Retinoblastoma Protein Is Frequently Absent or Phosphorylated in Anaplastic Large-Cell Lymphoma

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**The possible role of retinoblastoma protein (Rb) in
the pathogenesis of anaplastic large-cell lymphoma
(ALCL) is unknown. We investigated Rb protein ex-
pression, both total (phosphorylated and underphos-
phorylated) and active (underphosphorylated), in
four anaplastic lymphoma kinase (ALK)-positive
ALCL cell lines (Karpas 299, JB-6, SU-DHL1, and SR-
786) by Western blot analysis, and in 67 ALCL tumors
(30 ALK-positive, 37 ALK-negative) using immunohis-
tochemical methods. We also used fluorescence *in
situ* hybridization and polymerase chain reaction
methods to assess for loss of heterozygosity of the *rb*
gene. The findings were correlated with apoptotic
rate assessed by the terminal dUTP nick-end labeling
assay. Immunoblots showed high total Rb levels in
Karpas 299, SU-DHL1 and SR-786 and relatively lower
levels in and JB-6. Underphosphorylated Rb was neg-
ative or expressed at low levels in all cell lines. In
ALCL tumors, total Rb was detected in 44 (66%) and
absent in 23 (34%). The mean apoptotic rate was 3.2%
in Rb-negative tumors compared with 2.7%, 2.2%,
and 1.2% in tumors with <10%, 10 to 50%, and >50%
Rb-positive cells, respectively (*P* = 0.2, Kruskall-Wal-
lis test). In a subset of 25 total Rb-positive tumors we
assessed for underphosphorylated Rb, which was de-
tected in 12 tumors. The detection of only total Rb in
the remaining 13 tumors suggests that Rb was phos-
phorylated. Fluorescence *in situ* hybridization
showed allelic loss of the *rb* gene in 10 (40%) of 25
tumors analyzed and was significantly associated with
absence of Rb expression (*P* = 0.003). Similar results
were obtained for loss of heterozygosity of the 13q14
locus. Five-year progression-free survival for patients
with Rb-negative ALCL was 89.4% compared with
47.7% for patients with total Rb-positive ALCL (*P* =
0.006, log-rank test). Similar trends for progression-
free survival held true for patients with ALK-positive
and ALK-negative tumors analyzed separately. In con-
clusion, Rb is absent or phosphorylated in most ALCL
cell lines and tumors and absence of Rb expression is
associated with better clinical outcome in patients
with ALCL. (Am J Pathol 2004, 164:2259–2267)**

The retinoblastoma (*rb*) gene, located at 13q14, was the
first tumor suppressor gene identified by its involvement
in hereditary retinoblastoma.[1–3] Accumulating evidence
from *in vitro* and *in vivo* studies suggests that the *rb* gene
product, a 105-kd protein, is implicated in many cellular
functions including cell proliferation, apoptosis, and dif-
ferentiation.[4] The role of Rb protein as a major cell-cycle
inhibitor is mediated through its interaction with the E2F
transcription factors.[5,6] Rb directly binds and inhibits the
transcriptional activity of E2F family members by recruit-
ment of several chromatin-remodeling complexes to pro-
moter regions, resulting in chromatin condensation and
inhibition of transcription.[7–9] As a result, cells undergo
arrest in the G_1 phase of the cell cycle.[10] Although Rb is
normally expressed throughout the entire cell cycle, its
function depends on its phosphorylation status.[11] Under-
phosphorylated Rb is the active, growth suppressive
form of the protein, and phosphorylated Rb is inactive,
incapable of binding to E2F proteins. Progressive phos-
phorylation of Rb in mid to late G_1 phase of the cell cycle,
by cyclin/cyclin-dependent kinase (CDK) complexes, re-
sults in release of E2F proteins, transcription of numerous
target genes, and entry of the cell cycle into S phase.
CDKs, in turn, are negatively regulated by their inhibitors
including the INK4 (p14[ARF], p15, p16, p18) and Cip/Kip
(p21, p27, p57) families.[12] Rb is also involved in apopto-
tic mechanisms. Loss of Rb function is known to trigger
the p53-dependent apoptotic pathway,[13] possibly by re-
lease of free E2F proteins, which in turn activate p14[ARF]
and other apoptosis-related proteins.[14] However, recent
studies suggest that loss of Rb function can activate
apoptotic pathways that are independent of p53 as
well.[15]

Rb may be inactivated by gene mutation, or by protein changes including hyperphosphorylation, sequestration by viral oncoproteins, and degradation by a caspase-dependent proteolytic pathway.[10,16] In many tumor types, *rb* gene alterations may result in complete loss or low levels of Rb expression.[4] Homozygous deletions of the 13q14 locus have been detected in a small subset of lymphoid malignancies,[17–19] but these studies have failed to show a correlation between 13q14 deletions and Rb expression in these tumors.

A number of studies have investigated Rb protein expression in various types of non-Hodgkin's lymphomas with conflicting results.[19–24] Because the methods used in these studies did not assess Rb phosphorylation status, it is not known whether the Rb protein detected in these tumors was active (underphosphorylated) or inactive (phosphorylated).

In this study, we assessed Rb expression in anaplastic large cell lymphoma (ALCL), a distinct lymphoma type that frequently carries the t(2;5)(p23;q35) or variant abnormalities involving the 2p23 locus, resulting in overexpression of anaplastic lymphoma kinase (ALK).[25–27] We hypothesized that the Rb pathway may be altered in ALCL because previous studies of these tumors have shown deregulation of other cell-cycle-controlling proteins.[28–31] To investigate the functional status of Rb in ALCL, we assessed a subset of these tumors for expression of underphosphorylated Rb. We also used fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) methods to assess for possible loss of *rb* alleles. We correlated these results with tumor apoptotic rate (AR), proliferation index, and clinical outcome.

