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Differential Expression of Cyclin-Dependent Kinase 6 in Cortical Thymocytes and T-Cell Lymphoblastic Lymphoma/Leukemia

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Cyclin-dependent kinase-6 (CDK6) is the earliest inducible member of the CDK family in human T lymphocytes, involved in growth factor stimulation and cell cycle progression. CDK6 is one of the targets of p16 and p15, CDK inhibitors encoded by MTS1 and MTS2, two tumor suppressor genes that are frequently deleted in T-cell leukemia. In this study we have investigated CDK6 expression in normal and neoplastic lymphoid tissues using immunohistochemistry and flow cytometry. In normal (six samples) and hyperplastic (four samples) thymuses, strong CDK6 expression was observed in a discrete proportion of cortical thymocytes (10 to 15%), mainly located in the peripheral (subcapsular) zone of the cortex. All tested cases of T-cell lymphoblastic lymphoma/leukemia (T-LBL/ALL) showed strong CDK6 expression in the majority (up to 100%) of neoplastic lymphoid cells. Western blot analysis confirmed the expected CDK6 protein size (40 kd). According to Southern blot analysis, CDK6 overexpression in neoplastic T lymphoblasts was not due to gene amplification. In all other lymphomas investigated (28 peripheral T-cell non-Hodgkin's lympohomas (T-NHLs), 7 CD30$^+$ anaplastic NHLs, 22 high-grade B-NHLs, 15 low-grade B-NHLs, 25 B-cell precursor ALLs), CDK6 was not expressed or expressed at low levels, with the only exception of three nasal angiocentric T-NHLs, all exhibiting CDK6 immunoreactivity comparable to that observed in T-LBL/ALL. These data provide evidence that CDK6 is abnormally expressed in T-LBL/ALL and may be involved in the pathogenesis of this malignancy. In addition, the quantitative difference of CDK6 expression between neoplastic and non-neoplastic cortical thymocytes can be potentially useful in the differential diagnosis of thymic neoplasms on histological and cytological specimens. ***(Am J Pathol 1998, 152:209–217)***

Progression through the cell cycle in eukaryotic cells is mediated by positive regulators of cell proliferation, such as cyclin-dependent kinases (CDKs), and negative regulators, such as those encoded by the tumor suppressor genes p53 and retinoblastoma (pRB), as well as different CDK inhibitors, such as p21^{WAF1}, p27^{KIP-1}, p16^{MTS1}, p15^{MTS2}, and others.[1] Intense investigation is currently devoted to understanding to what extent the deregulation of this array of molecules is involved in the pathogenesis of human neoplasias, searching possible molecular targets for therapy or diagnostic and prognostic markers to be applied in clinical practice.[1–6]

CDK4 and CDK6, which are the most homologous among the various CDKs, after coupling with cyclins of the D family, regulate the cell cycle entry and/or transit through very early G1 phase by phosphorylating pRB.[1,7–10] In T lymphocytes, CDK4 and CDK6 are able to associate with proliferating cell nuclear antigen and the regulatory subunit cyclins D2 and D3 after cell activation, thus forming the catalytically active kinases necessary for cell proliferation.[11–13] CDK6 seems to play a particular role in human T cell activation as it is the earliest inducible member of the CDK family in T lymphocytes, first appearing in mid-G1, before the up-regulation of any other CDK, and independently of the key T cell progression factor interleukin (IL)-2.[8,13] Interestingly, CDK6 shows a striking differential expression among normal tissues, being detected at high levels only in primary cultures of T lymphocytes.[14] CDK6 is one of the targets of p16^{MTS1} and p15^{MTS2}, two highly homologous CDK inhibitors that exert their inhibitory function by directly binding CDKs, thus specifically interfering with the formation of catalytically active kinase complexes.[15–17] p16^{MTS1} and p15^{MTS2} are encoded by two genes (MTS1 and MTS2) that are frequently deleted and/or mutated in T-cell leukemias.[18–23] MTS1 inactivation, observed in at least 80% of T-cell acute lymphoblastic leukemias (T-ALLs), is the most con-

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sistent genetic defect found in this disease.[24] Taken together, these data suggest that the molecular mechanism centered on the interaction of p16 and p15 inhibitors and their target CDK6 plays a fundamental role in the regulation of the G1 checkpoint in T cells and that deregulation of this molecular system is primarily involved in the development of T-cell lymphoblastic neoplasias. The aim of this study was to investigate the pattern of CDK6 expression in normal and neoplastic mature and immature T cells, analyzing a series of T-cell lymhoblastic lymphomas (T-LBLs)/ALLs and peripheral T-cell lymphomas (together with a series of B-cell lymphomas as controls), using immunophenotypical and molecular analyses.

