

Expression of Cyclin-Dependent Kinase Inhibitor p27^{Kip1} in AIDS-Related Diffuse Large-Cell Lymphomas Is Associated with Epstein-Barr Virus-Encoded Latent Membrane Protein 1

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Knowledge of the role of cell-cycle regulators in the pathogenesis of acquired immune deficiency syndrome-related non-Hodgkin's lymphomas (AIDS-NHLs) is scarce. Here we analyzed 86 systemic AIDS-NHLs and 20 AIDS-primary central nervous system lymphomas for expression of p27^{Kip1}, a negative regulator of cell-cycle progression belonging to the Kip family of cyclin-dependent kinase inhibitors. In parallel, we investigated the relationship between p27^{Kip1}, the lymphoma proliferation index, Epstein-Barr virus status, expression of cellular cyclin D3 and cyclin D1, and B-cell differentiation stage. We report that AIDS-immunoblastic lymphomas (AIDS-IBLs), either systemic or primarily localized to the central nervous system, consistently express p27^{Kip1} protein (19 of 24 and 10 of 14, respectively) despite the high proliferative rate of the lymphoma clone, suggesting a failure of p27^{Kip1} to inhibit the cell cycle in AIDS-IBL. Conversely, the remaining systemic AIDS-NHLs and AIDS-primary central nervous system lymphomas preferentially fail to express p27^{Kip1}. Expression of p27^{Kip1} in Epstein-Barr virus-positive AIDS-NHLs generally associates with latent membrane protein 1 (LMP1) expression and is related to a late stage of B-cell differentiation, characterized by the BCL-6-

MUM1+/syn-1± phenotypic profile, whereas it seems to be unrelated to the expression of cellular cyclins. In B cells *in vitro*, induction of LMP-1 expression under the control of inducible promoters up-regulates expression of p27^{Kip1}, thus providing a putative mechanistic explanation for the association between LMP1 and p27^{Kip1} observed *in vivo*. Overall, these data show that AIDS-IBL pathogenesis is characterized by loss of the inverse relationship between p27^{Kip1} positivity and tumor growth fraction that is otherwise generally observed in normal lymphoid tissues and in most other types of NHLs. (*Am J Pathol* 2002, 161:163-171)

Acquired immune deficiency syndrome-related non-Hodgkin's lymphomas (AIDS-NHLs) represent a significant source of morbidity and mortality among human immunodeficiency virus (HIV)-infected individuals. All AIDS-NHLs are characterized by extreme clinical aggressiveness, display a predilection for extranodal sites, and derive from B cells. Pathologically, however, AIDS-NHLs are markedly heterogeneous.^{1,2} The pathological spectrum of AIDS-NHLs includes systemic AIDS-NHLs, primary central nervous system lymphomas (AIDS-PCNSLs), primary effusion lymphoma (AIDS-PEL), and plasmablastic lymphoma (AIDS-PBL) of the oral cavity.¹⁻⁷ Systemic AIDS-NHLs are histologically classified into AIDS-related Burkitt's lymphoma (AIDS-BL) and AIDS-related diffuse large cell lymphomas that include AIDS-related large noncleaved cell lymphoma (AIDS-LNCCL) and AIDS-related immunoblastic lymphoma (AIDS-IBL).^{4,5,8} AIDS-PCNSLs are classified into AIDS-LNCCL and AIDS-IBL.^{2,4,5}

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The pathological heterogeneity of AIDS-NHL is consistent with the existence of multiple molecular pathways selectively associated with a given type of AIDS-NHL and implicated in the pathogenesis of these disorders. Despite their pathological heterogeneity,⁹ most cases of AIDS-NHL are histogenetically related to germinal center (GC) or post-GC B cells, as indicated by the expression pattern of several B-cell phenotypic markers corresponding to different physiological stages of mature B-cell development.^{1,10}

The biological research performed on AIDS-NHLs during the last decade has led to significant discoveries that have clarified several topics regarding the pathogenesis of these lymphomas.² However, knowledge of the role of cell-cycle regulators in AIDS-lymphoma development and growth is more limited.¹¹ The cellular cyclins contribute to cell-cycle control by forming complexes with catalytic subunits termed cyclin-dependent kinases (CDKs).^{12,13} The activation of the CDK/cyclin complex is controlled by CDK inhibitors that regulate cell cycle.¹² A major inhibitor of the CDK/cyclin complex is represented by p27^{Kip1}, a nuclear phosphoprotein belonging to the Kip family of CDK inhibitors.^{14–18} In physiological conditions, expression of p27^{Kip1} is highest in quiescent cells and declines as cells re-enter the cell cycle. In lymphoid tissues, p27^{Kip1} is expressed in nonproliferating lymphocytes, whereas activated lymphocytes, eg, GC cells, score consistently negative for p27^{Kip1} expression.¹⁹ The inverse relationship between p27^{Kip1} expression and cell proliferation that is physiologically observed in normal lymphoid tissues is also encountered in most subtypes of NHL of immunocompetent hosts.^{19–21}

The aims of the present study were to establish the expression pattern of p27^{Kip1} in systemic AIDS-NHL and AIDS-PCNSL and to address the relationship between p27^{Kip1} expression, proliferation index, B-cell differentiation stage, viral status for Epstein-Barr virus (EBV), and expression of cellular cyclin D3 and cyclin D1 in these lymphomas.

