Clinical Implications of Decreased Retinoblastoma Protein Expression in Acute Myelogenous Leukemia

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ABSTRACT

The retinoblastoma (RB) protein levels in blast-enriched mononuclear fractions from the peripheral blood of 33 newly diagnosed patients with acute myelogenous leukemia were studied. Ten patients who had previously been treated were also analyzed, nine of whom had achieved prior complete remission. Low RB protein expression was found in 13 of 43 (30%) of the acute myelogenous leukemia patients as determined by Western blotting and immunochemical analysis. Of particular interest among the 20 newly diagnosed patients treated with the same therapeutic regimen, the median survival was 39 days for those with low RB protein expression compared to 333 days for those with high levels of RB protein expression in their leukemic cells (P < 0.02). This preliminary study suggests that decreases of RB protein expression in peripheral blood of myeloid leukemic cells occur frequently and may be associated with shortened survival of acute myelogenous leukemia patients.

INTRODUCTION

Most patients with acute myelogenous leukemia AML ultimately succumb to their disease. Although remissions are common in AML, long-term survival following chemotherapy occurs in only 20% of the patients (1). In an attempt to understand the biology of this malignancy, we are studying molecular changes which could provide clues to both the etiology and prognosis of this disease. Functional loss of the RB gene has been demonstrated in a variety of solid tumors, including carcinomas of the bladder (2, 3), prostate (4), lung (5-7), and breast (8, 9) as well as sarcomas of soft tissues (10) and bone (11). Moreover, for high-grade soft tissue sarcomas, loss of RB function has been correlated with a poor prognosis (10). In addition, alteration of RB protein expression has been demonstrated both in cell lines (12) and clinical samples of T-cell acute lymphoblastic leukemia (13), as well as in some cases of AML (14), implying that abnormalities in RB may also occur in certain leukemias. We believed that examination of the RB protein expression in patients with AML would be warranted to determine the frequency of functional RB loss and should alterations be common, whether such functional loss was also correlated with a shorter overall survival. Indeed, absence or low levels of RB protein expression was found in 30% of the AML patients studied, suggesting that RB abnormalities are quite frequent in AML. Of particular interest was the fact that among 20 newly diagnosed AML patients treated with the same therapeutic regimen (GM-CSF/DA/ara-C), the median survival for patients with RB-negative leukemic cells was 39 days compared to 333 days for patients whose leukemia cells had high levels of RB expression.

MATERIALS AND METHODS

Western Blotting. Mononuclear cells enriched in blasts from the peripheral blood of 39 AML patients were separated by continuous flow centrifugation and were frozen in liquid nitrogen. Fresh peripheral blood samples from four patients with high leukemic cell counts were also used. Fresh or thawed samples were separated on a Ficoll gradient (Organon Teknika Corp.) with centrifugation at 1400 rpm for 20 min to yield mononuclear fractions. The cells were then washed in phosphate-buffered saline and the viability of thawed cells was determined by the trypan blue exclusion test. Viability exceeded 80% in all samples. A total of 4 x 10^6 cells were diluted to 2 ml with phosphate-buffered saline and mixed with 2 ml of 2X sample lysis buffer [0.25 M Tris-chloride (pH 6.8)-2% sodium dodecyl sulfate-4% β-mercaptoethanol with 0.01% bromophenol blue] to yield a final concentration of 1 x 10^7 cells/ml. Western blot analysis was done as previously reported (15). The equivalence of protein loading into each lane was verified by probing duplicate membranes with an anti-actin monoclonal antibody (data not shown).

Single-Cell Immunochemical Analysis. Immunohistochemical analysis was performed as previously described (15). Briefly, cells were bound to polylysine-coated coverslips, fixed, permeabilized, and then preincubated with 1.5% normal goat serum in phosphate buffer for 4 h and incubated with monoclonal anti-RB antibody 1 (Triton Biosciences, Inc., Alameda, CA) overnight. The coverslips were processed for further staining using the avidin-biotinylated peroxidase complex method (Vector Laboratories, Burlingame, CA).

Statistical Analysis. The Kaplan-Meier method was used to generate the survival curves. Differences in survival were compared using the log-rank test. Survival curves were plotted by the Kaplan-Meier method and compared using the log-rank test. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 3/30/92; accepted 6/24/92.

The abbreviations used are: AML, acute myelogenous leukemia; RB, retinoblastoma; GM-CSF, granulocyte macrophage-colony stimulating factor; pRB, underphosphorylated Mr, 110,000 RB protein; ppRB, phosphorylated Mr, 110,000-116,000 RB protein; ara-C, 1-0-β-d-arabinofuranosylcytosine; DA, daunomycin.
the Wilcoxon test unless otherwise indicated. χ² and Mann-Whitney tests were used, respectively, to compare the distribution of individual prognostic features and the overall hazard ratios between the altered and normal RB protein expression groups. Individuals involved in the protein analysis were unaware of the clinical outcome of the patients.

