the tumor (Fig. 2*A*) and an uninvolved lymph node from the same patient (Fig. 2*B*). Case 3 (Fig. 2*C*) demonstrates the heterogeneous background interspersed with macrophages of the mixed immunophenotype lymphoma.

Analysis by Southern blot was performed on Cases 2–4. Case 2 has been previously described showing the presence of monoclonal integration of HIV within the lymphoma specimen (7). Similarly, clonal integration of HIV was demonstrated in Cases 3 and 4 (data not shown).

IPCR identified two amplified fragments from the four cases as shown in Fig. 3*A*. One of the amplified fragments from Case 2 has been successfully sequenced; Fig. 4 shows that the integration of the 3′ LTR

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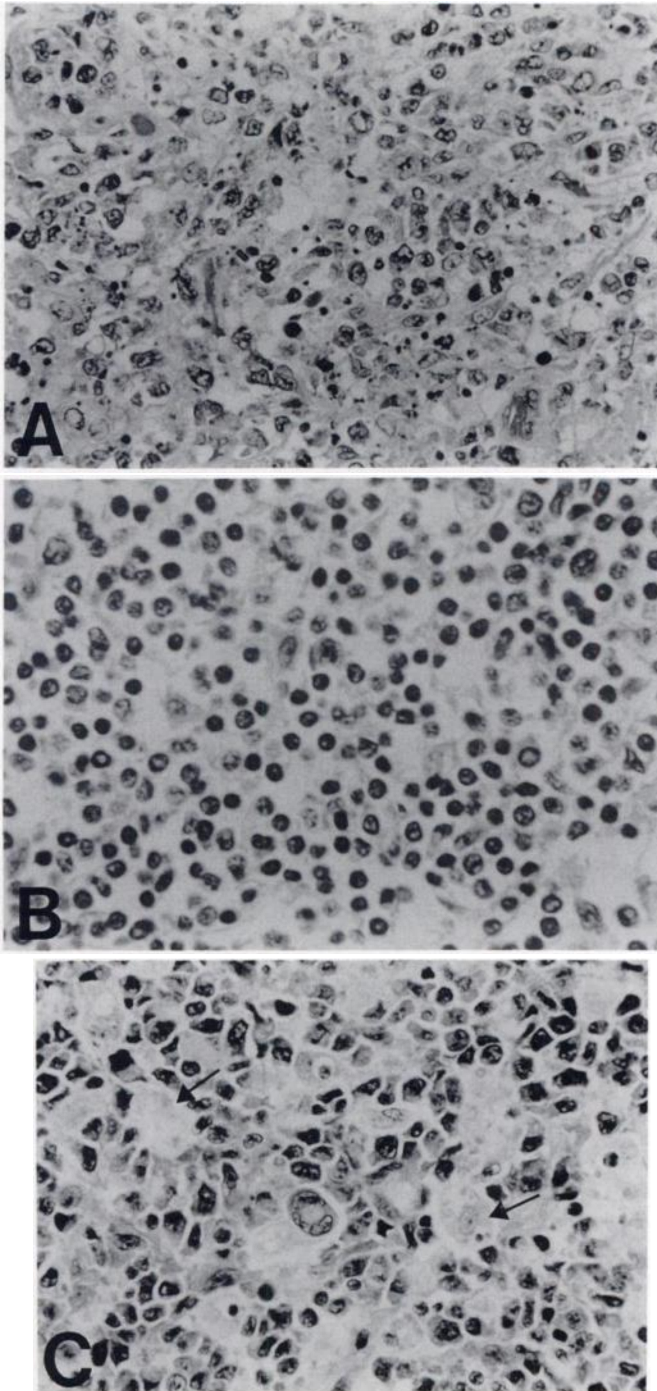

Fig. 2. Representative histology of mixed immunophenotype lymphoma. (*A*) Tumor and (*B*) uninvolved lymph node from Case 2. × 300. (*C*) Tumor from Case 3. × 375. *Arrows*, macrophages within the tumor.

lies within a region previously identified as *fur*. Fig. 4 shows the sequence analysis of the 300-base pair fragment. The sequence of the first 100 bases aligns to the LTR regions of known HIV strains. The latter part of the sequence corresponds to the exon Z of the *fur* gene. The *fur* gene codes for a protein with receptor-like properties and is located approximately 1 kilobase upstream to an oncogene known as c-*fes*/*fps* (15). The DNA map of this region is shown in Fig. 1. c-*fes*/*fps* is associated with myeloid and lymphoid malignancies and is also expressed in Hodgkin's disease-associated Reed-Sternberg cells (16, 17).

