Levels of Retinoblastoma Protein Expression in Newly Diagnosed Acute Myelogenous Leukemia

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The relationship between the level of retinoblastoma protein (RB) expression and the survival of 113 newly diagnosed acute myelogenous leukemia (AML) patients was studied. Western blotting was used to determine the level of RB protein present in peripheral blood leukemia cells and results were confirmed in 26 patients by immunohistochemistry. The leukemic cells from 22/113 AML patients (19%) contained RB protein at levels that were equal to or less than the level of RB observed in the mononuclear cell fraction of peripheral blood from normal individuals (Low RB). Levels of RB greater than that of normal blood (Elevated RB) were seen in 91 patients (81%). The median survival of patients with low RB was significantly shorter than that seen in patients with elevated RB, 12 weeks versus 40 weeks (P = .02).

Remission induction frequency was 36% in low RB patients compared with 68% in AML patients with elevated RB (P = .01). Multivariate analysis showed that low RB protein level was an independent prognostic factor predictive of poor survival after allowing for other known prognostic factors. These data suggest that a low level of the RB protein at the time of diagnosis is associated with shortened survival in AML patients because of inferior response to conventional therapy. Monitoring of the RB level could identify a subgroup of AML patients with an extremely poor prognosis when treated with chemotherapy alone, who would be eligible for alternative therapeutic strategies. © 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Western blotting. Western blotting using an anti-RB monoclonal antibody (Triton Biologicals, Almeda, CA) was performed as previously described using cell lysates derived from the mononuclear fraction of peripheral blood (PB) of 113 newly diagnosed patients with AML (two were fresh, nonpheresed, PB samples and the remainder were cryogenically preserved PB pheresis samples) and 15 normal individuals. The only difference in technique is that a colorimetric assay based on alkaline phosphatase and nitro blue tetrazolium was used in the previous study, whereas in this study the membranes were incubated with sheep antimouse IgG conjugated to horseradish peroxidase and then exposed to chemiluminescence mixture for 1 minute according to the directions of the manufacturer (Amersham, Arlington Heights, IL). Films were then exposed at intervals of 15 seconds to 2 minutes until maximum saturation of the film had occurred. Representative examples of the Western blot assays are presented in Fig 1.

The level of RB expression was scored by densitometry (Joyce-Loebl Ephortec densitometer, Gateshead, England) and normalized by dividing the numerical value of a sample’s RB signal by that of the RB signal from the K562 positive control on the same blot (hereafter called the patient RB/K562 RB level). To insure standardization of the positive control all of the K562 used was from a single large protein preparation that had been aliquoted into single use vials. The membranes were also incubated with an antiantigen antibody (Oncogene Science, Uniondale, NY) to verify protein loading and to monitor for protein degradation. A normal range for RB protein was determined from the PB of 15 normal individuals. The mean

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value was calculated and three times the standard error of the mean added to this to define a cutoff point for elevated RB levels. To ensure that the results were linear for both the amount of protein loaded and the time of exposure, these studies were repeated in some patients using three dilutions of sample. The normalized patient RB/K562 RB level for each concentration of sample, from each different exposure time, was plotted to assess the linearity of the results. The dilution results for the positive control cell line and patient samples were linear on a log plot for the three different concentrations of protein at each of the different exposure times up to the point of film saturation. For the normal control samples, the signal of the quarter-strength protein mixture was often too weak to show up on the shorter exposure times. When all three dilutions were detected, the results were linear. Additionally, a serial dilution of the positive control cell line, K562, mixed with the retinoblastoma-derived negative control cell line, WERI-Rb-1 (ATCC HTB169), was performed (Fig 1B). Linear regression of the densitometry results from this serial dilution study on a log plot gave a correlation coefficient of .99. Therefore, we concluded that the results are quantitative and determination of the patient RB/K562 RB level is not subject to arbitrary differences dependent on the time of exposure or minor variations in the amount of protein loaded.

Single cell immunochemical analysis. Immunohistochemical analysis was performed as previously described in a separate lab that was blinded to the Western blot results on 17 patients with low RB levels and 16 patients with elevated RB levels as defined by Western blotting. Representative cases with no or strong nuclear pattern staining are shown in Fig 2.

Patient data. A total of 113 newly diagnosed, untreated, nonconsecutive AML patients were studied. This study includes 32 of the 33 newly diagnosed patients from the pilot study, with insufficient sample precluding inclusion of 1 patient. Eligibility for RB analysis was based on the availability of cryopreserved or fresh sample and the passage of at least 1 year from diagnosis. The median follow-up of survivors is just under 5 years and all but 5 surviving patients have more than 2 years of follow-up from the time of diagnosis. All samples produced technically interpretable and reproducible Western blot assays. Samples for analysis were obtained during regularly scheduled diagnostic evaluations as part of protocols approved by the Human Subjects Committee of the University of Texas M.D. Anderson Cancer Center.