Materials and Methods

Samples Composition

Formalin-fixed, paraffin-embedded samples were collected from the files of the Departments of Pathology of Verona University and New York University. The investigated tissue samples included fetal thymus (2 samples), infantile thymus (4 samples), T-LBL/ALL (16 bone marrow trephine biopsies, 5 mediastinal masses, and 4 involved lymph nodes), thymoma samples with a rich lymphoid component (7 samples), and thymic hyperplasia from patients with myasthenia gravis (4 samples). Control tissues included normal bone marrow (10 samples, obtained during staging procedures) and reactive lymph node (4 samples). A series of other non-Hodgkin's lymphomas (NHLs) were also studied, including peripheral T-cell NHL (28 cases), $CD30^+$ anaplastic T/null NHL (7 cases), B-cell NHL (22 high-grade and 15 low-grade), and B-cell precursor lymphoblastic leukemia (BCP-ALL; 25 cases). All samples were collected at diagnosis before any treatment. T-LBL/ALL cases were diagnosed on the basis of morphological and immunophenotypical features (blasts exhibiting nuclear terminal deoxynucleotidyl transferase together with T-cell markers such as membrane expression of CD7, CD5, CD1, and cytoplasmic CD3).[25] The thymomas investigated were of the cortical (4 samples) or mixed (3 samples) types according to recent classifications[26] and had a large lymphoid component characterized by the cortical thymocyte immunophenotype (TdT^+, $CD1^+$, $CD3^+$).[27–29] Peripheral T-cell NHLs and B-cell NHLs were diagnosed on the basis of morphological and immunophenotypical features according to the criteria of the Kiel and the Revised European-American Lymphoma classifications.

Sample Immunostaining

The immunohistochemical analysis for CDK6 was performed on formalin-fixed (<24 hours of fixation), paraffin-embedded material using a biotin-streptavidin immunoperoxidase technique (LSAB peroxidase, Dako, Carpinteria, CA). Briefly, sections were deparaffinized in xylene, rehydrated, washed in PBS, immersed in 0.01 mol/L citrate buffer, pH 6, and irradiated in a microwave oven for 5 minutes at 750 W (three times) and 10 minutes at 600 W (once). Sections were then kept for 15 minutes at room temperature before additional PBS washing and immunostaining. CDK6 immunostaining was performed using an affinity-purified rabbit polyclonal antibody recognizing CDK6 (C-21 antibody, Santa Cruz Biotechnology, Santa Cruz, CA) raised against a peptide corresponding to amino acid residues 306 to 326 mapping at the carboxy terminus of CDK6.

A quantitative evaluation of antigen expression was performed, assigning to each case a different score, obtained evaluating the percentage of positive cells counted in different high-power microscopic fields. A thorough analysis of serial sections was used with a selected panel of antibodies recognizing different cell types to precisely define the nature of CDK6-positive cells. A CDK6/Ki67 combination was performed on thymic samples to investigate the proliferative status of CDK6-positive cells, at the single-cell level, using a previously described double marker technique.[30]

Flow Cytometric Analysis

For flow cytometric detection of nuclear CDK6, 1×10^6 cells in suspension were first treated with the FACS permeabilizing solution (Becton Dickinson, San Jose, CA), according to the manufacturer's instructions. After incubation with a rabbit anti-CDK6 antibody (Santa Cruz; 1:100) for 30 minutes (4°C) and washing, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated (F(ab')2 fragments of sheep anti-rabbit immunoglobulin (1:300; Organon Teknika-Cappel, Malvern, PA). For two-color analysis, the cells were first stained with anti-CDK6 or hyperimmune rabbit sera and then incubated with the appropriately diluted phycoerythrin-conjugated monoclonal antibody (CD19 and CD5, Becton Dickinson) for 30 minutes at 4°C. Multiple combinations of isotype-matched monoclonal antibodies with irrelevant specificity were used as negative controls. Cells stained with single positive fluorescence were used as single positive controls to perform a manual electronic compensation. In mixed experiments, highly purified cells from a patient with chronic lymphocytic leukemia (B-CLL; $CD19^+/CD5^+$, >95%) were mixed with ALL cells ($CD4^+/CD8^+/CD5^+$, >95%) in different ratios (CLL/ALL: 100%/0%, 75%/25%, 50%/50%, 25%/75%, and 0%/100%). Mixed cells were stained as described above. Flow cytometric analysis was performed using the FACScan cytometer (Becton Dickinson) as previously described.[31]

Western Blot Analysis

To confirm the specificity of CDK6 immunoreactivity in our experimental conditions, tissue lysates obtained from six samples of T-LBL/ALL (two from mediastinal mass and four from peripheral blood leukemic samples) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions, followed by Western blotting.[32] The same antibody to CDK6 used for immunohistochemistry was used for Western blotting.