Materials and Methods

Cell Lines and Western Blot Analysis

Four ALK-positive ALCL cell lines, all known to carry the t(2;5) were used, including Karpas 299 (a gift from Dr. M. Kadin, Beth-Israel-Deaconess Medical Center, Boston, MA), SR-786, SU-DHL-1 (both from DSMZ, Braunschweig, Germany), and JB-6 (a gift from Dr. D. Jones, M.D. Anderson Cancer Center, Houston, TX). The cell lines were maintained in RPMI 1640 medium supplemented with 1% nonessential amino acids, 10% fetal calf serum (Invitrogen Corp., Grand Island, NY), and 1% streptomycin-penicillin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For the serum starvation experiments, Karpas 299 and SU-DHL-1 cells were maintained in RPMI 1640 medium supplemented with only 1% fetal calf serum (Invitrogen Corp.), and 1% streptomycin-penicillin. Lysates from serum-starved cells were prepared before the experiment (control or time point 0 hours) and after 48 and 72 hours.

Total protein was extracted from these cell lines and Western blot analysis performed using methods described previously.[31] Primary antibodies specific for total Rb (clone Rb1; DAKO, Carpinteria, CA) and underphosphorylated Rb (clone G99-549; BD Biosciences Pharmingen, San Diego, CA) were used. We further tested the specificity of the underphosphorylated Rb antibody using Western blot analysis and lysates of previously serum-starved Karpas 299 cells. Because the underphosphorylated Rb migrates faster in 6% polyacrylamide gels than the phosphorylated form of the protein (higher molecular weight), immunoblots using the underphosphorylated Rb antibody revealed the presence of a band corresponding only to a lower molecular weight (underphosphorylated) protein product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX) was used as a control for protein load and integrity.

ALCL Tumors

This group included 67 cases of systemic ALCL obtained from previously untreated patients accessioned at The University of Texas M.D. Anderson Cancer Center and the University of Freiburg, Germany from 1982 to 2001. The diagnosis of ALCL was based on morphological and immunohistological criteria as specified by the World Health Organization classification.[25] ALK-positive lymphomas, regardless of morphological features (ie, classical, monomorphous, lymphohistiocytic), were diagnosed as ALCL. ALK-negative ALCL tumors were composed predominantly of large anaplastic cells. Some investigators, as acknowledged in the World Health Organization classification, believe that ALK-negative ALCLs are better classified as high-grade peripheral T-cell lymphoma unspecified. All ALCL tumors were uniformly positive for CD30 and negative for B-cell antigens, including CD20, CD79a, and PAX-5. Fifty-three (79%) tumors were T cell and 14 (21%) were null cell. ALK was assessed using the ALK-1 antibody (1:30, DAKO) and was positive in 30 (45%) cases.

All patients were treated with doxorubicin-based chemotherapy. The clinicopathological features of most of these patients have been reported previously.[32] The median age of patients with ALK-positive tumors was 29 years compared with 52 years for patients with ALK-negative tumors ($P < 0.0001$ by Mann-Whitney *U*-test). Patients with ALK-negative tumors were more frequently associated with anemia ($P = 0.03$, Fisher's exact test). All other clinicopathological parameters between the two groups were comparable.

Design and Construction of the Tissue Array

Tissue sections of ALCL tumors, 5 μm thick, were cut from whole paraffin blocks (19 tumors) or a tissue microarray (48 tumors). The tissue microarray included triplicate or quadruplet tumor cores from all 48 tumors and two reactive lymph nodes and was constructed using a manual tissue arrayer (Beecher Instruments, Silver Springs, MD) as described previously.[33]

Immunohistochemical Methods and Terminal dUTP Nick-End Labeling (TUNEL) Assay

The immunohistochemical methods used in this study have been described previously.[32] For all antibodies,

heat-induced epitope retrieval was performed. Rb was assessed using monoclonal antibodies specific for total Rb (clone Rb1, dilution 1:50; DAKO) and underphosphorylated Rb (clone G99-549, dilution 1:25; BD Biosciences). The latter antibody recognizes an epitope between amino acids 514 to 610 of human Rb and does not recognize the phosphorylated forms of the protein. Proliferation rate was assessed using the MIB-1 (Ki-67) antibody (dilution 1:120; Immunotech, Westbrook, ME). The slides were incubated with the total and underphosphorylated Rb monoclonal antibodies at 4°C overnight, and with MIB-1 at room temperature for 1 hour. Detection of the immunoreaction was performed using the LSAB+ kit (DAKO). Reactive small lymphocytes in all tissue sections served as internal positive controls for total Rb and underphosphorylated Rb. Slides stained with normal rabbit serum (DAKO) without primary antibody were used as negative controls.