Samples were collected between January 1983 and October 1991. Patients with a white blood cell (WBC) count of ≥ 4,000/mm³ were eligible for pheresis. These 113 patients represent 17% of the 681 newly diagnosed AML patients seen at our institution over the accrual period and 24% of the 472 patients with a WBC ≥ 4,000/mm³. Therefore, patients included had a higher mean WBC count compared with excluded patients. Except for this difference, the patients in this study were similar to those not included in terms of other clinical and laboratory findings predictive for survival and clinical course. Models predicting for the probability of achieving remission (P > .5), the probability of death during the induction period (P > .5), and for long-term survival (P > .5) showed no difference between included and excluded patients. Compared with the excluded patients, the patients studied had a similar survival experience (median survival of 37 weeks for included patients and 34 weeks for excluded patients, similar complete remission (CR) rates of 62% compared with 58%, and overlapping remission duration curves). There were no differences in response rates, survival experience or remission duration experience between those included and the excluded patients with a WBC ≥ 4,000/mm³ either. These similarities suggest that the clinical experience of the patients studied was representative of all AML patients seen at this institution during the time period.

There were 62 males and 51 females in this study. The median age was 52 years, with a range from 17 to 83 years of age. There were patients in all French-American-British (FAB) classification categories except M6. There were 15 M1, 26 M2, 7 M3, 39 M4, 12 M5, 1 M7, and 13 M0 or unclassifiable patients. In this cohort, there were no karyotypic abnormalities of the RB locus on chromosome
13q14 by standard cytogenetic analysis, although 1 patient had an additional chromosome 13, and one had a monosome for chromosome 13. Neither patient with chromosome 13 abnormalities had altered expression of the RB protein. These patients received a variety of regimens because of the changing protocols in use during the period of collection. High-dose ara-C was used alone in 23 patients, and in combination in 55 patients (20 with asparagin, 2 with daunorubicin, 22 with daunorubicin plus granulocyte macrophage colony-stimulating factor, 9 with idarubicin, and 2 with fludarabine). Anthracyclines were used alone in 6 cases and in combination with standard-dose ara-C in 3 cases, or ara-C plus vincristine and prednisone in 21 cases. Four patients received vincristine, daunorubicin, and dexamethasone and 1 patient died before starting therapy.

Statistical analysis. Distributions of prognostic features between RB groups were compared by chi-square tests or Mann-Whitney tests. Survival distributions were estimated by the method of Kaplan and Meier, and comparisons were based on log-rank tests. Regression methods based on a proportional hazards model were used to provide a test of association between RB category and survival outcomes, while simultaneously adjusting for small differences in other prognostic factors. Characteristics were selected for inclusion on their previous recognition as important prognostic factors and evidence of association with survival in this population. Evaluation of covariates was based on likelihood ratio tests.

RESULTS

RB levels in normal individuals. Densitometric analysis of the Western blots of RB protein from the PB mononuclear cells of normal individuals or AML patient samples were conducted as outlined above and previously. As shown in Fig 3, the mean RB levels of all samples from normal subjects, when divided by the level of RB in K562 cells (so as to be able to compare values from different blots), were below 0.1, whereas the RB levels from AML patients, when divided by the K562 RB level, extended over a wide range of values, above and below those seen in normal subjects. Each of the PB mononuclear cell fractions from the 15 normal subjects was analyzed more than once with a mean subject RB to K562 RB ratio of 0.071 and a standard error of 0.019 (Fig 3A). The mean value plus three times the standard error of the RB levels in the PB cells of normal donors was used to establish an upper limit of 0.128 for the ratio of normal RB/K562 RB. Only one of the 52 determinations from the 15 normal individuals was above the cutoff (.141). This boundary was used to divide AML patients into low-or elevated-RB groups. Representative examples for each level and group are shown in Fig 1A.

RB levels in AML patients. Twenty-two AML patients (19%) had patient RB/K562 RB levels less than or equal to that observed in the normal individuals (<0.128), and in many of these cases, the RB level was zero or undetectable, whereas low levels of RB were always observed in the normal samples. Ninety-one patients (81%) had patient RB/K562 RB levels in excess of that seen in the normal controls analyzed (Fig 3), ranging from .13 to 1.224. Some degree of RB protein phosphorylation (110 to 116 kD) was observed on the Western blot analysis of 37 of these 91 patients, and a significant amount of hyperphosphorylated RB (116 kD) was observed in 16 of these cases. Phosphorylation of any degree was never seen in the PB cells of the 15 normal individuals studied and has not been reported in PB by others. Phosphorylated RB is elevated in the normal dividing cell and all tumor-derived cell culture lines tested. Therefore, those patients with normalized patient RB/K562 RB levels higher than that found in normal PB cells were considered to have the expected level of PB protein expression for rapidly proliferating malignant cells that contain a functional RB gene.