RESULTS

Western Blot Analysis of RB Protein. Western blot analysis indicated that the peripheral blood cells of 16 normal individuals contained only low levels of the pRB form of RB protein (Fig. 1, Lane 3), and none of the peripheral blood cells of normal individuals contained ppRB forms. This was consistent with the findings reported previously that normal, nonproliferating adult human cells in vivo have very low levels of pRB only, which is not usually sufficient for producing RB nuclear staining at the single-cell level (7, 15). The RB protein levels in the peripheral blood cells of the newly diagnosed AML patients as determined by Western blotting are summarized below. These experiments were repeated in two separate laboratories with identical results.

Four levels of RB protein expression were observed. In level A, RB protein was undetectable as seen in four patients (Fig. 1, Lanes 4 and 7). In level B, underphosphorylated M₁, 110,000 RB protein (pRB) levels were lower than those seen in peripheral blood cells of normal individuals as was documented in nine AML patients (Fig. 1, Lane 9). In level C, pRB levels were considerably higher than those seen in normal peripheral blood cells, whereas no phosphorylated M₁, 110,000–116,000 RB protein (ppRB) was readily detectable. Four AML patients were found to have this RB pattern as shown in Fig. 1, Lanes 5, 8, 18, and 19. Finally, in level D, there were higher levels of both pRB and ppRB isoforms as observed in 16 AML patients (Fig. 1, Lanes 6, 13, 17, and 20. In addition, RB expression in all of the ten samples taken from previously treated patients were elevated.

In summary, 13 of the newly diagnosed AML patients had altered RB protein expression based on the Western blot assay which was either absent or lower than that seen in normal peripheral blood cells. In the samples of the remaining 20 newly diagnosed AML patients and the 10 previously treated patients, the RB protein level was higher than that found in nonmalignant peripheral blood cells and this was considered to be a normal RB protein expression for malignant cells with a functional RB gene as previously described (7, 15).
Immunohistochemical Analysis. Since the Western blot assay determines an average level of RB protein for the entire population of cells, it was likely that the low levels of pRB expression observed among the level B group of patients resulted from residual normal cells present in the specimen and that the RB protein in the AML blasts was actually absent for both level A and B cases. To determine if this assumption was correct, available samples from 23 of the AML patients were studied at the single-cell level. These samples were sent for immunocytochemical analysis without previous knowledge of the Western blot results by those reviewing the slides. As shown in Fig. 2, the leukemic blasts in the peripheral blood of AML patients with low levels of RB protein as defined by Western blotting were totally negative for RB nuclear protein staining (Fig. 2, A and B). The 100% correlation between results of the RB chemical analysis without previous knowledge of the Western blot results by those reviewing the slides. As shown in Fig. 2, the leukemic blasts in the peripheral blood of AML patients with low levels of RB protein by Western blotting had typical heterogeneous RB nuclear protein staining (Fig. 2, A and B), whereas many of the leukemic blasts of other AML patients which showed high levels of RB protein by Western blotting had typical heterogeneous RB nuclear protein staining (Fig. 2, C and D), whereas many of the leukemic blasts of other AML patients which showed high levels of RB protein by Western blotting had typical heterogeneous RB nuclear protein staining (Fig. 2, A and B). The 100% correlation between results of the RB Western blot and single-cell assays (23 of 23 cases) indicates that the leukemic blasts cells of the AML patients in whom the RB levels were undetectable (level A) or lower than normal (level B), as determined by Western blotting, had altered RB protein expression.

Survival of Patients with Low or Absent versus Normal RB Protein Expression in Their Malignant Cells. The median survival for the 13 newly diagnosed AML patients in whom the RB protein was absent in their leukemic cells was 94 days compared to 248 days for the 20 remaining patients whose leukemic cells expressed high levels of RB protein indicative of a normal functional RB gene in these cancer cells. The difference in survival approached statistical significant at the 0.05 level (P = 0.07). Of particular interest, among the 20 newly diagnosed AML patients who were treated with the same therapeutic regimen, the difference between the median survival of the low RB group of patients (40 days) and the normal RB group of patients (333 days) was significant (P < 0.02), although this is a small cohort of patients (Fig. 3). In addition, nine patients who had initially achieved complete remission expressed high RB protein levels and had a median survival of 415 days. Although not included in the statistical analysis, the fact that the patients in this latter group had the greatest overall survival and were all RB positive also suggests that altered RB protein expression adversely affects survival in patients with AML.