Materials and Methods

Neoplastic Samples

This study was based on 106 AIDS-NHLs. All cases were of B-cell origin and included 86 systemic AIDS-NHLs and 20 AIDS-PCNSLs. Systemic AIDS-NHLs were classified according to the revised European-American classification of lymphoid neoplasms (REAL).²² AIDS-BLs (from 29 patients) include cases displaying the histological features of classical endemic BLs and the so-called Burkitt-like lymphomas.²² AIDS-LNCCLs (from 32 patients) usually contain large noncleaved cells that are intermediate in size between those of Burkitt's and IBL. They have scant to moderately abundant cytoplasm and round nuclei containing one or more small distinct nucleoli adjacent to the nuclear membrane. Occasionally LNCCLs contain variable proportions or are composed entirely of neoplastic cells containing cleaved or multilobated nuclei.⁹ AIDS-IBLs (from 24 patients) contain large neoplas-

tic cells that usually have abundant acidophilic cytoplasm with evidence of plasmacytoid differentiation. All AIDS-PCNSLs were classified as LNCCL or IBL (a total of 16 patients).⁹ AIDS-PCNSL cases containing a mixture of large noncleaved cells and large cells, immunoblastic plasmacytoid were classified separately as LNCCL/IBL (four patients).²³ Thus, the subject of the analysis is mainly a series of diffuse large-cell lymphoma patients. Tissues were fixed in Bouin solution or neutral-buffered formalin. In most cases, a portion of unfixed tissue was snap-frozen in liquid nitrogen and stored at -80°C . Detailed characterization of part of these cases has been reported previously.^{1,10}

Sixteen samples of IBL (similar in morphology and immunophenotype to the AIDS-IBL) from nonimmunosuppressed (HIV-seronegative) patients were also included in the study for comparative purposes.

Nonneoplastic Samples

Nonneoplastic lymph node ($n = 93$), nasopharynx ($n = 30$), and tonsil ($n = 4$) samples from a total of 100 HIV-infected patients with persistent generalized lymphadenopathy were also included in the study. The histopathological pattern of lymph nodes and tonsils was predominantly represented by hyperplastic changes of the lymphoid follicles. The nasopharynx exhibited the nasopharyngeal lymphoid tissue hypertrophy pattern related to HIV infection.²⁴

Immunohistochemical Studies

Immunohistochemistry was performed by the avidin-biotin-peroxidase complex (ABC-px) or alkaline phosphatase anti-alkaline phosphatase methods.^{25,26}

The expression of p27^{Kip1} was investigated with the monoclonal antibody (mAb) Kip-1 (Transduction Laboratories, Lexington, KY) or the mAb 1B4 (Novocastra Laboratories Ltd., Newcastle on Tyne, UK). The reactivity pattern of both antibodies recognizing p27^{Kip1} was tested in selected cases and was superimposable.

The proliferation index was assessed using the MIB-1 mAb (Immunotech, Marseille, France) directed against the Ki-67 nuclear proliferation antigen.

In selected cases (ie, p27^{Kip1}-positive cases and a fraction of p27^{Kip1}-negative cases), the expression of cyclin D1 and D3 was assayed. Cellular cyclin D1 was detected with the mAbs AM29 (Zymed Laboratories, San Francisco, CA); cyclin D3 was detected with the mAb DCS-22 (NeoMarkers, Inc., Fremont, CA).

All these antigens were tested on paraffin-embedded sections from cell blocks with a previous step of antigen retrieval. For p27^{Kip1} assessment, sections were treated twice in a microwave oven for 5 minutes in citrate buffer (pH 6); for Ki-67, sections were first treated with trypsin (Sigma Chemical Co., St. Louis, MO) (0.33 mg/ml) for 1 minute and then twice for 5 minutes in citrate buffer (pH 6) in a microwave oven at 650 W; for cyclin D1, Bouin-fixed sections were treated with trypsin (Sigma Chemical Co.) (0.2 mg/ml) for 5 minutes whereas formalin-fixed

sections were treated for 30 minutes in citrate buffer (pH 7) in microwave oven at 250 W; for cyclin D3, sections were treated for 30 minutes in TEC (Tris-ethylenediaminetetraacetic acid-citrate) solution (pH 7.8) in a microwave oven at 250 W.

Immunocytochemical staining for p27^{Kip1}, Ki-67, and cyclin D3 was performed using the ABC method²⁵ (ABC-Elite kit; Vector, Burlingame, California), whereas immunocytochemical staining for cellular cyclin D1 was performed on an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ) according to the company's protocols.

The expression of BCL-6, MUM1/IRF4, and CD138/syndecan-1 (syn-1), which are well-defined phenotypic markers of B-cell lymphoma histogenesis,^{1,10} was tested on paraffin-embedded tissue sections as previously described.¹⁰

In all AIDS-NHLs tested, the percentage of neoplastic cells showing positive staining for the different antigens (nuclear staining for p27^{Kip1}, Ki-67, cyclins D1 and D3, BCL-6, and MUM1; cytoplasmic staining for syn-1) was assessed in each case. The percentage of antigen-positive neoplastic cells was assigned to one of the following categories: 0, less than 10%, 10 to 24%, 25 to 49%, 50 to 74%, and $\geq 75\%$. Only definite and unambiguous staining on unequivocal malignant cells was accepted as positive.

All neoplastic samples included in this study were subjected to determination of tumor infection by EBV as previously described.^{27,28} The expression of EBV-encoded latent membrane protein 1 (LMP1) was tested on paraffin-embedded tissue sections of EBER-positive samples as previously described.^{1,10} Immunostaining for LMP1 was performed with a LMP1-specific antibody (Dakopatts A/S, Glostrup, Denmark).^{1,10} The percentage of LMP1-positive neoplastic cells was assigned to one of the following categories: 0, less than 10%, 10 to 24%, 25 to 49%, 50 to 74%, and $\geq 75\%$.