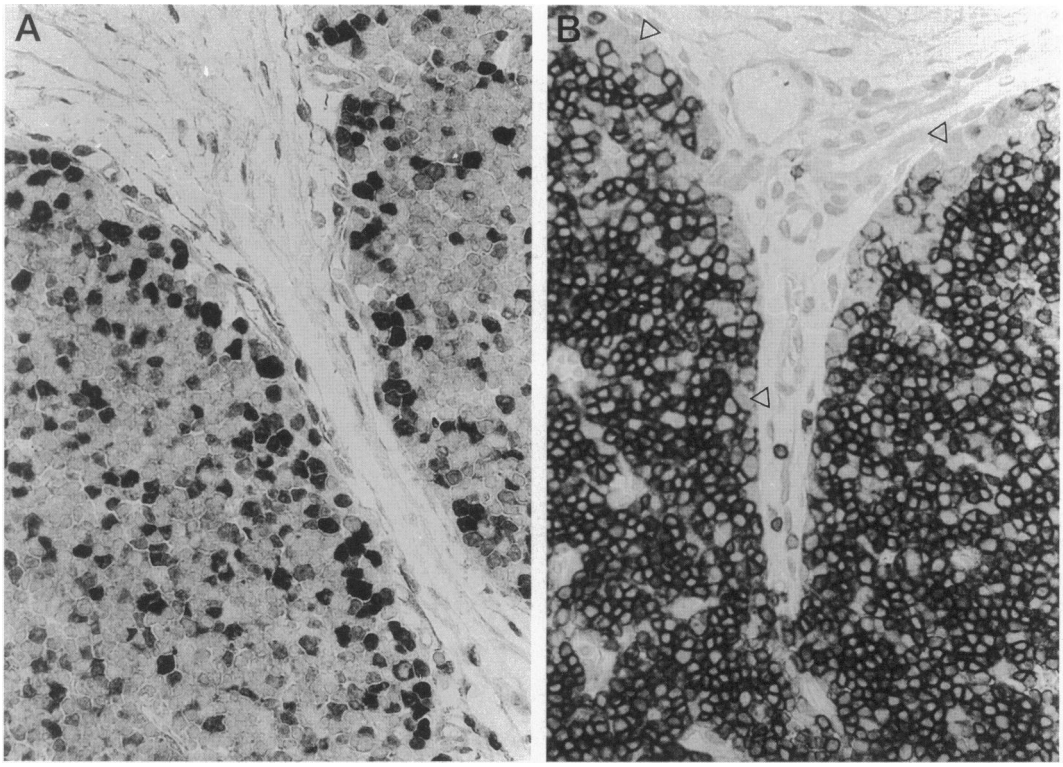

Figure 1. Infant thymus. Immunoreactivity for CDK6 (**A**) is evident in a proportion of cortical thymocytes (10 to 15%), with some clustering in the subcapsular zone where immature thymocytes predominate as shown by the lack of CD8 expression (**arrowheads** in **B**). LSAB-immunoperoxidase on consecutive paraffin sections; magnification, ×250.

Southern Blot Analysis

Ten-microgram aliquots of genomic DNA were digested, electrophoresed, denatured, and transferred to nitrocellulose filters according to Southern. The genomic *Eco*RI- and *Bgl*II-digested DNAs were hybridized with the CDK6 cDNA.[8] The organization of the c-*myc* (MC413RC)[33] and *bcl*-1 major breakpoint (MTC)[34] loci were also investigated as control genes. Filters were scanned using ID Image Analysis software, Kodak Digital Science (Eastman Kodak Co., Rochester, NY), to evaluate the optical density of each corresponding band. Normalization was achieved by dividing the CDK6 values (individual bands) by the values obtained from the hybridization of the same filters using c-*myc* and/or *bcl*-1 MTC probes as controls.

Results

Reactive Lymph Nodes

In all samples, CDK6 immunostaining was confined to a few large lymphoid cells scattered in paracortical areas. The staining appeared as both nuclear and cytoplasmic. On the basis of double-marker and serial section analysis with B- and T-cell markers (CD3, CD8, and CD20) $CDK6^+$ cells could be recognized as T cells. A weak CDK6 expression was observed in germinal center cells of lymphoid follicles (not shown).

Normal Bone Marrow

Extremely few scattered large blasts were observed showing strong CDK6 immunostaining, again characterized by nuclear and cytoplasmic distribution.