The two bands from each sample are the result of amplifications of the 5′ and 3′ LTR integration sites, as evident by hybridization to the LTRP probe (Fig. 3*B*). Because the first case had HIV clonally integrated within the *fur* gene, the membrane containing the amplified fragments was rehybridized to a cocktail of *fur* probes, FUR1 and FUR2 (Fig. 1). The results show that one of the bands in each case hybridized to the probes (Fig. 3*C*). This result provides evidence that the integration of one of the LTRs in the other three cases lies within the *fur* gene because of the cohybridization to the LTRP and *fur* probes.

IPCR is a sensitive technique that in theory could amplify a rare cell or clone within the lymphoma sample. To circumvent this possibility, Southern analysis, which is less sensitive, was performed and confirmed the presence of HIV in Cases 2–4. As additional controls, no clonal IPCR products were amplified from nontumorous lymphoid tissues from 2 cases (Fig. 3, *Lanes 2* and *6*).

Recent molecular studies of HIV-associated lymphomas have found these lymphomas to be a heterogeneous set of disease processes (18). Although the majority of HIV-associated lymphomas are of B-cell origin, we and others have identified another subset which may have a different pathogenic pathway (6–10). Analysis of these T-cell and mixed immunophenotype lymphomas by IPCR provides evidence that HIV-1 has integrated within the genomic DNA, specifically within the *fur* gene which is located 5′ to a known oncogene, c-*fes*/*fps*. This oncogene encodes a M_r 92,000 protein-tyrosine kinase which is expressed in immature and differentiated hematopoietic cells and has been associated with cellular transformation but through an unclear mechanism (15–17). The discovery of four HIV-associated non-B-cell lymphomas with integration of HIV-1 upstream to the same oncogene suggests that the event probably occurred nonrandomly. What role the

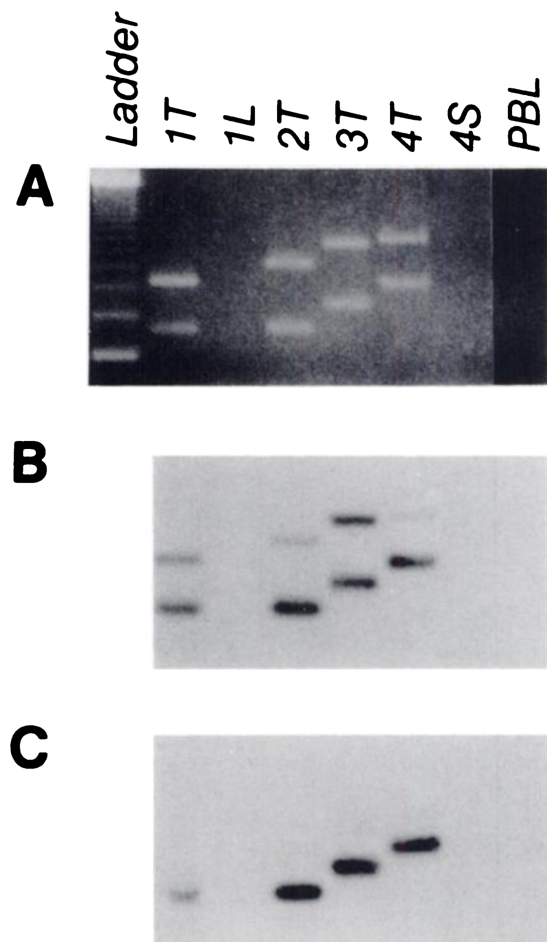

Fig. 3. (*A*) Ethidium-stained gel (1% agarose-1.5% NuSieve gel) of IPCR products (123-base pair ladder). *Lane 1*, Case 1, tumor (*1T*); *Lane 2*, Case 1, uninvolved lymph node (*1L*); *Lane 3*, Case 2, tumor (*2T*); *Lane 4*, Case 3, tumor (*3T*); *Lane 5*, Case 4, tumor (*4T*); *Lane 6*, Case 4, uninvolved spleen (*4S*); *Lane 7*, normal peripheral blood mononuclear cells (*PBL*). (*B* and *C*) Hybridization of membrane from gel in *A*. Blots are hybridized separately to oligomers: *B*, Long terminal repeat probe; *C*, FUR1 and FUR2.