Expression of p27^{Kip1} in Nontransformed Lymphoid Tissues and Neoplastic Samples

The expression of p27^{Kip1} in reactive B cells within lymphoid tissues and in AIDS-NHLs was referred to the stages of physiological B-cell differentiation as defined by the combined expression of BCL-6, MUM1, and syn-1.¹⁰ The expression pattern of p27^{Kip1} was also compared with that of LMP1 in AIDS-NHL. Serial sections were used to compare the immunoreactivity of these antibodies.

Cell Lines and Cell Transfection

Ramos cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum. To generate cells expressing LMP1, 20 μ g of plasmid DNA (pMEP4 or pMEP4-LMP1; EBV-based episomally replicating vector) were first mixed with 1.0×10^7 cells in 0.4 ml of IMDM medium with 10% fetal bovine serum and then transfected into Ramos cells by electro-

poration using the Bio-Rad Gene Pulser apparatus (960 μ F, 200 V) (Bio-Rad Laboratories, Hercules, CA). After transfection, cells were transferred to T25 flasks, incubated at 37°C for 48 hours, and then selected for transfected cells in IMDM with 10% fetal bovine serum containing 450 μ g/ml of hygromycin and 0.5 mmol/L of ethylenediaminetetraacetic acid. Approximately 2 weeks later, the hygromycin-resistant populations were collected for analysis. For LMP1 induction, hygromycin and ethylenediaminetetraacetic acid were withdrawn from cell culture by washing with phosphate-buffered saline (PBS) for three times, then 1 μ mol/L of cadmium chloride (CdCl₂) was added to the cell culture, and the cells were harvested at various time points as indicated.

Western Blot Analysis

Proteins were extracted from cells by lysing with RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 1.0% Nonidet P-40, 0.5% deoxycholate (DOC), 0.1% sodium dodecyl sulfate), separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and immunoassayed with anti-LMP1 (1:250, DAKO monoclonal anti-EBV, LMP), anti-p27^{Kip1} (1:1000, Transduction Laboratories), or anti- β -actin (1:1000, Sigma) antibodies. Secondary antibodies (anti-mouse Ig, horseradish peroxidase-linked whole antibody; Amersham Pharmacia Biotech, Piscataway, NJ) were used at 1:3000 dilution and the results were developed by enhanced chemiluminescence (Amersham).

Molecular Studies

Genomic DNA was isolated by cell lysis followed by digestion with proteinase K and purified by salting-out extraction and precipitation by ethanol.²⁹ Mutations of the p53 gene (exons 5 through 9) were investigated by a combination of polymerase chain reaction-single chain conformation polymorphism and DNA direct sequencing, as previously reported.³⁰ DNA hypermethylation in the CpG islands of the p16^{INK4a} and p73 genes was determined by methylation-specific polymerase chain reaction using previously described primers and strategies.^{31,32}

Statistical Methods

Fisher exact test was used to compare differences in discrete data, whereas correlations were computed by means of Spearman (*r*) rank-order coefficients.³³

Results

Relationship between p27^{Kip1} Expression and B-Cell Differentiation Stage as Defined by MUM1, BCL-6, and syn-1 in Nontransformed Lymphoid Tissues

Expression of p27^{Kip1} was referred to the stages of mature B-cell differentiation identified by the coordinated