Normal Infant and Fetal Thymuses

A discrete number of CDK6-immunoreactive cells were observed in the thymic cortex, mainly localized in the outer (subcapsular) zone (Figure 1A). CDK6-immunoreactive cells were mainly large lymphoid blasts, characterized on serial section and double-marker analysis by the TdT^+, $CD8^-$ immunophenotype (Figure 1B). A quantitative evaluation of $CDK6^+$ cortical thymocytes performed on immunohistochemical preparations and by flow cytometry revealed figures varying between 10 and 15% (Table 1). These figures clearly contrasted with the very high proportion of thymocytes expressing the proliferation-related antigen Ki67.[29] Low proportions of $CDK6^+$ thymocytes, similar to those observed in normal cortex, were observed in thymic samples from myasthenic patients with follicular thymic hyperplasia.

Thymoma Samples

The proportion of $CDK6^+$ thymocytes (recognized by CD1, CD8, and/or terminal deoxynucleotidyl transferase

Table 1. Expression of CDK6 Revealed by Immunohistochemical Analysis in Normal and Pathological Samples of Lymph Node, Bone Marrow, and Thymus

Sample	Number of samples	CDK6 reacting cells	%
Reactive lymph node	4	T cells in PCA	<1%
Normal bone marrow	10	Large blasts	<1%
Normal thymic samples			
Fetal thymus	2	Cortical thymocytes	10–15%
Infant thymus	4	Cortical thymocytes	10–15%
Hyperplastic thymus	4	Cortical thymocytes	10–15%
Thymoma (total 7)			
Cortical	4	Thymocytes	10%
Mixed	3	Thymocytes	10–30%
T-LBL/ALL (total 25)			
T-LBL (mediastinum)	6	T blasts	90–100%
T-LBL (lymph node)	3	T blasts	90–100%
T-ALL (bone marrow)	16	T blasts	90–100%

PCA, paracortical areas.

immunostaining on serial sections) was similar to that observed in infant thymic cortex (Figure 2). The proportion of CDK6$^+$ cells was heterogeneous in different tumor areas, with higher figures in areas where thymocytes were less represented and epithelial cells predominated. Two cases of thymoma of the mixed (cortical and medullary) histological type exhibited higher proportions of CDK6$^+$ cells (up to 30%; Table 1).

T-Cell Lymphoblastic Lymphoma/Leukemia

A completely different pattern of CDK6 expression was observed in all 25 cases of T-LBL/ALL analyzed on lymph node, mediastinal, or bone marrow samples. In fact, most neoplastic lymphoblasts (up to 100%) exhibited strong nuclear and cytoplasmic immunostaining in all cases. (Table 1 and Figures 3 and 4).

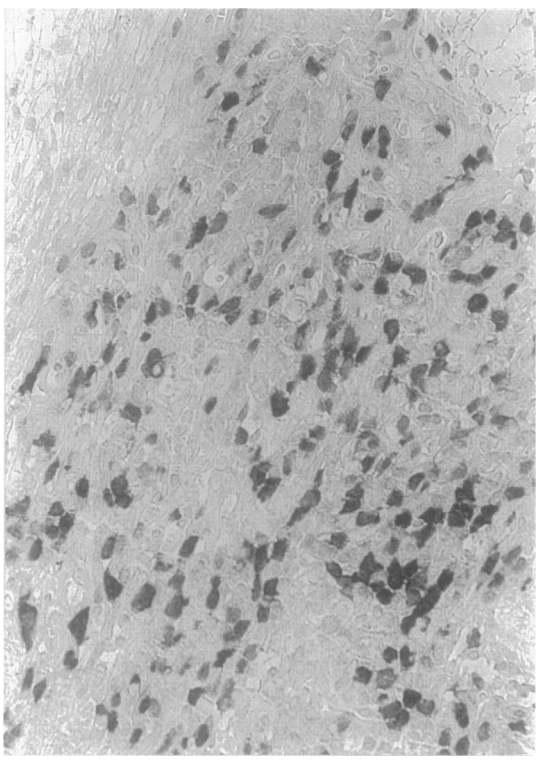

Figure 2. Cortical thymoma with abundant lymphoid component immunostained for CDK6, showing scattered positive thymocytes. LSAB-immunoperoxidase; magnification, ×250.

Peripheral T-Cell Lymphomas

In 19 of 28 cases of peripheral T-NHL, CDK6 expression was either very low or absent. In only three cases was the expression of CDK6 similar to that observed in T-LBL/ALL for both intensity and proportion of positive cells. All of these three cases were diagnosed as nasal/angiocentric lymphomas, exhibiting T-cell markers (CD3 and/or CD5 expression), together with strong CD56 and granzyme expression. In 7 of 28 cases, the number of CDK6-immunoreactive cells was within the 10 to 30% range, with a single case in the 30 to 50% range (Table 2). Among these latter cases, three were diagnosed as mycosis fungoides and four were characterized by a predominance of large cells (Table 2).