Any nuclear staining of ALCL cells was considered positive, irrespective of intensity. Expression levels for Rb were determined by counting the percentage of positive tumor cells and, for the purpose of statistical analysis, the tumors were divided into five groups as follows: negative, <10% positive, 10 to 50% positive, 50 to 90% positive, and >90% positive. Proliferation index was designated as the percentage of MIB1-positive tumor nuclei. AR was assessed using a modified TUNEL assay and designated as the percentage of TUNEL-positive tumor nuclei as described elsewhere.[32]

FISH

FISH was performed on a 5-μm paraffin section from the tissue microarray used in this study after appropriate deparaffinization in fresh xylene. Our FISH methods have been described in detail elsewhere.[34,35] We used the Spectrum Orange LSI 13/RB-1 and the CEP 8 probes purchased from Vysis (Downers Grove, IL) to assess the *rb* gene and the centromeric region (8p11.1-q11.1) of chromosome 8 (control), respectively. The tissue microarray slide was counterstained with 4,6-diamidino-2-phenylindole in an anti-fade mounting fluid before it completely dried. The signal was viewed at ×1000 magnification with a Zeiss fluorescence microscope (Carl Zeiss, Thornwood, NY) using Vysis FISH filters specific for 4,6-diamidino-2-phenylindole/Spectrum Orange. We analyzed at least 100 evaluable nuclei of each tumor. Only isolated cells with nonoverlapping nuclei were counted. To avoid overestimation of tumors with allelic loss of *rb* because of sectioning of the tumor cells, we defined allelic loss of *rb* as being present using a conservative cutoff of 50% or more cells that had only one signal with the RB1 probe. Most small reactive lymphocytes cut in full cross-section in tumor cores showed two signals and served as internal positive controls for the presence of both *rb* alleles.

Loss of Heterozygosity (LOH) Studies

After histological examination of 37 ALCL tumors (12 ALK-positive, 25 ALK-negative), areas of tumor infiltration were delineated microscopically, and most of the uninvolved lymph node or other normal tissue in the paraffin blocks was removed with sterile surgical blades. Subsequently, 20-μm sections were cut from the trimmed blocks and genomic DNA was extracted using the QIAamp DNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

To determine LOH, genomic DNA was amplified using a PCR method and two microsatellite markers, D13S153 and D13S263, spanning the 13q14 locus. Both microsatellite markers are known to have a high frequency of heterozygosity (82% and 84%, respectively). The D13S153 and D13S263 markers were labeled with the fluorescent dyes TET and HEX, respectively, and were purchased from Research Genetics (Huntsville, AL). In addition, a control microsatellite marker derived from the 13q32 locus and labeled with HEX (Research Genetics) was used. The 50-μl reaction mixture contained 500 ng of DNA (5 μl), 5 μl of 10× PCR buffer (Invitrogen Corp.), 1.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 200 nmol/L each primer pair (Research Genetics) and 2.5 U of *Taq*DNA polymerase (Invitrogen Corp.). The *Taq*DNA polymerase was added to the reaction mixture after the denaturation step (hot start). The thermal cycler was programmed as follows: 95°C for 10 minutes (denaturation); 30 cycles of 45 seconds at 94°C, 45 seconds at 54°C, and 60 seconds at 72°C; and finally 10 minutes at 72°C. The presence of the PCR products was confirmed by electrophoresis on a 1.5% agarose gel. Amplified products were subsequently analyzed by high-resolution capillary electrophoresis using the 310-Genetic Analyzer (PE/Applied Biosystems, Foster City, CA), as previously described.[36] Fluorescence data were analyzed using GeneScan and Genotyper software (PE/Applied Biosystems). Informative cases were heterozygous for the RB1 locus. Cases homozygous for this locus were considered unevaluable for LOH. In heterozygous cases, a decrease in signal intensity of more than 50% between the two alleles was required to establish the presence of LOH.

Statistical Analysis

The chi-square and Fisher's exact tests were used to compare total and underphosphorylated Rb expression (positive *versus* negative) with various clinicopathological parameters. The Mann-Whitney *U*-test and the Kruskal-Wallis test were chosen for the nonparametric correlation of proliferation index and AR between various Rb expression levels. Progression-free survival (PFS) was defined as time from initiation of therapy to last clinical follow-up, primary treatment failure, or relapse. Univariate survival analysis was based on the method of Kaplan and Meier using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. All computations were performed using the StatView statistical program (Abacus Concepts, Inc., Berkeley, CA).
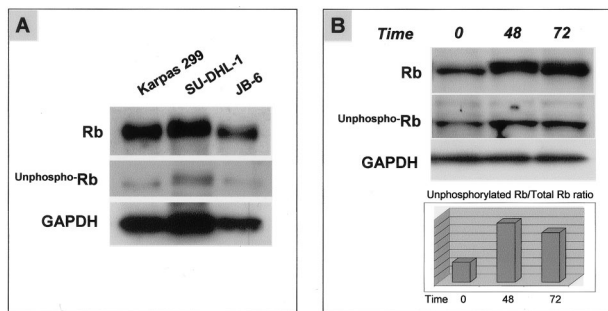
Figure 1. Expression of Rb in ALCL cell lines. **A:** Immunoblot showing three cell lines that express total Rb, with Karpas 299 and SU-DHL-1 cells having higher levels compared with JB-6 cells. Underphosphorylated Rb was detected at low levels in all three ALCL cell lines. Similar results were obtained for the SR-786 cell line (data not shown). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as a control for protein load and integrity, and its levels are similar among all three ALCL cell lines. **B:** Immunoblot showing the levels of total and underphosphorylated Rb protein after serum starvation of SU-DHL-1 cells. Densitometry showed that the underphosphorylated Rb/total Rb ratio was higher in cells treated with RPMI and only 1% of FBS (serum starvation) at 48 and 72 hours compared with control cells.