A comparison of results from the Western blot analysis of RB levels based on the alkaline phosphatase method, used in the prior study, and the more sensitive chemiluminescence method, used in the present study, showed identical results in all but three cases. These latter patients, considered to have low RB in the previous study, were found to have elevated patient RB/K562 RB ratios by the chemiluminescence detection method.

The in situ immunohistochemistry gave technically interpretable results in 26 of the 33 patients analyzed (Fig 2).
There was complete concordance of results between Western blot and in situ immunohistochemistry in the 26 patients studied by both techniques. In samples considered to have low RB levels by Western blot analysis, all of the cells of the low-RB and elevated-RB categories of AML exhibited a wide range of variable positivity for RB by the in situ assay. This suggested that the low levels of RB seen by Western blot analysis were caused by residual normal cells in the populations. Again, this indicated that a qualitative as well as a quantitative difference existed between the cells of the low-RB and elevated-RB categories of AML patients.

Relationship of RB level to clinical outcome. The percentage of patients who achieved a CR in the low-RB group, (36%, 8/22), was significantly lower than the elevated-RB group (68%, 62/91) \( (P = .01) \). The median survival from the time of diagnosis was shorter in the low RB group (12 weeks) compared with patients in the elevated RB group (40 weeks) \( (P = .01) \) (Fig 3B). The median duration of remission was similar in both groups. In a separate analysis of hazard rates (not presented), there was evidence that the risk of death in the low-RB group exceeded that for the remaining patients throughout the study period, though effective sample size was insufficient for precise estimates. This suggests that the survival difference between the groups is not merely caused by an excessive early induction period mortality among patients in the low-RB group. No major differences in the cause of death during the first 16 weeks were noted between the low- and elevated-RB groups although hemorrhage, often late in the induction period, was a more frequent cause of death in the low-RB patients and infection a more frequent cause of death among patients with elevated RB.

Comparison of clinical features by univariate and multivariate analysis. The patients in the two groups were very similar with respect to the distribution of previously identified prognostic features for patients with AML \(^{13,14}\) as shown in Table 1. There were no significant differences in age, gender, cytogenetics, hemoglobin, platelet count, neutrophil count, albumin, bilirubin, fibrinogen, percentage marrow blasts, or history of antecedent hematologic disorders between the low-RB and elevated-RB groups. There were fewer patients with poorer performance status at the time of diagnosis in the elevated-RB group compared with the low-RB group. Categories of favorable and unfavorable cytogenetic abnormalities were based on results of earlier studies at our institution. \(^{14}\) Briefly, patients with t(8;21) or inversion 16 were classified as having a favorable prognosis, those with t(15;17), insufficient metaphases or a diploid karyotype were considered to have an intermediate prognosis and all others were felt to have an unfavorable prognosis. To compare the distribution of several of these prognostic features simultaneously, two previously developed models were used, one to predict the probability of achieving CR, \(^{13}\) and the other an overall “hazard ratio” for death caused by leukemia (E. Estey, personal communication, March 1992). Using either model, both the mean and median values for each group were similar. These data suggest that the two groups were well balanced for previously identified prognostic factors.

Although there were no substantial differences in other pretreatment characteristics among patients grouped by RB levels, a proportional hazards regression model was fitted to adjust for small existing differences. Survival time served as the endpoint and the model included terms for RB cate-

Relationship of RB level to cell cycling. The percentage of cells that were in S, G2, or M phase of the cell cycle \( (%S + G2M) \) was available in 79 patients, 13 with low RB and 66 with elevated RB. There was no difference in the mean and range of the \( %S + G2M \) cells between low RB \( (11.58 \pm 5.55, \text{range } 5 \text{ to } 20) \) and elevated RB \( (12.44 \pm 5.84, \text{range } 3 \text{ to } 28) \). Linear regression showed no correlation between the RB/K562 level and the \( %S + G2M \) \( (R^2 = 0.018) \).

There was complete concordance of results between Western blot and in situ assay.
gory and other prognostic features as follows: age (years), performance status (categories 3 and 4 combined), antecedent hematologic disorder (present or absent), favorable cytogenetic abnormalities (present or absent), and unfavorable cytogenetic abnormalities (present or absent). Table 2 summarizes results for a regression model including these six covariates. The term for RB category remained statistically significant after adjustment for other terms in the model ($P = .02$). It is important to note that although these patients received a variety of therapy regimens, the type of treatment was not an important factor as determined by regression analysis.