B-Cell Precursor Lymphoblastic Leukemia

Twenty-five BCP-ALL samples, analyzed using the same technique on paraffin-embedded bone marrow trephine biopsies, were either negative for CDK6 expression or showed faint immunoreactivity in most cells. In a single case, a relatively high proportion of CDK6-positive cells was demonstrated, which nevertheless did not reach the intensity of staining observed in T-LBL/ALL.

B-Cell Lymphomas

Only 2 of 37 cases, both characterized by high-grade morphology, showed CDK6 immunoreactivity (nuclear and cytoplasmic) in the 30 to 50% range. In most high-grade cases, cytoplasmic immunoreactivity could be observed in 50 to 80% of neoplastic cells, but nuclear CDK6 expression was <10%. All 15 cases of low-grade B-NHL (including follicular centroblastic/centrocytic NHL, B-cell chronic lymphocytic leukemia, and hairy cell leukemia) were negative.

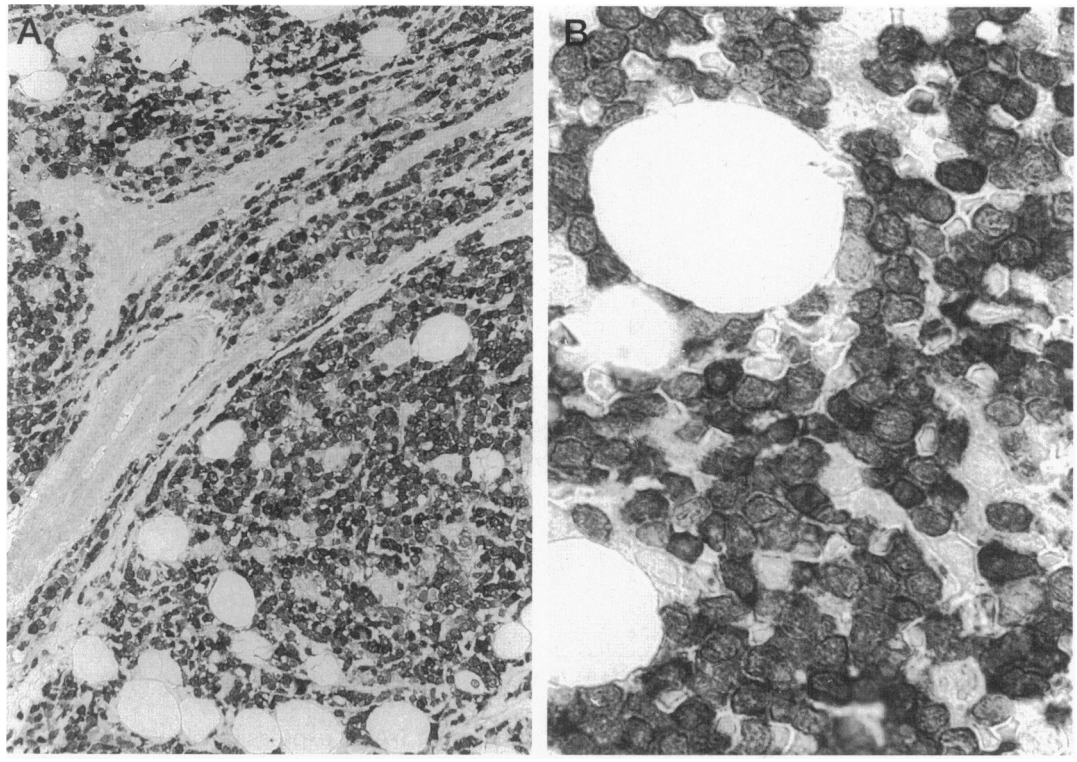

Figure 3. Mediastinal T-lymphoblastic lymphoma. Most cells show strong CDK6 expression (**A**), with nuclear and cytoplasmic location at higher magnification (**B**). LSAB-immunoperoxidase; magnification, ×100 (**A**) and ×250 (**B**).