Results

Cell Lines

Total Rb was expressed at high levels in Karpas 299, SU-DHL1, and SR-786 cells and at lower levels in JB-6 cells (Figure 1A). Immunoblots showed a thick band in positive cell lines, consistent with phosphorylated and hyperphosphorylated forms of Rb.[19] Underphosphorylated Rb was detected at low levels in all four ALCL cell lines (Figure 1A). Serum starvation of SU-DHL1 and Karpas 299 cells resulted in an increased underphosphorylated Rb/total Rb ratio indicating that a subset of these cells underwent cell-cycle arrest through activation of Rb protein (Figure 1B).

Expression of Total Rb in ALCL Tumors

In reactive lymph nodes, total Rb was detected predominantly in germinal center cells as described previously.[37] Total Rb was detected in 44 (66%) ALCL, including 18 of 30 (60%) ALK-positive and 26 of 37 (70%) ALK-negative tumors ($P = 0.4$, Fisher's exact test). Rb immunoreactivity was localized in the tumor nuclei with variable staining intensity (Figure 2). Three tumors had >90% cells positive, 11 tumors had >50 to 90% cells positive, 15 tumors
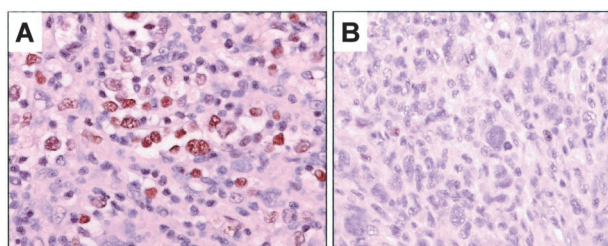
Figure 2. Total Rb expression in ALCL tumors. **A:** A Rb-positive ALCL case with strong nuclear immunoreactivity in most tumor cells. **B:** A Rb-negative ALCL case. Occasional small reactive lymphocytes were Rb-positive and served as internal positive controls. DAB, hematoxylin counterstain, for all panels.
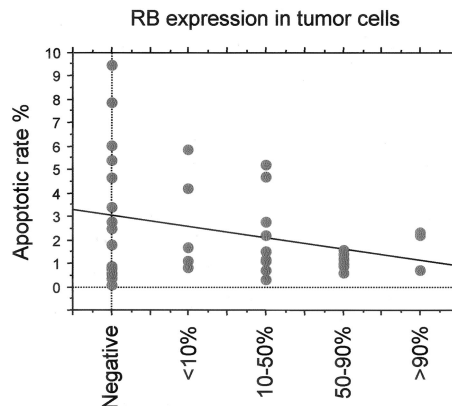
Figure 3. Association of total Rb expression with apoptotic rate (AR) in ALCL tumors. AR appears to correlate inversely with percentage of Rb-positive cells.

had 10 to 50% cells positive, and 15 tumors had <10% cells positive. Twenty-three (34%) ALCLs showed complete absence of Rb (Figure 2) including 12 ALK-positive and 11 ALK-negative. Rb expression in ALCL did not significantly correlate with clinical and laboratory features in these patients.

Correlation of Total Rb Expression with AR and Proliferation Index

AR was assessed previously for 43 of these ALCL tumors.[32] In this group, higher AR was commonly associated with absence or lower numbers of total Rb-positive cells as shown in Figure 3. The mean AR was 3.2% for Rb-negative ALCLs and 1.9% for Rb-positive ALCLs. In Rb-positive tumors, the percentage of positive cells also appeared to be inversely correlated with AR although this was not statistically significant ($P = 0.2$, Kruskal-Wallis test; Figure 3). More specifically, the mean AR was 2.7%, 2.2%, and 1.2% in tumors with <10%, 10 to 50%, and >50% Rb-positive cells, respectively. Correlation between AR and functional status of Rb, showed that the mean AR was 3.2% for Rb-negative tumors, compared with 1.9% for underphosphorylated Rb-positive tumors and 1.6% for phosphorylated Rb-positive tumors ($P =$ not significant, Kruskal-Wallis test). Proliferation index was evaluated in 51 ALCL tumors and ranged from 20 to 99%, with a mean of $75 \pm 17\%$. No correlation between proliferation index and total Rb expression was observed.

Table 1. Total Rb and Underphosphorylated Rb Expression in 40 ALCL Tumors

	Underphosphorylated Rb expression	
	Positive	Negative
Total Rb expression		
Positive	12	13
Negative	0	15
P value*	$P = 0.001$	

*By Fisher's exact test.
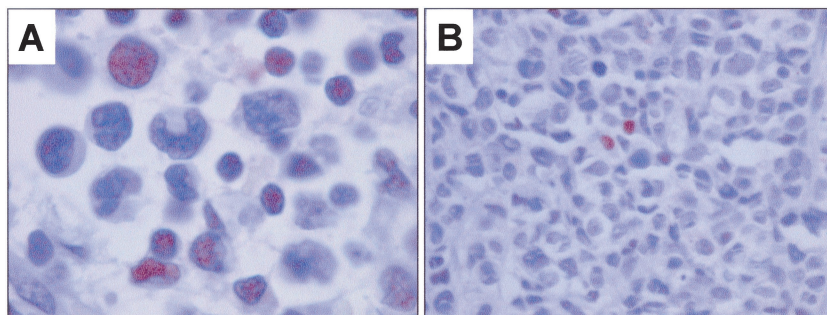
Figure 4. Expression of underphosphorylated Rb in ALCL tumors. **A:** An ALK-positive ALCL case positive for underphosphorylated Rb in a small number (<10%) of tumor cells. Intensity of underphosphorylated Rb immunostaining was generally weaker compared with total Rb. **B:** An ALK-positive ALCL case negative for underphosphorylated Rb. Occasional small reactive lymphocytes show nuclear immunoreactivity and are considered as internal positive controls. DAB, hematoxylin counterstain, for all panels.