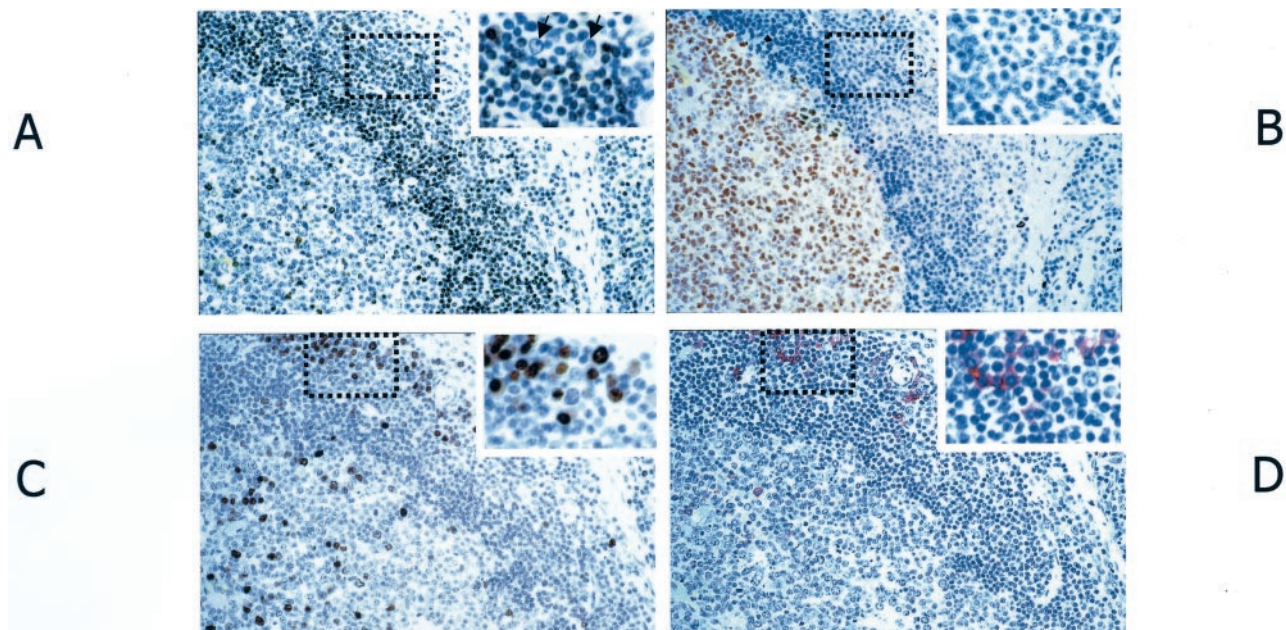


Figure 1. Relationship between p27^{Kip1} expression and B-cell differentiation stage as defined by BCL-6, MUM1, and syn-1 in nontransformed lymphoid tissues. Serial sections from hyperplastic lymph node from an HIV-infected individual with persistent generalized lymphadenopathy. **A:** A follicle with a large GC (left) and perifollicular/interfollicular areas (right) is shown. Numerous small lymphocytes in the follicular mantle zone exhibit nuclear staining (brown) for p27^{Kip1}. Several plasma cells in the perifollicular areas (dotted area and inset) also score positive. p27^{Kip1} is absent in large blast cells in the perifollicular area (inset, arrows). **B:** Within the same follicle, numerous GC cells (centroblasts and centrocytes) exhibit nuclear staining (brown) for BCL-6, whereas B cells in the mantle zone and perifollicular/interfollicular areas score negative (see also dotted area and inset). **C:** Expression of MUM1 is restricted to a small subset of cells located in the GC and to a group of perifollicular plasma cells (dotted area and inset); the staining is nuclear (brown). **D:** In the same field, cells with a plasma cell morphology show a strong cytoplasmic and membrane staining (red) with the anti-syn-1 mAb. They are present within the perifollicular areas (dotted area and inset). Conversely, intrafollicular or extrafollicular cell populations other than plasma cells are devoid of syn-1 staining. Thus, the coordinated expression of BCL-6, MUM1, and syn-1 in reactive B cells shows that the expression of p27^{Kip1} occurs early before B-cell entry in the GC, because it is found in mantle zone B cells but not in GC B cells. During the phases of B-cell transit through the GC, B cells undergo a decrease in p27^{Kip1} expression, switch off BCL-6, and start to express MUM1. Finally, on GC exit and differentiation to plasma cells, B cells express p27^{Kip1} and syn-1. Paraffin-embedded tissue sections, immunoperoxidase method (A–C), alkaline phosphatase anti-alkaline phosphatase method (D), hematoxylin counterstain. Original magnifications, ×250.

expression of BCL-6, MUM1, and syn-1 in reactive B cells within lymphoid tissues from HIV-infected individuals with persistent generalized lymphadenopathy (Figure 1, A to D). According to this model, expression of p27^{Kip1} occurs early before B-cell entry in the GC, because it is found in resting B cells of the mantle zone but not in centroblasts and centrocytes of the GC (Figure 1A). On B-cell activation and cell-cycle entry, topographically corresponding to the phases of B-cell transit through the GC, B cells undergo a decrease in p27^{Kip1} expression, switch-off BCL-6 and start to express MUM1 (Figure 1, B and C). On GC exit, immunoblasts are still p27^{Kip1}-negative (Figure

1A), MUM1-positive, but lack plasma cell morphology. Finally, differentiation to plasma cells associates with expression of p27^{Kip1} and syn-1 (Figure 1D) though retaining expression of MUM1 (Figure 1, A to D).

p27^{Kip1} and Ki-67 Expression in AIDS-NHL

Results of the expression pattern of p27^{Kip1} in systemic AIDS-NHL are detailed in Table 1. Representative examples are shown in Figures 2 and 3. Overall, expression of p27^{Kip1} was detected in 24 of 86 (28%) cases of systemic AIDS-NHL and clustered with AIDS-IBL (19 of 24, 79%) (Figure 2),

Table 1. p27^{Kip1} Protein Expression in Acquired Immune Deficiency Syndrome-Related Non-Hodgkin's Lymphomas (AIDS-NHL)

	Percentage of neoplastic cells p27 ^{Kip1} -positive					
	0%	<10%	10–24%	25–49%	50–74%	>75%
Systemic AIDS-NHL*						
BL (n = 29)	25	0	0	0	2	2
LNCCCL (n = 33)	32	1	0	0	0	0
IBL (n = 24)	5	2	1	5	5	6
AIDS-PCNSL†						
LNCCCL (n = 2)	1	0	0	1	0	0
LNCCCL/IBL (n = 4)	2	1	0	1	0	0
IBL (n = 14)	4	0	2	3	5	0

Abbreviations: BL, Burkitt's lymphoma; LNCCCL, large noncleaved cell lymphoma; IBL, immunoblastic lymphoma; PCNSL, primary central nervous system lymphoma.

*P value <0.01 comparing p27^{Kip1} expression (negative versus positive) for AIDS-IBL versus all other systemic AIDS-NHL.

†P value = 0.61 comparing p27^{Kip1} expression (negative versus positive) for AIDS-IBL versus all other AIDS-PCNSL.