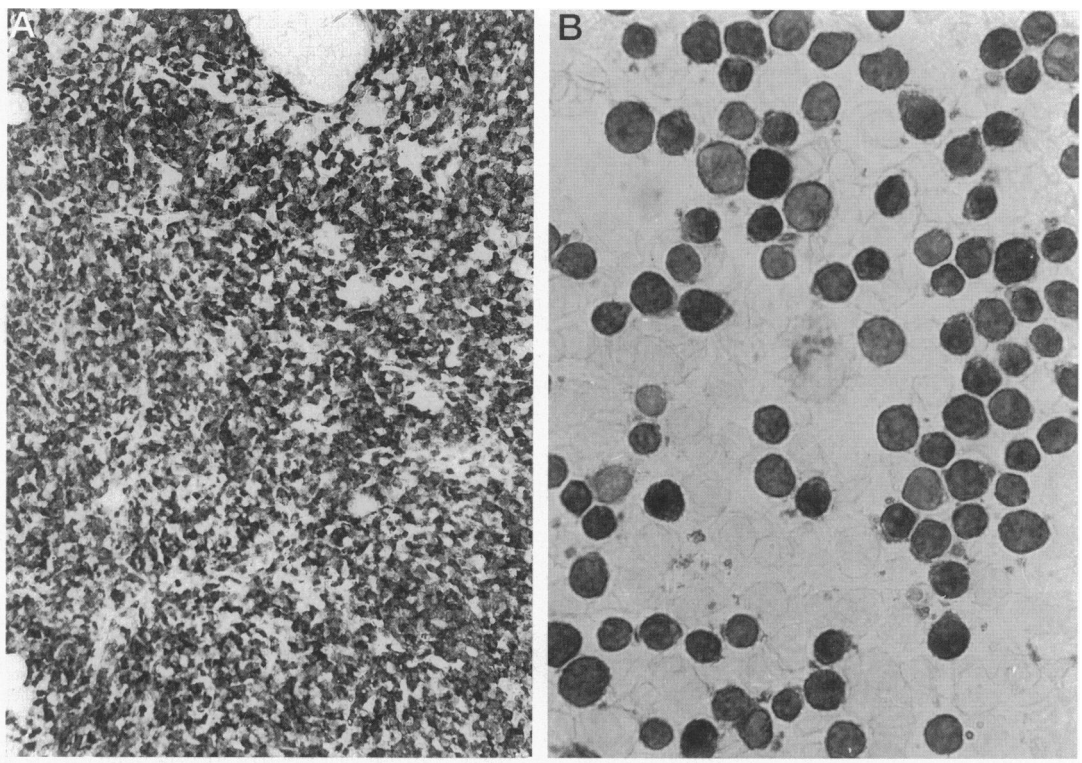

Figure 4. T-ALL. Most blastic cells infiltrating the bone marrow (**A**) and peripheral blood (**B**) express high levels of CDK6. LSAB-immunoperoxidase; magnification, ×100 (**A**) and ×400 (**B**).

Table 2. Expression of CDK6 in T-Cell Lymphomas and B-Cell Lymphomas

	Number of cases	CDK6 Immunoreactivity			
		+++	++	+	−
T-cell lymphomas					
T-LBL/ALL	25	25	0	0	0
NHL T peripheral (total)	28	3	1	5	19
Immunoblastic/large cell	6	0	1	2	3
Mycosis fungoides/Sezary	7	0	0	3	4
Nasal angiocentric T-cell l.	3	3	0	0	0
T-CLL (CD4+)	2	0	0	0	2
AILD	1	0	0	0	1
Unspecified	9	0	0	0	9
NHL T/null $CD30^+$	7	0	0	0	7
B-cell lymphomas					
BCP ALL	25	0	1	4	20
NHL B high-grade	22	0	2	1	19
Diffuse large cell	19	0	2	1	16
Burkitt's	2	0	0	0	2
Richter	1	0	0	0	1
NHL B low-grade	15	0	0	0	15
Follicular	5	0	0	0	5
B-CLL	5	0	0	0	5
Hairy cell leukemia	2	0	0	0	2
Mantle cell lymphoma	2	0	0	0	2
Lymphoplasmacytoid	1	0	0	0	1

Quantitative evaluation of nuclear CDK6 expression: −, 0 to 10%; +, 10 to 30%; ++, 30 to 50%; +++, 50 to 100% positive.

Flow Cytometry

Flow cytometric analysis and dilution experiments performed on permeabilized samples of T-ALL and B-CLL confirmed the high expression of CDK6 in neoplastic thymocytes (Figures 5 and 6) when compared with normal controls.

Western Blotting Preparations

Figure 7 shows discrete bands in all T-LBL/ALL samples, corresponding to the expected CDK6 protein size (40 kd). No reactivity was detected under the same experimental conditions using an isotype-matched antibody (not shown).