Expression of Underphosphorylated Rb in ALCL Tumors

In reactive lymph nodes, underphosphorylated Rb was detected in a small subset of germinal center cells and small reactive lymphocytes. Expression of underphosphorylated Rb was assessed in a subset of 40 ALCL, including 25 total Rb-positive and 15 total Rb-negative. As shown in Table 1, 12 (30%) of these 40 tumors, all total Rb-positive, expressed underphosphorylated Rb (Figure 4). In the group of 12 total Rb-positive tumors, 5 tumors had >50 to 90% cells positive, 5 tumors had 10 to 50% cells positive, and 2 tumors had <10% cells positive. In four tumors, the number of underphosphorylated Rb-positive cells approximated the number of total Rb-positive cells. Hence, in eight tumors the number of underphosphorylated Rb-positive cells was clearly less than the number of total Rb-positive cells, suggesting that Rb in these tumors is predominantly phosphorylated (inactive) (Table 1). However, our methods do not allow us to quantify the amounts of total Rb and underphosphorylated Rb in individual tumor cells. Expression of underphosphorylated Rb did not correlate with ALK expression (Table 2).

FISH Analysis of the rb Gene

To investigate the mechanism of relatively low level of Rb expression in JB-6 cells, we performed FISH analysis using cytospin preparations, a probe specific for the RB1 locus, and a control chromosome 8 centromeric probe. FISH analysis revealed the presence of an equal number of alleles for the RB1 and control probes suggesting no deletion of the *rb* gene locus (data not shown).

The *rb* gene was assessed for allelic loss in 25 ALCL (11 ALK-positive, 14 ALK-negative). Deletion of at least one *rb* allele was found in 10 (40%) tumors (Figure 5), 5 ALK-positive and 5 ALK-negative (Table 2). Nine of these

tumors had hemizygous allelic loss at *rb* and one ALK-negative tumor had homozygous deletion of *rb*. Loss of *rb* correlated significantly with absence of total Rb protein. Eight of ten (80%) tumors with *rb* allelic loss were negative for Rb and two tumors expressed Rb in less than 50% of cells. In contrast, only 1 of 15 (7%) tumors that retained both *rb* alleles were negative for Rb (*P* = 0.003, Fisher's exact test).

LOH at 13q14

Using PCR and two microsatellite markers specific for the 13q14 locus, LOH was found in 9 (45%) of 20 informative ALCL tumors; 2 ALK-positive and 7 ALK-negative (Figure 6, Table 2). All 11 ALCL tumors with no evidence of LOH were total Rb-positive whereas 6 of 9 (67%) ALCL tumors with LOH were total Rb-negative (*P* = 0.002, Fisher's exact test).

Correlation of rb Allelic Loss and RB Phosphorylation Status with p27 Labeling Index

RB1 allelic loss as shown by FISH correlated with p27 levels assessed previously in this ALCL series.[31] The mean percentage of p27-positive tumor cells was 30% for ALCL tumors with *rb* allelic loss (either hemizygous or homozygous) compared with 9% for tumors without *rb* deletion (*P* = 0.09, Mann-Whitney test; Figure 7). A correlation between underphosphorylated Rb expression and p27 labeling index was also suggested but this did not reach statistical significance (*P* = 0.2 by Mann-Whitney test).

## Clinical Outcome

Complete follow-up data were available for 62 patients analyzed in this study. The median follow-up period was

Table 2. Total Rb Expression, Underphosphorylated Rb Expression, and Allelic Loss of the *rb* Locus in ALK-positive and ALK-negative ALCL
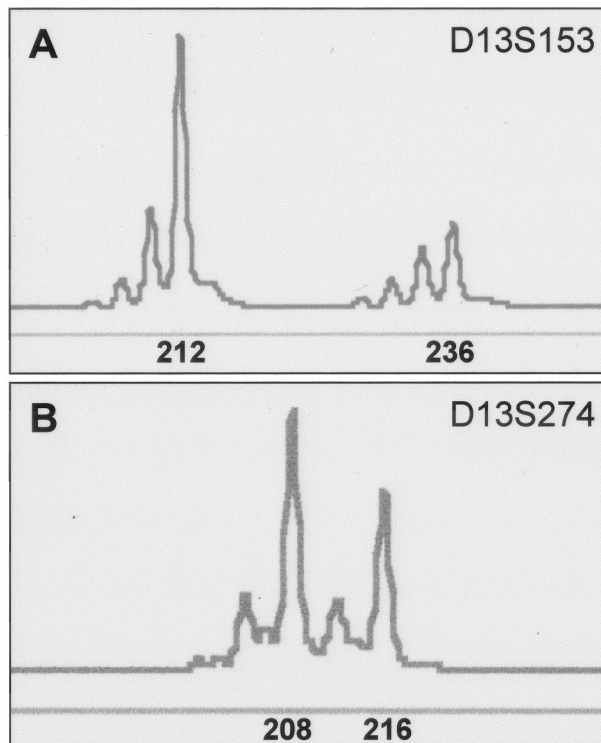
|	Total Rb expression (n = 67)	Underphosphorylated Rb expression (n = 40)	Allelic loss of *rb* locus	
			FISH (n = 25)	LOH (n = 20)
ALK-positive	18/30 (60%)	4/17 (24%)	5/11 (45%)	2/6 (33%)
ALK-negative	26/37 (70%)	8/23 (35%)	5/14 (36%)	7/14 (50%)
P value*	*P* = 0.4	*P* = 0.5	*P* = 0.7	*P* = 0.6