The survival of the 33 newly diagnosed AML patients studied was similar to that observed for the total 523 new AML patients accrued over the time period (January 1985 through March 1991) when the patients subjected to RB analysis were selected (data not shown; P = 0.58). Therefore, the 33 patients selected for inclusion in our study were representative of the total newly diagnosed AML population with regard to survival outcomes.

Of note also was the fact that there was a rapid descent of the survival curve within the first 50 days after initiating treatment for AML among the patients with altered RB expression. During this period, 6 of the 13 AML patients in the low RB group died (46%). Over the same period, 4 of 20 (20%) patients with high RB levels died. However, these results were not statistically significant in this small cohort of patients (Table 1). Only 37% (5 of 13) of patients with altered RB achieved complete remission following their initial course of induction chemotherapy while in the normal RB group 60% (12 of 20) of patients achieved complete remission. However, again, these results were not statistically significant (Table 1).

Numerous studies have identified the patient's pretreatment age, cytogenetics, bilirubin, albumin, absolute neutrophil count, and history of antecedent hematologic disease to be prognostic factors for survival (17). The two groups of newly diagnosed AML patients with normal or altered RB patterns were compared to determine the distribution of these previously identified features. The two groups did not differ significantly with

![Figure 3](http://cancerres.aacrjournals.org)
regard to French-American-British classification, sex, age, initial induction chemotherapeutic agents, bilirubin, albumin, absolute neutrophil count, or the frequency of antecedent hematological disorders. The \( P \) values for most of these parameters are shown in Table 1. The low RB group had more patients with either favorable or unfavorable cytogenetics while the normal RB group had a greater percentage of patients with cytogenetics of intermediate prognosis (18) (Table 1). To determine the overall effect, we calculated the overall hazard value using a regression model worked out previously that is based on the aforementioned clinical and laboratory parameters. The distribution of the hazard ratio between the two groups did not differ statistically (Mann-Whitney test, \( P = 0.87 \)) and the median hazard ratios were identical for the two groups (Table 1). The two groups were, therefore, balanced with respect to previously identified prognostic features.

**DISCUSSION**

Our RB Western blotting and immunohistochemical studies have shown that the peripheral blood myeloblasts of 13 of the 43 AML patients analyzed exhibited lowered levels of RB protein. These results are consistent with the percentage of AML patients having alteration in RB protein expression as recently reported by others (14) and confirms that loss of RB expression is a frequent event in AML.

The peripheral blood mononuclear cell fraction used for our studies contained a small portion of normal monocytes and lymphocytes in addition to leukemic myeloblasts. The low levels of pRB found in some leukemic samples represented RB expression from normal peripheral WBC in these samples, whereas the myeloblasts were RB negative. This was shown by the fact that at the single-cell level those leukemic samples with low levels of RB protein on Western blots lacked any detectable nuclear RB protein in their leukemic cells (Fig. 2, A and B). The molecular basis for the absence of RB protein expression in these AML patients is not yet known. Nevertheless, loss of RB mRNA has also recently been reported in two cases of AML, although no abnormalities were detected by Southern analysis (14).

The majority of leukemic samples showing significant levels of RB protein expression had high levels of both pRB and ppRB. Since, in general, we have found that normal nondividing human cells in vivo express only pRB, the presence of ppRB from in vivo samples may be in general considered a marker for the presence of a population of cancer cells (7, 15). For the purpose of our analysis, we considered leukemic cells with high RB protein expression to have no alteration of the \( R^B \) gene but rather to reflect their malignant nature.

The estimated median survival of newly diagnosed AML patients who received the same therapy (GM-CSF/DA/ara-C) was 39 days for the patients with altered RB protein expression, whereas the median survival was 333 days for patients with normal RB expression in their leukemic cells (\( P < 0.02 \)). Since no important differences in prognostic characteristics were detected between patients in the two groups, we postulate that differences in RB protein expression may represent an additional important prognostic marker for this disease. Consistent with this belief was the finding that all of ten previously treated patients who initially achieved a complete remission had normal RB protein expression in their leukemic cells as well as the longest median survival (415 days). However, because of the small number of patients available for study, it is also possible that the differences in survival were attributable to chance or to differences in other unrecognized prognostic features. A larger, prospective study is in progress to confirm that indeed patients with altered RB protein expression in their AML cells have a poorer prognosis resulting in overall decreased survival.

**ACKNOWLEDGMENTS**

The authors wish to thank Rosemarie Lauzon and Trish Waitschies for their expert editorial assistance.

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