CDK6 Southern Blot Analysis

The precise mechanisms leading to the overexpression/deregulation of CDK6 in T blasts is unclear. To gain some insight in this process, we evaluated the possible presence of CDK6 gene amplification. Toward this end, seven cases of T-ALL, expressing high levels of CDK6 protein, demonstrated either by flow cytometry and/or immunohistochemistry, were evaluated by Southern blot analysis. As the control group, BCP-ALL cases were used. CDK6 gene amplification was assessed by comparing CDK6 numerical values with those obtained after c-*myc* or *bcl*-1, after ID-Image Analysis software analysis (numbers are expressed as the ratio of *Eco*RI-digested DNAs after hybridization with PLSTIRE (CDK6)/MC413RC (c-*myc*); mean ± SD: 1.31 ± 0.112/1.277 ± 0.108 or CDK6/*bcl*-1/MBR (data not shown) values (Figure 8, A and B)). Using this approach, we observed no significant differences

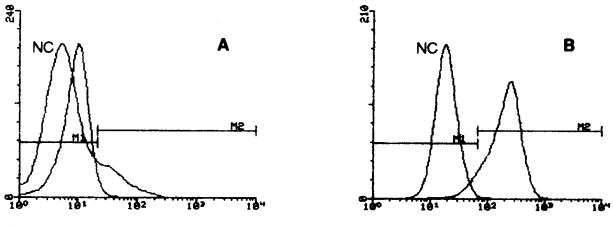

Figure 5. Intracellular CDK6 expression in normal thymocytes and neoplastic T-ALL cells by flow cytometry. Normal thymocytes (**A**) and neoplastic T-ALL cells (**B**) were incubated with appropriate amounts of hyperimmune rabbit (NC) and anti-CDK6 rabbit antisera (right histogram) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody as described in Materials and Methods (see above) and analyzed using FACScan.

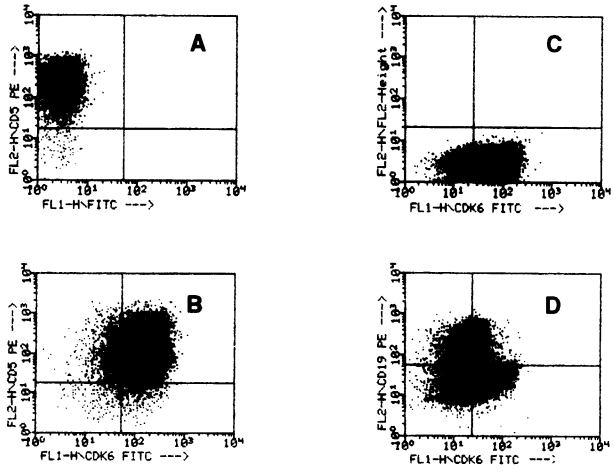

Figure 6. Specific intracellular expression of CDK6 in T-ALL cells by flow cytometry. The CDK6 gene expression was investigated (blasts were >95%) after staining with anti-CD5 (**A**) and anti-CDK6 (**B**) antibodies. To confirm the specificity of the CDK6 binding, T-ALL cells (50%) were mixed with monoclonal B cells obtained from a patient with chronic lymphocytic leukemia (50%) and then incubated with anti-CDK6 (**C**) and anti-CD19 (**D**) antibodies. The results demonstrate that flow cytometry can specifically identify intracellular CDK6 antigens in T-ALL cells.

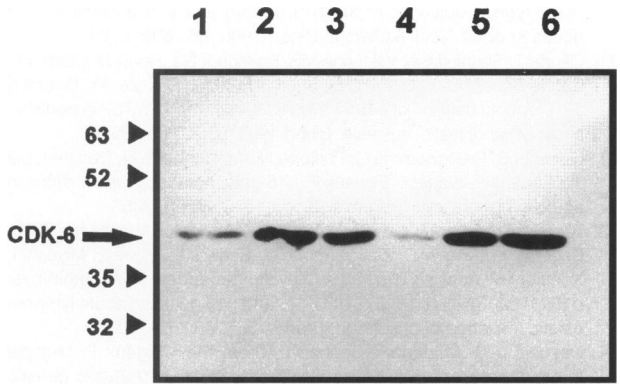

Figure 7. Western blotting preparations showing discrete bands in all T-cell lymphoblastic leukemia/lymphoma samples (**lanes 1** to **6**), corresponding to the expected CDK6 protein size (40 kd).

among T-ALLs and/or with BCP-ALL as the control group. Thus, these results tend to exclude the possibility that CDK6 overexpression/deregulation in T-ALL is due to CDK6 gene amplification.

Discussion

In this study we demonstrate that all cases of T-LBL/ALL are characterized by strong expression of CDK6 in the large majority of neoplastic cells (up to 100%), at variance with most other types of lymphomas investigated. The expression of CDK6 in neoplastic T lymphoblasts, demonstrated by immunohistochemical analysis and flow cytometry in our study and by immunochemical analysis on cell lysates of human leukemias by Wolowiec et al,[35] can be considered as abnormal overexpression as $CDK6^+$ thymocytes represented less than 15% in all control thymic samples (including fetal, infant, and hyperplastic thymuses). Similarly, the non-neoplastic lymphoid component of thymomas, which is known to exhibit cortical immunophenotypic features,[25–28] showed figures never exceeding 30% $CDK6^+$ lymphoid cells. In normal thymic samples, $CDK6^+$ cells were mainly located within the peripheral areas of the cortex, corresponding to the subcapsular region where immature prothymocytes are frequent.[36] This peculiar localization suggests that CDK6 can play a major role in the control of the cell cycle during the early phases of T cell ontogeny.