*By Fisher's exact test.
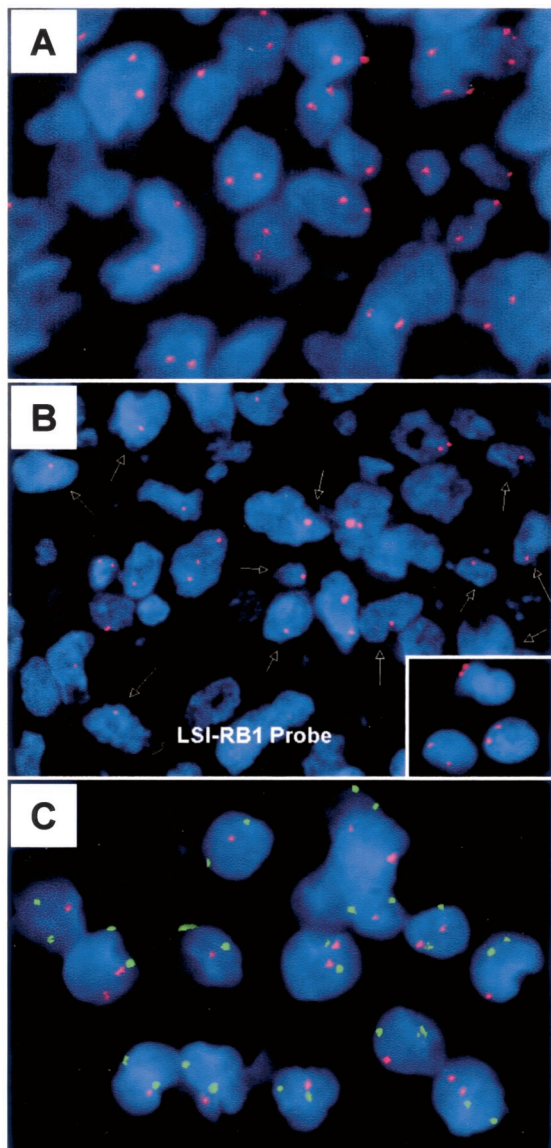
Figure 5. FISH analysis for allelic loss at the *rb* locus using the Spectrum Orange LSI 13/RB-1 probe (Vysis). **A:** An ALK-positive ALCL case with most tumor cells showing two signals indicating the presence of both *rb* alleles. **B:** An ALK-negative ALCL case with hemizygous allelic loss of *rb*. Most tumor nuclei show a single signal indicated by **arrows**. **Inset**: Small reactive lymphocytes present in all tumor samples show two distinct signals for *rb* gene locus. **C:** An ALK-positive ALCL case with deletion of the *rb* locus. The red and green signals indicate the RB1 and centromeric (chromosome 8) probes, respectively.


Figure 6. Representative cases of ALCL tumors analyzed for LOH using the GeneScan and Genotyper software. **A:** An ALK-positive ALCL tumor with LOH of the 13q14 locus assessed with the D13S153 microsatellite marker. The expected size of the alleles (212 and 236) is shown. **B:** An ALK-negative ALCL tumor with heterozygous 13q32 locus assessed with the D13S274 microsatellite marker that served as a control. The expected size of the alleles (208 and 216) is shown.

46 months (1 to 129 months). For the entire group, 5-year PFS was 89.4% for patients with total Rb-negative tumors compared with 47.7% for patients with total Rb-positive tumors ($P = 0.006$ by log rank, Figure 8A). Survival analysis was also performed separately for the ALK-positive and ALK-negative groups. For 29 patients with ALK-positive ALCL, 5-year PFS was 88.9% for patients with total Rb-negative tumors compared with 48.6% for patients with total Rb-positive tumors ($P = 0.08$ by log rank, Figure 8B). For 33 patients with ALK-negative ALCL, 5-year PFS was 90.9% for patients with total Rb-negative tumors *versus* 46.5% for patients with total Rb-positive

tumors ($P = 0.03$ by log rank, Figure 8C). In 53 patients with available data, functional loss of Rb protein was correlated with clinical outcome. Patients with total Rb-negative tumors because of allelic loss of *rb* locus were associated with a significantly higher PFS compared to those patients with underphosphorylated Rb expression or phosphorylated (functionally inactive) Rb ($P = 0.007$, log rank; Figure 9). Multivariate analysis for PFS using the Cox proportional hazards model demonstrated the independent prognostic value of total Rb expression in this
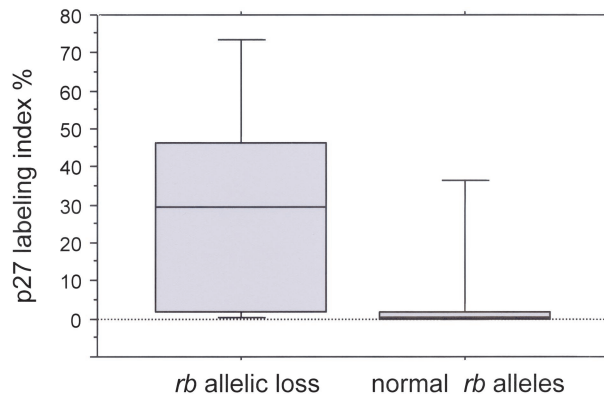

Figure 7. Association of rb allelic loss with p27 labeling index. Box plot showing higher levels of p27 expression in ALCL tumors with loss of the *rb* gene locus compared to tumors with normal *rb* alleles.