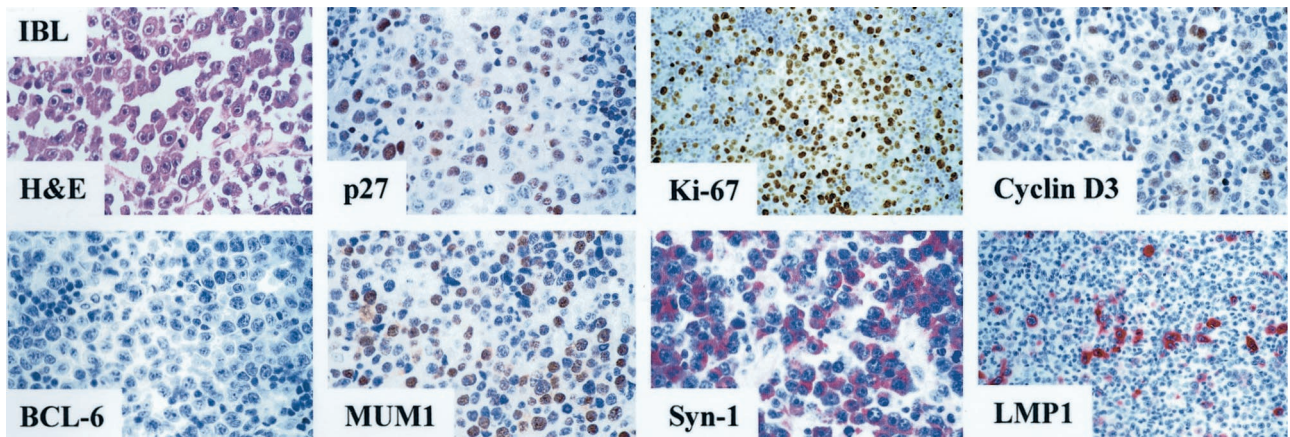


Figure 2. Relationship between p27^{Kip1} expression and B-cell differentiation stage as defined by MUM1, BCL-6, and syn-1 in AIDS-related IBL. The figure shows a p27^{Kip1}-positive IBL case that is associated with the BCL-6⁻/MUM1⁺/syn-1⁺ phenotypic pattern reflecting post-GC B cells. LMP1 is expressed by a fraction of large tumor cells. The staining is cytoplasmic and intense. In the figure numerous immunoblastic-plasmacytoid tumor cells show nuclear immunoreactivity with the anti-Ki-67 (MIB1) mAb. Furthermore, neoplastic cells display strong nuclear immunoreactivity with the anti-cyclin D3 mAb. Paraffin-embedded tissue sections, immunoperoxidase method (p27^{Kip1}, Ki-67, cyclin D3, BCL-6, MUM1), alkaline phosphatase anti-alkaline phosphatase method (syn-1, LMP1), hematoxylin counterstain. Original magnifications, $\times 250$.

whereas it was restricted to 4 of 29 (14%) cases of AIDS-BL (not shown) and to 1 of 33 (3%) cases of AIDS-LNCCL (Table 1). Difference between AIDS-IBL and all other systemic AIDS-NHLs was statistically significant ($P < 0.01$). In the majority of positive cases, nuclear p27^{Kip1} staining consistently was of moderate to strong intensity.

Expression of the proliferation marker Ki-67 among cases of systemic AIDS-NHL occurred both in the presence and in the absence of p27^{Kip1} expression (Tables 1 and 2). In most AIDS-IBL (17 of 24, 71%), the proliferative index of the neoplastic population was high (Ki-67 $> 50\%$) (Table 2, Figure 2) regardless of the high p27^{Kip1} expression. In the remaining systemic AIDS-IBL cases (7 of 24, 29%), the proliferative index of the neoplastic population was lower (25 to 49%, six cases and 10 to 24%, one case) (Table 2) and the expression of p27^{Kip1} was extremely variable ranging from negative (0%, two cases) to positive (25 to 49%, one case; 50 to 74%, one case;

$\geq 75\%$, three cases). In contrast to AIDS-IBL, the histological types AIDS-BL and AIDS-LNCCL generally failed to express p27^{Kip1} (Figure 3) and displayed an inverse relationship between the proliferative index and p27^{Kip1} positivity (Tables 1 and 2).

The results observed in systemic AIDS-NHL were also confirmed in AIDS-PCNSL (Table 1). In particular, expression of p27^{Kip1} was detected in 13 of 20 (65%) cases of AIDS-PCNSL, including 10 of 14 AIDS-IBL (71%), 1 of 2 AIDS-LNCCL, and 2 of 4 AIDS-LNCCL/IBL. In the majority of positive cases, nuclear p27^{Kip1} staining was of moderate to strong intensity. Analogous to systemic AIDS-IBL, the proliferation index of the majority of AIDS-PCNSL with IBL morphology (10 of 14, 71%) was high (Ki-67 $> 50\%$) regardless of p27^{Kip1} expression (Tables 1 and 2).

For comparative purposes, 16 IBL cases from nonimmunosuppressed patients were included in the study. Expression of p27^{Kip1} was detected in 2 of 16 (12.5%)