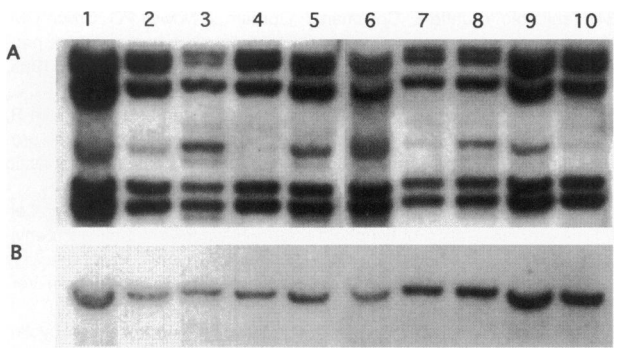

Figure 8. CDK6 normal gene configuration in T-ALL and B-ALL by Southern blot analysis. Genomic DNAs of seven T-ALL (**lanes 1** to **7**) and three BCP-ALL (**lanes 8** to **10**) were digested with *Eco*RI restriction endonuclease, electrophoresed, transferred, and hybridized with ^{32}P-radiolabeled CDK6 (PLSTIRE; **A**) and c-*myc* (MC413RC; **B**) cDNA probes. Samples are indicated as case numbers.

The demonstration of CDK6 overexpression in T-LBL/ALL can be an interesting finding for different reasons. First, several studies have revealed the central role of G1 dysregulation in lymphoblastic leukemia by demonstrating the frequent loss of function of p15 and p16 CDK6 inhibitors, due to deletions of MTS1 and MTS2 genes.[18–24] In T-LBL/ALL oncogenesis, it can be hypothesized that the acquired loss of these potent CDK inhibitors can trigger the emergence and perpetuation of a malignant clone accounting for the malignant transformation of T lymphoblasts. In this context, the overexpression of CDK6 could be directly related to the lack of functional inhibitors, providing the abnormal signals necessary for neoplastic proliferation and block of cell differentiation (as recently shown for CDK4 in erythroleukemia cells).[37] In fact, G1 cell cycle deregulation can be achieved not only by MTS1 gene loss but also by CDK6 amplification, as recently demonstrated in human gliomas, another malignancy characterized by frequent MTS1 gene deletion and CDK4 gene amplification.[38,39] A similar role of CDK6 gene amplification as a mechanism accounting for the observed CDK6 overexpression/deregulation in leukemic T blasts was ruled out by our Southern blot analysis.

According to our study, CDK6 overexpression seems mainly restricted to lymphoblastic T-cell neoplasms, as most peripheral T-cell and B-cell lymphomas of our series were characterized by either low expression or complete lack of nuclear immunoreactivity for CDK6. The only tumor exhibiting CDK6 expression comparable to that observed in T-LBL/ALL was nasal angiocentric lymphoma (all three cases of our series were strongly reactive for CDK6), a neoplasm characterized by peculiar immunophenotypic and clinical features.

A more precise understanding of cell cycle dysregulation taking place in T-cell malignancy could provide new targets for drug design. In fact, at variance with other members of the CDK family, the expression of CDK6 is not under glucocorticoid regulation[40]; thus, compounds specifically inhibiting CDK6 could be searched and rationally tested for developing alternative protocols for lymphoblastic T-LBL/ALL cases refractory to conventional therapy.[41–44]

Finally, the different immunophenotypic level of CDK6 expression between neoplastic and non-neoplastic cortical thymocytes could represent a useful diagnostic marker in the differential diagnosis of mediastinal masses. In fact, in different types of thymic lesions, including thymic hyperplasia, thymoma with rich lymphoid component, and T-LBL/ALL, a large number of lymphoblasts are usually found at histological and cytological analysis, which exhibit a common immature phenotype (TdT^+, $CD3^+$, $CD1^+$, etc).[27–29] Consequently, the differentiation between these lesions may be difficult or impossible, especially in fine-needle aspiration preparations.[45] Gene rearrangement analysis can be a valuable diagnostic tool in these cases by proving the clonality in T-LBL,[46] providing that sufficient numbers of fresh cells are avail-

able, but this is not always the case. As shown in our study, the analysis of CDK6 can be performed on routine paraffin-embedded material or cytological smears and also by flow cytometry, thus providing a simple and reliable additional tool to distinguish neoplastic from non-neoplastic cortical thymocyte populations.

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