A. All ALCL

B. ALK+ ALCL

C. ALK- ALCL
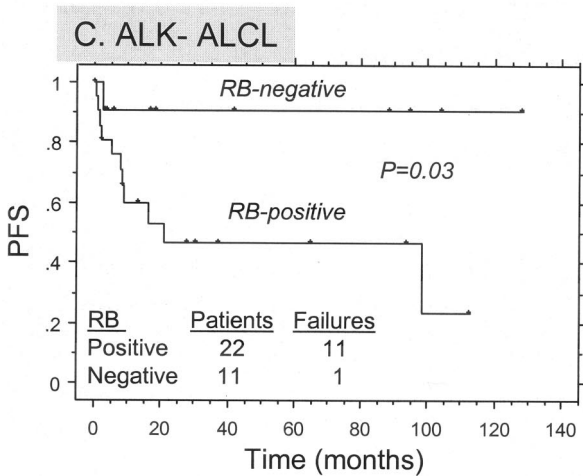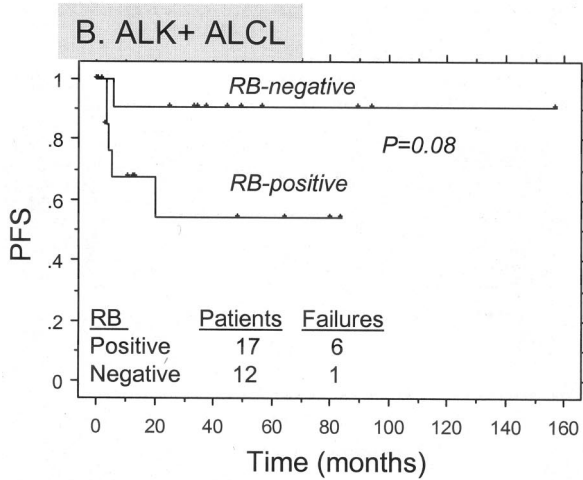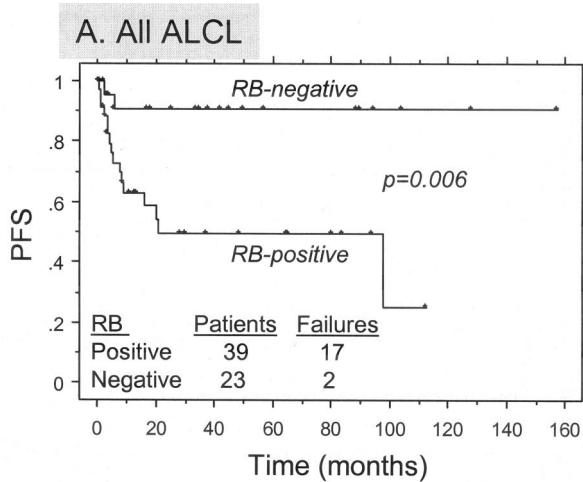
Figure 8. Correlation of total Rb expression in ALCL with progression-free survival (PFS). **A:** All patients; **B:** ALK-positive ALCL patients; **C:** ALK-negative ALCL patients.

series of ALCL patients, along with other known clinical prognostic factors such as Ann Arbor stage IV, elevated serum lactate dehydrogenase (LDH) levels, and age >60 years (Table 3).
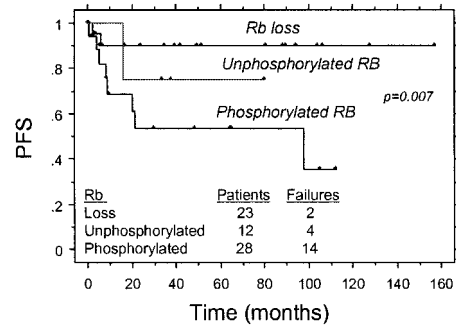
Figure 9. Correlation of functional loss of Rb in ALCL with PFS. Patients with ALCL tumors exhibiting loss of *rb* locus have a higher PFS compared to patients with phosphorylated or unphosphorylated Rb expression.

Discussion

In this report, we have shown that complete absence of Rb expression, as shown by immunohistochemistry, is found in 40% of ALCL tumors. Absence of Rb expression has been reported in many tumor types including non-Hodgkin's lymphomas.[18,37,38] Complete loss of Rb may be because of chromosomal alterations involving the *rb* gene at 13q14. Homozygous deletions of 13q14 have been detected in a subset of non-Hodgkin's lymphomas,[18,19] but these studies failed to show a correlation between 13q14 chromosomal alterations and Rb expression. In our study we assessed a subset of 25 ALCL tumors for deletions of the *rb* gene using FISH methods. Ten (40%) of these tumors showed loss of one or both *rb* alleles and this finding significantly correlated with absence of Rb expression. In addition, we performed LOH studies that showed allelic imbalances of the 13q14 locus in 45% of the informative ALCL cases, confirming the FISH results. Using similar FISH methods, Wada and co-workers[39] also detected deletions of the 13q locus in 40% of aggressive lymphomas including one case of ALCL of T-cell lineage. Rosenwald and colleagues[40] also used FISH methods and reported allelic loss at 13q14 in 3 of 13 peripheral T-cell lymphomas.