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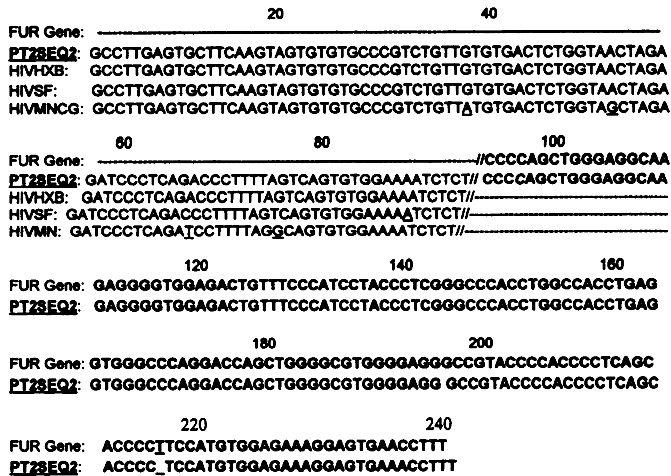

Fig. 4. Sequence analysis of IPCR product from PT2SEQ2 (Case 2). Sequence from the *FUR* gene is aligned above and sequences from representative HIV-1 strains are aligned below the IPCR product (*PT2SEQ2*). *Underlined nucleotides* correspond to mismatches compared to PT2SEQ2.

virus plays in the regulation, if any, of c-*fes*/*fps* and lymphomagenesis can be speculated based on other models.

In the proviral form, the retroviral genome is flanked by two LTRs. Although the 5′ and 3′ LTRs maintain sequence homology, they have different functions in retroviral replication and oncogene activation. Mechanisms by which retroviruses activate protooncogenes include LTR promotor and enhancer insertion (4). With LTR promotor insertion, a protooncogene is transcribed from either the 5′ or 3′ LTR promotor. In the case of ALV, c-*myc* is activated with insertion of the provirus upstream of the oncogene, allowing the 3′ LTR to initiate transcription of the downstream *myc* sequences while transcription from the 5′ LTR is reduced. Boerkoel and Kung (19) have shown that the 5′ and 3′ LTR regions probably do not function independently of each other. They demonstrated that although the 5′ and 3′ LTR regions contain similar internal sequences, transcription can occur from either LTR, depending on the transcriptional interaction between the two LTRs. In the wild-type proviruses, transcription initiates predominantly in the 5′ LTR, whereas in the c-*myc*-associated ALV system there exists a predominance of transcription from the 3′ LTR with little transcription from the 5′ LTR. Boerkoel and Kung (19) have identified a leader sequence that is required for transcriptional dominance of the 5′ over the 3′ LTR.

The HIV-associated lymphomas analyzed in this study demonstrated HIV integration upstream to the c-*fes*/*fps* protooncogene, suggesting that these lymphomas arose through a nonrandom process. Although these results do not prove that these cells were transformed by HIV, several mechanisms could explain how HIV infection may result in a lymphoma: (*a*) HIV could have integrated near an oncogene and, through insertional mutagenesis, could have up-regulated the oncogene; (*b*) HIV could have picked up an oncogene similar to transforming viruses that cause tumors in animals; or (*c*) HIV could have mutated a regulatory gene and function similarly to the *tax* gene that is involved in immortalization of HTLV-I-infected T-cells (20). Additionally, Laurence and Astrin (21) have recently shown that HIV has properties of a transforming retrovirus. Their data indicate that the virus augments the expression of Epstein-Barr virus and c-*myc* in human B-lymphoblasts *in vitro*, two events which are linked to B-cell neoplasia. Because the immunocytochemical studies do not demonstrate exactly the cell or cells containing the integrated form of HIV, further studies will be required to identify the responsible cell containing the virus and to determine mechanistically how these tumors arose.

As the acquired immunodeficiency syndrome epidemic continues, the incidence of lymphomas continues to increase. The finding of a nonrandom integration of HIV upstream to a known oncogene is strong evidence that HIV may be involved in causing cellular transformation in a subset of HIV-associated lymphomas. Although HIV is normally cytopathic to T-cells, the process of integrating near c-*fes*/*fps* may have allowed for a selective survival and growth advantage for that particular clone. Analysis of the integrated HIV sequences may reveal mutations, implying that a strain of virus has emerged with direct transforming potential. As the incidence of HIV infection continues to increase and the life span of HIV-infected individuals lengthens, the combination of potentially transforming variants of HIV, integration, and selection could result in a parallel increase in the incidence of T-cell and mixed immunophenotype lymphomas in these patients.

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