**DISCUSSION**

This study, using an improved technique for determining the presence of RB protein in a larger cohort than that in our previous report,\textsuperscript{10} showed that absent or low RB-protein expression is a common event in the development of AML, with significant clinical consequences. The leukemic cells of 19% of the newly diagnosed AML patients were categorized as having an RB level equal to or lower than that found in normal individuals, and all of the AML blasts from the 13 patients in the low RB category studied by in situ analysis were negative for RB. A similar rate (25%) of absent RB expression in AML patients was observed by Furukawa et al.\textsuperscript{11} Loss of RB expression was observed in all of the FAB classifications studied and occurred most frequently in the M0, M2, and M3 subtypes.

Patients in the low RB group were significantly more likely to fail their initial induction therapy and had an inferior survival experience compared with patients with elevated RB levels. The negative effect of low RB expression on survival would appear to be secondary to a failure to achieve remission with standard induction therapy. All 5-year survivors in this population have emerged from patients in whom no decrease of RB protein was present at diagnosis. The prognostic value of the RB level was confirmed in regression analysis that included other previously identified features. We selected a cutoff point based on a population of normals compared with a standard because this could be reproduced in other laboratories. In fact, the RB level remained an independent predictor when the population was divided evenly into three groups, and the best fit, yielding the most significant $P$ value, included the next six patients above the .128 cutoff point. Thus, the finding that RB is an independent predictor of outcome in AML was not dependent on the selection of one particular cutoff point.

The patients in the low RB category appear to have a change in the RB gene locus or its relevant cellular regulatory elements that results in either absent or lower than normal RB expression, and this change is associated with a very poor prognosis. It is not yet known through what mechanism this decrease in RB expression is generated. Mutations leading to absent RB expression may have occurred in some patients. However, it seems that in other cases there may be an uncoupling of cellular proliferation and induction of the level of RB protein expression. This is based on the observation that the level of RB protein was increased in vitro in cells from some of the patients with low RB when these cells were stimulated by hematopoietic growth factors.\textsuperscript{21}

Many of the patients in the elevated-RB group expressed phosphorylated RB protein, which is known to be present in actively cycling cells.\textsuperscript{18,22} Thus, the observation of greater than normal levels of RB among the patients with elevated-RB levels suggests that their leukemic cells were cycling, or that the regulatory environment of most of the cells was permissive for proliferation through posttranslational modification of the RB protein. An additional finding was a separate pattern of poor survival for patients with evidence of hyperphosphorylated RB. The small subgroup with this finding had a shorter median survival of 35 weeks compared with the rest of the elevated patients. This seems to arise from trends for shorter remission durations and higher relapse rates. Whereas hyperphosphorylated RB is commonly seen in rapidly proliferating cultured cells, similar degrees of phosphorylation were rare in primary solid-tumor specimens from other human malignancies.\textsuperscript{20} Hyperphosphorylated RB could be a marker of a higher proliferation rate in the leuke-

<table>
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<tr>
<th>Characteristic</th>
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<th>$P$ Value</th>
<th>Risk Ratio</th>
<th>95% CI</th>
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</tr>
</tbody>
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Abbreviation: CI, confidence interval.
mia cells of these patients. Alternatively, these leukemia cells could develop an insensitivity to RB through the ability to hyperphosphorylate RB into its inactive form.

These samples contained residual normal lymphocytes and monocytes in addition to the leukemic blasts and promyelocytes. One concern is that the RB level could be related to the percentage of blasts present in the sample, but there was no discernable correlation between the RB/KS62 level and the percent blasts and promyelocytes ($R^2 = .13$) or the absolute blast count ($R^2 = .15$). The RB level also showed no correlation with the WBC count ($R^2 = .15$) suggesting that the results were not affected by the higher mean WBC count present in patients included in this study compared with all AML patients.

Although the mechanisms through which alterations in RB expression occur are unknown, and indeed, the way in which this reduces survival is unclear, our studies clearly showed that these changes have a major impact on survival. A study to confirm these observations in a prospective fashion is currently underway. If confirmed, these results would suggest that patients with low RB levels and either intermediate or unfavorable cytogenetic classification should receive alternative therapy at the time of initial induction, as no patient with these findings has achieved long-term survival and most do not survive the initial induction period. Confirmation of a higher and more rapid relapse rate for patients with hyperphosphorylated RB may warrant an early referral for bone marrow transplant for patients in this group.

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Levels of retinoblastoma protein expression in newly diagnosed acute myelogenous leukemia

SM Kornblau, HJ Xu, W Zhang, SX Hu, M Beran, TL Smith, J Hester, E Estey, WF Benedict and AB Deisseroth