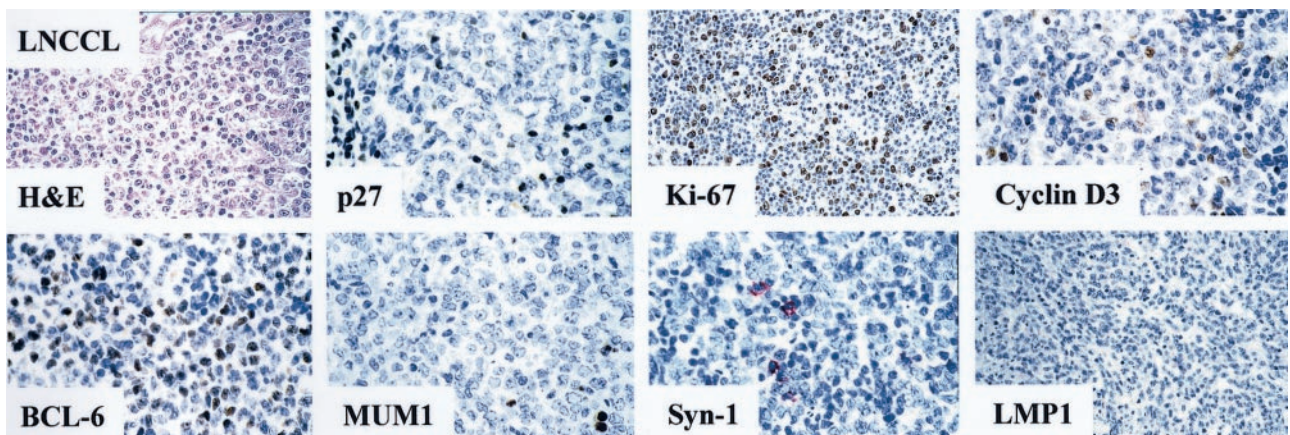


Figure 3. Relationship between p27^{Kip1} expression and B-cell differentiation stage as defined by MUM1, BCL-6, and syn-1 in AIDS-related large noncleaved cell lymphoma (LNCCL). The figure shows a p27^{Kip1}-negative LNCCL case that is associated with the BCL-6⁺/MUM1⁻/syn-1⁻ phenotypic pattern reflecting GC B cells. LMP1 is not expressed by tumor cells. In the figure several large tumor cells show nuclear immunoreactivity with the anti-Ki-67 (MIB1) mAb. Only few neoplastic cells display weak nuclear immunoreactivity with the anti-cyclin D3 mAb. Paraffin-embedded tissue sections, immunoperoxidase method (p27^{Kip1}, Ki-67, cyclin D3, BCL-6, MUM1), alkaline phosphatase anti-alkaline phosphatase method (syn-1, LMP1), hematoxylin counterstain. Original magnifications, $\times 250$.

Table 2. Ki-67 Expression in Acquired Immune Deficiency Syndrome-Related Non-Hodgkin's Lymphomas (AIDS-NHL)

	Percentage of neoplastic cells Ki-67-positive					
	0%	<10%	10–24%	25–49%	50–74%	>75%
Systemic AIDS-NHL						
BL (<i>n</i> = 29)	0	0	0	0	6	23
LNCCCL (<i>n</i> = 33)	0	1	1	7	13	11
IBL (<i>n</i> = 24)	0	0	1	6	5	12
AIDS-PCNSL						
LNCCCL (<i>n</i> = 2)	0	1	0	0	0	1
LNCCCL/IBL (<i>n</i> = 4)	0	3	0	0	1	0
IBL (<i>n</i> = 14)	0	4	0	1	9	0

Abbreviations: BL, Burkitt's lymphoma; LNCCCL, large noncleaved cell lymphoma; IBL, immunoblastic lymphoma; PCNSL, primary central nervous system lymphoma.

cases, whereas expression of the proliferation marker Ki-67 occurred in all cases. The proliferation index was high (Ki-67 > 50%) regardless of p27^{Kip1} expression.

Relationship between p27^{Kip1} Expression, LMP1, and Phenotypic Profile in AIDS-NHL

Among systemic AIDS-NHLs carrying EBV infection (38 of 73 cases, 52%), expression of the EBV-encoded LMP1 antigen was detected in 10 of 38 cases (26%) (Table 3). Most cases of LMP1-positive systemic AIDS-NHL were morphologically classified as AIDS-IBL (9 of 10, 90%) (Table 3 and Figure 2), scored positive for p27^{Kip1} (6 of 10, 60%) (Table 3), expressed both MUM1 and syn-1 (8 of 10, 80%), and stained negative for BCL-6 (10 of 10, 100%) (Table 4 and Figure 2). Conversely, expression of LMP1 was absent in 28 EBV-positive systemic AIDS-NHLs, which usually expressed BCL-6 (21 of 28, 75%) and failed to express p27^{Kip1} (20 of 28, 71%) (Figure 3). Overall, among systemic AIDS-NHL carrying EBV infection, p27^{Kip1} positivity correlates with expression of LMP1 (*r* = 0.24, *P* = 0.02) and preferentially associates with the BCL-6–/MUM1+/syn-1+ profile (Table 4).

With respect to AIDS-PCNSL, expression of LMP1 was detected in 18 of 20 cases. All LMP1-positive AIDS-PCNSLs were morphologically classified as AIDS-IBL or LNCCCL/IBL. Most of these cases expressed p27^{Kip1} (12 of 18, 67%), MUM1 (14 of 18, 78%) and/or syn-1 (13 of 18, 72%), and stained negative for BCL-6 (14 of 18, 78%). Expression of LMP1 was negative in the AIDS-PCNSL with AIDS-LNCCCL morphology.

All IBL from nonimmunosuppressed patients were EBV-negative and, in most cases, displayed the BCL-6–/MUM1+/syn-1– phenotype.

LMP1 Induces the Expression of the CDK Inhibitor p27^{Kip1}

To determine whether LMP1 expression affects the expression of p27^{Kip1} in B cells, we established a Ramos cell line (MT-LMP1) stably transfected with a vector expressing LMP1 under the control of the CdCl₂-inducible metallothionein promoter (MT). Figure 4 shows that, on treatment with CdCl₂, LMP1 expression was readily detected in cells transfected with MT-LMP1, but not in control cells transfected with the same vector lacking LMP1. As shown in Figure 4, induction of LMP1 expression in B cells is associated with induction of p27^{Kip1} expression. Overall, these results show that LMP1 is capable of inducing p27^{Kip1} expression in B cells.

Cyclin D1 and Cyclin D3 Expression in Systemic AIDS-NHL

Cyclin D1 was tested in 71 systemic AIDS-NHLs (26 AIDS-BLs, 24 AIDS-LNCCCLs, 21 AIDS-IBLs). All cases were considered negative for cyclin D1 expression. Sixty-three cases did not show any staining. Conversely, eight cases (1 of 26 AIDS-BLs, 3 of 24 AIDS-LNCCCLs, and 4 of 21 AIDS-IBLs) displayed cyclin D1 positivity in scattered and occasional cells.