Absence of Rb protein expression may be associated with a higher AR in ALCL tumors (Figure 3) because the data in this series seem to show a trend, however, this association did not reach statistical significance. Accumulating evidence suggests that Rb is an inhibitor, not only of cell growth, but also of cell death. This was first revealed in *rb*-null mice that developed phenotypes with an increased rate of apoptosis.[16] Because inactivation of Rb stimulates both cell proliferation and apoptosis, it

Table 3. Multivariate Analysis for Progression-Free Survival in Patients with Anaplastic Large-Cell Lymphoma

| Prognostic factor | Relative risk of failure | | |
	Relative risk	Standard error	P value*
Total Rb-positive	2.6	1.1	0.019
Performance status = >2	1.9	0.8	0.013
Secondary skin involvement	1.1	0.5	0.039
Age >60 years	1.1	0.5	0.044

*Cox's proportional hazards model.

seems likely that cells respond to mitogenic or apoptotic signals, respectively, by activating different molecular pathways.

For the entire study group of ALCL, absence of total Rb expression significantly correlated with favorable PFS (Figure 8). This was also true when patients with ALK-positive or ALK-negative ALCL were analyzed separately. Multivariate survival analysis confirmed the independent prognostic significance of Rb expression in ALCL. Loss of Rb at the DNA level also correlated with clinical outcome. Patients with Rb-negative tumors because of allelic loss of the *rb* locus were associated with a significantly higher PFS compared to patients whose tumors expressed underphosphorylated or phosphorylated Rb (Figure 9). There is little precedent for this finding in the literature except in chronic lymphocytic leukemia. Dohner and colleagues[41] have reported that patients with chronic lymphocytic leukemia and deletion of the 13q14 locus are associated with the longest median survival times. The explanation of these findings is uncertain. However, the possibility of loss of other unknown genes within the region of deletion cannot be excluded.

Rb is expressed in approximately two thirds of ALCL tumors at a variable level, but its active (underphosphorylated) form is immunohistochemically undetectable in almost half of these tumors. Thus, where Rb is present in ALCL cells, it is often phosphorylated and therefore cannot bind and inhibit E2F family proteins. This was also true in the ALK-positive ALCL cell lines we studied. As a result, E2F transcriptional activity is uncontrolled leading to expression of a number of target genes that promote cell cycle progression from G_1 to S phase.[10] These results suggest that deregulation of Rb function may contribute to tumor cell proliferation and have a pathogenetic role in ALCL. This is also supported by the fact that ALCL cells may alter the phosphorylation status of Rb, as a response to growth inhibition stimuli.[42]

It is well established that Rb function depends on its phosphorylation by multiple CDKs, which in turn are regulated by CDK inhibitors, including p27[Kip1]. Because p27 is not expressed in most ALCL tumors,[31] it seems reasonable to speculate that low expression levels of p27 lead to increased kinase activity of cyclin/CDK complexes that directly phosphorylate and inactivate Rb in ALCL, resulting in cell proliferation. This is further supported by our findings here, because the presence of *rb* alleles correlated with significantly lower levels of p27 (Figure 7). The high proliferation index in ALCL tumors supports this concept. Cooperation of Rb and p27 proteins to suppress tumor growth, through different regulatory signals, also has been proposed.[43]

It is of interest that phosphorylated Rb, but not its underphosphorylated counterpart, has been shown to translocate to the nucleolus through direct binding with nucleophosmin in normal human fibroblasts in late S or G_2 phase of the cell cycle.[44] Nucleophosmin is a component of the nucleophosmin-ALK fusion protein resulting from the t(2;5), the most frequent translocation in ALK-positive ALCL tumors. Nucleophosmin is a nonribosomal RNA-binding protein involved in bi-directional shuttling of
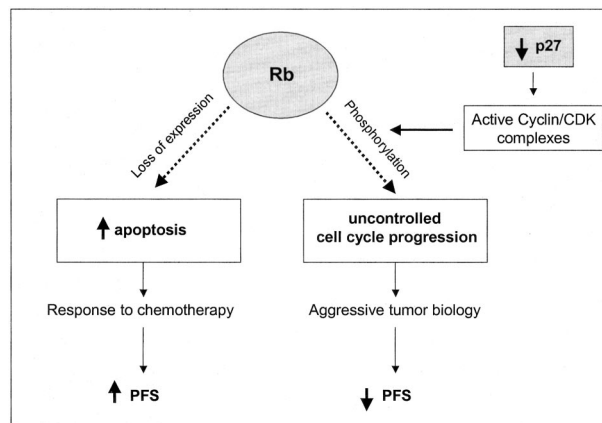
Figure 10. Schematic showing the possible role of Rb expression in the biology and prognosis of ALCL. As previously reported,[31] expression levels of the CDK inhibitor p27 are low in ALCL, thus allowing phosphorylation of Rb by cyclin/CDK complexes. When phosphorylated, Rb is not capable of binding to E2F transcriptional factors leading to uncontrolled cell proliferation and possibly more aggressive tumor biology. Loss of Rb expression, commonly by deletion of the *rb* gene, results in increased tumor apoptosis and may sensitize tumor cells to chemotherapy. This is a possible explanation for the better PFS observed in patients with total Rb-negative ALCL.

proteins between the nucleolus and the cytoplasm[45] and it has the ability to oligomerize. Whether nucleophosmin-ALK chimeric protein (and not only nucleophosmin) also can interact with phosphorylated Rb is unknown and the potential biological significance of this interaction in ALCL needs to be explored in future functional studies.

In conclusion, loss of Rb expression, most likely because of allelic loss of the *rb* gene, is a common event in ALCL tumors and independently predicts a better prognosis in these patients. When Rb is present in ALCL, the protein is commonly phosphorylated, perhaps the result of low levels of p27[31] (Figure 10). Experimental therapeutic approaches targeting the Rb pathway may be useful in treating patients with ALCL.

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