Table 3. Relationship between p27^{Kip1} Expression and Latent Membrane Protein 1 (LMP1) in Acquired Immune Deficiency Syndrome-Related Non-Hodgkin's Lymphomas (AIDS-NHL) Carrying EBV Infection

	LMP1+		LMP1–	
	p27 ^{Kip1} +	p27 ^{Kip1} –	p27 ^{Kip1} +	p27 ^{Kip1} –
Systemic AIDS-NHL				
BL (<i>n</i> = 12)	0	0	1	11
LNCCCL (<i>n</i> = 10)	0	1	1	8
IBL (<i>n</i> = 16)	6	3	6	1
AIDS-PCNSL				
LNCCCL (<i>n</i> = 2)	0	0	1	1
LNCCCL/IBL (<i>n</i> = 4)	2	2	0	0
IBL (<i>n</i> = 14)	10	4	0	0

Abbreviations: BL, Burkitt's lymphoma; LNCCCL, large noncleaved cell lymphoma; IBL, immunoblastic lymphoma; PCNSL, primary central nervous system lymphoma.

Table 4. Relationship between p27^{Kip1} Expression and B-Cell Differentiation Stage as Defined by MUM1, BCL-6, and syn-1 in Acquired Immune Deficiency Syndrome-Related Non-Hodgkin's Lymphomas (AIDS-NHL)

	Major phenotypic patterns							
	BCL6 ⁺ /MUM1 ⁻ / syn-1 ⁻		BCL6 ⁺ /MUM1 ⁺ / syn-1 ⁻		BCL6 ⁻ /MUM1 ⁺ / syn-1 ⁻		BCL6 ⁻ /MUM1 ⁺ / syn-1 ⁺	
	p27+	p27-	p27+	p27-	p27+	p27-	p27+	p27-
Systemic AIDS-NHL								
BL (n = 29)	4	21	0	4	0	0	0	0
LNCCCL (n = 33)	0	26	1	4	0	1	0	0
IBL (n = 24)	0	0	0	0	3	3	16	2
AIDS-PCNSL								
LNCCCL (n = 2)	1	0	1	0	0	0	0	0
LNCCCL/IBL (n = 4)	0	0	1	0	0	0	0	0
IBL (n = 14)	0	0	0	0	2	1	8	2
EBV-positive (n = 58)	1	17	3	3	4	4	18	4
LMP1-positive (n = 28)	0	0	1	0	2	3	14	4

Abbreviations: BL, Burkitt's lymphoma; LNCCCL, large noncleaved cell lymphoma; IBL, immunoblastic lymphoma; PCNSL, primary central nervous system lymphoma.

Minor phenotypic patterns included: BCL6⁻/MUM1⁻/syn1⁻ in one systemic NHL (with LNCCCL morphology) that scored negative for p27^{Kip1}; BCL6⁻/MUM1⁻/syn1⁺ in one PCNSL (with IBL morphology) that scored negative for p27^{Kip1}; BCL6⁺/MUM1⁺/syn1⁺ in two PCNSL (with LNCCCL/IBL morphology) one of which scored positive for p27^{Kip1}; and BCL6⁺/MUM1⁻/syn1⁺ in one PCNSL (with LNCCCL/IBL morphology) that scored negative for p27^{Kip1}.

Cyclin D3 was tested in 55 systemic AIDS-NHLs (16 AIDS-BLs, 16 AIDS-LNCCCLs, 23 AIDS-IBLs). Cyclin D3 scored positive in a large fraction of cases (47 of 55, 85%), including 14 of 16 (87.5%) AIDS-BLs, 15 of 16 (94%) AIDS-LNCCCLs, and 18 of 23 (78%) AIDS-IBLs. Cyclin D3 positivity occurred both in the presence (4 AIDS-BLs, 15 AIDS-IBLs) and in the absence of p27^{Kip1} expression (10 AIDS-BLs, 15 AIDS-LNCCCLs, and 3 AIDS-IBLs) (Figures 2 and 3). Cyclin D3 was also tested in 10 AIDS-PCNSLs (2 AIDS-LNCCCLs, 6 AIDS-IBLs, 2 AIDS-LNCCCL/IBLs). Cyclin D3 could be observed only in two AIDS-IBLs that scored positive also for p27^{Kip1} expression (not shown).

Molecular Alterations of the p53, p16^{INK4a}, and p73 Genes

Mutations of the p53 tumor suppressor gene were detected in 4 of 22 tested cases of AIDS-NHL. CpG island hypermethylation of the p16^{INK4a} and p73 genes were observed in 7 of 21 and 2 of 17 AIDS-NHLs, respectively. The occurrence of molecular lesions of p53, p16^{INK4a}, and p73 genes in AIDS-NHL did not show a preferential association with a given expression pattern of p27^{Kip1} expression.

Discussion

In this study, we analyzed a large panel of systemic AIDS-NHLs and AIDS-PCNSLs for the expression of p27^{Kip1} in relation to the lymphoma proliferation index, B-cell differentiation stage, EBV status, and expression of cellular cyclins D3 and D1. We report that AIDS-IBLs, either systemic or primarily localized to the central nervous system, consistently express p27^{Kip1} despite the high proliferative rate of the lymphoma clone, suggesting a failure of p27^{Kip1} to inhibit the cell cycle in AIDS-IBL cells. The co-existence of p27^{Kip1} expression and high proliferative index is a selective feature of AIDS-IBLs among systemic AIDS-NHLs and AIDS-PCNSLs and may be because of concomitant expression of the EBV-encoded LMP1 and/or to peculiarities in the differentiation stage of the lymphoma clone.

The role of EBV in determining the co-existence of p27^{Kip1} and high proliferative index in AIDS-IBLs is supported by the observation that LMP1 is generally expressed by p27^{Kip1}-positive AIDS-IBLs *in vivo* and is able to induce p27^{Kip1} expression in B cells *in vitro*. Conceivably, up-regulation of p27^{Kip1} may be mediated by reg-

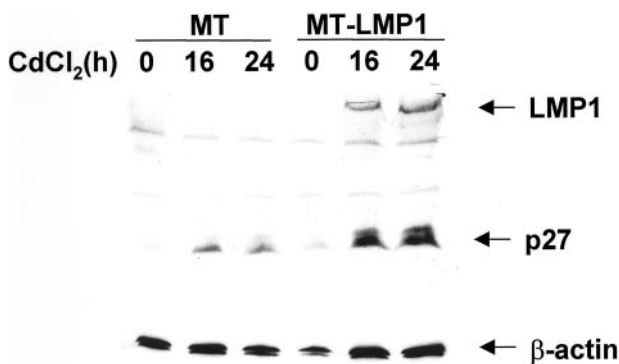


Figure 4. Induction of LMP1 expression activates p27^{Kip1} expression. Ramos cells were stably transfected with an expression vector (MT-LMP1) in which LMP1 expression was dependent on the metallothionein promoter, or with empty control vector (MT). The stably transfected cells were treated with CdCl₂ for 16 hours or 24 hours as indicated, and then LMP1 expression (**top**), p27^{Kip1} expression (**middle**), and β-actin expression (**bottom**, as a loading control) were analyzed by Western immunoblotting using specific antibodies. The results show that p27^{Kip1} is induced on LMP1 induction. A modest p27^{Kip1} induction is observed also in control cells because of CdCl₂ treatment.

ulation of BCL-6 expression. In fact, LMP1 is able to down-regulate expression of BCL-6,^{1,34} that, in turn, physiologically exerts a negative effect on p27^{Kip1} expression.³⁵ Thus, by removing a negative regulator of p27^{Kip1}, namely BCL-6, LMP1 allows for up-regulation of p27^{Kip1} expression. According to this model, it may be argued that LMP1 is able to stimulate the lymphoma growth and cell-cycle progression despite the high expression of the cell-cycle inhibitor p27^{Kip1}. In particular, it may be envisaged that expression of LMP1 by AIDS-IBLs induces and simultaneously overcomes the p27^{Kip1}-mediated inhibition of cell growth. The precise molecular mechanism allowing LMP1 to overcome p27^{Kip1}-mediated cell-cycle inhibition in lymphoma cells is not known and may be related to currently unexplored molecular derangements proper of the tumor clone. However, because some p27^{Kip1}-positive AIDS-IBL cases are LMP1-negative, it is conceivable that EBV infection may determine the co-existence of p27^{Kip1} and high proliferative index in AIDS-IBL also by mechanisms that are independent of LMP1 expression.

The co-existence of p27^{Kip1} expression and high proliferative index in AIDS-IBL may also be related to the differentiation stage of the lymphoma clone, that is generally characterized by the BCL-6-/MUM1+/syn-1+ phenotype of preterminally and terminally differentiated B cells. Indeed, in normal physiology, expression of p27^{Kip1} associates with B cells that have exited the GC and differentiate to plasma cells, as indicated by acquisition of the BCL-6-/MUM1+/syn-1+ phenotype. Conversely, GC centroblasts and centrocytes, characterized by the BCL-6+/MUM1-/+syn-1- phenotype, fail to express p27^{Kip1}. The hypothesis that p27^{Kip1} expression in AIDS-IBL may be related to the tumor differentiation stage is reinforced by the notion that, among AIDS large-cell lymphomas, co-expression of p27^{Kip1} and Ki-67 is virtually restricted to AIDS-IBL (this study) and to primary effusion lymphoma,^{10,11} a rare lymphoma type associated with HHV-8 infection and constituted of preterminal B cells displaying the BCL-6-/MUM1+/syn-1+ phenotype typical also of AIDS-IBL.

Co-expression of p27^{Kip1} and Ki-67 is a preferential feature of AIDS-IBL also considering tissue-based large B-cell lymphomas of the immunocompetent host. In fact, with the exception of few B-diffuse large-cell lymphomas,³⁶ the blastic variant of mantle cell lymphoma²¹ and a fraction of Burkitt's lymphoma,³⁶ NHL of the immunocompetent host generally display an inverse relation between p27^{Kip1} and growth fraction.¹⁹⁻²¹ In particular, our data indicate that regulation of p27^{Kip1} differs markedly in IBL of immunocompetent hosts compared to AIDS-IBL, because IBL of immunocompetent hosts display the inverse relation between p27^{Kip1} expression and growth fraction and, accordingly, score negative for p27^{Kip1} in virtually all cases. Differences in p27^{Kip1} expression in AIDS-IBL *versus* IBL of immunocompetent hosts may reflect differences in the virological features and/or the differentiation stage of the lymphoma. In fact, expression of the EBV-encoded LMP-1, which is capable of up-regulating p27^{Kip1}, is restricted to AIDS-IBL, whereas it is consistently negative in IBL of immunocompetent hosts.

Also, AIDS-IBLs generally display the BCL-6-/MUM1+/syn-1+ phenotype, that associates with p27^{Kip1} expression in B-cell physiology, whereas IBL of immunocompetent hosts show a lesser degree of differentiation, identified by the BCL-6-/MUM1+/syn-1- phenotype that normally fails to express p27^{Kip1}.

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