Low and Maximally Phosphorylated Levels of the Retinoblastoma Protein Confer Poor Prognosis in Newly Diagnosed Acute Myelogenous Leukemia: A Prospective Study

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ABSTRACT

A prior retrospective study suggested that the level of retinoblastoma protein (RB) expression was prognostic for survival in acute myelogenous leukemia (AML). Individuals with no/low RB protein expression were considered to have loss of RB function, and those with maximally phosphorylated (maxphos) RB were also felt to have nonfunctional RB. To confirm this, we prospectively investigated whether the level of RB expression was prognostic in AML in a larger cohort of patients. RB level was measured by Western blot and immunohistochemical analysis on peripheral blood samples from 210 newly diagnosed AML patients. Patients were divided into three groups based on the level of RB protein expression (i.e., no or low, elevated, and maxphos) or into two groups on the basis of presumed RB function, altered function (AF-RB, low and maxphos RB), versus normal function (NF-RB, elevated RB). By combined results of Western blot and immunohistochemical analysis, 20%, 65%, and 15% of patients had low, elevated, and maxphos RB, respectively. Most patients with acute promyelocytic leukemia (APL) with a French-American-British classification of M3 were in the low RB group, likely reflecting a lower proliferative rate of promyelocytes. Analysis was performed with and without these APL patients. The median survival was significantly shorter for both patients with low RB expression (48 weeks, \( P = 0.05 \), including APL patients; 34 weeks, corrected \( P = 0.008 \), with APL patients excluded) and maxphos RB expression (51 weeks, \( P = 0.007 \)) compared to those with elevated RB expression (122 weeks including and 98 weeks excluding APL patients). Differences were greatest among patients with nonfavorable prognosis cytogenetics (median survival, 34 weeks versus 85 weeks; corrected \( P = 0.001 \) for AF-RB versus NF-RB). Remission duration was also significantly shorter for non-APL patients with AF-RB versus NF-RB (median survival, 36 weeks versus not reached; corrected \( P = 0.02 \)). In multivariate analyses, including cytogenetics, performance status, age, antecedent hematological disorder, and RB status, with and without APL patients included, no/low and maxphos-RB protein expression were independent predictors for poorer survival. Therefore, therapeutic decisions based on the level of RB expression may be indicated, and protocols to incorporate this are currently under development.

INTRODUCTION

Leukemia arises from a combination of increased proliferation associated with a loss of differentiation. This results in an increased mass of leukemic blasts and a deficiency of functional differentiated cells. In addition to regulation of proliferation, the normal process of hematopoiesis involves a decision point between differentiation and apoptosis (1). The leukemic cell mass is therefore determined by the proliferative rate, the proportion in differentiation, and the proportion in apoptosis.

Recent investigations have defined proteins and pathways involved in the regulation of proliferation and apoptosis. The complex of a cyclin (D1 or D3) and a cdk (most prominently, cdk4 or cdk6), possible in combination with PCNA, regulates the activity of the RB protein by phosphorylation (2–7). The activity of this triad and the activities of others, composed of different cyclins and cdk5s, are regulated by cdk inhibitors such as p15, p16, p21 (waf1), and transforming growth factor-\( \beta \) (8–12). When RB is phosphorylated, it releases the sequestered transcription factor E2F (13–17), which in turn induces transcription of genes required to progress past the G1 blockpoint (18). RB phosphorylation (inactivation) is thus a key point in the
control of cell proliferation (19). Functional inactivation of RB could also occur from increased phosphorylation resulting from mutations of cdks, overexpression of cyclins D or E, or loss of expression of p21, p16, or p27.

Alterations of expression in proliferation and apoptosis-regulating proteins would be expected to influence the proliferation and apoptosis rates and hence the leukemic cell mass. Our group (5, 20–22) and others (23, 24) have demonstrated a wide heterogeneity between patients in the level of expression of these regulatory proteins in AML cells, which is associated with differences in response to chemotherapy and outcome. Overexpression of p21 (21) and increased levels of PCNA protein (5) have been associated with resistance to induction therapy and with poor outcome.

The loss of RB expression or its inactivation by hyperphosphorylation would be expected to result in increased proliferation because each would lead to functional inactivation and increased unbound E2F. Retrospective studies (20, 25) of patients with newly diagnosed untreated AML suggested that patients with low levels of RB or maxphos RB had worse survival compared to those with “normal” levels of RB. To conclusively confirm the prognostic importance of RB in AML, we conducted a prospective study of 210 patients that evaluated whether low RB and/or maxphos RB levels had prognostic significance.

PATIENTS AND METHODS

Study Group. Between October 1, 1991, and July 15, 1995, 376 patients with newly diagnosed, untreated AML were seen at the University of Texas M. D. Anderson Cancer Center; peripheral blood samples were obtained prior to the initiation of therapy from 218. Emergency initiation of therapy at night or on weekends (before samples could be obtained) or absence of circulating blasts were the predominant reasons for non accrual. Eight patients opted for no therapy and were excluded from analysis, leaving a sample size of 210. None of these patients were included in our prior studies (20). As might be expected, the patients who were not sampled because of emergency or weekend initiation of therapy did worse than those studied. The median follow-up of patients alive on study is 123 weeks. 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.

Patients received induction therapy according to institutional protocols. Therapy consisted of high-dose ara-C-based regimens combined with idarubicin alone, fludarabine alone, or both as described previously. Filgrastim (granulocyte colony-stimulating factor) was given in 152 patients per existing din-...
Definition of RB categories and representative Western blot results. A. Examples of different RB levels observed and definition of RB groups. Patients with no RB signal or with a signal less than or equal to normal are scored as 0 and 1 (110 kDa), respectively. Patients with RB signals with scores of 2, 3, or 4 had levels of RB in excess of the normal controls: without significant phosphorylation (Lane 2), with less than maxphos RB (Lane 3), or with evidence of hyperphosphorylated RB (maxphos RB; Lane 4). Phosphorylation was determined to be "maximal" (110–116 kDa; Lane 4) if the shape of the band was rectangular, versus trapezoidal for submaximal (Lane 3) phosphorylation and if the height of the band was similar to that of the K562 control cell line. Elevated RB protein expression patterns were scored as 2 or 3 and are considered to represent the normal RB pattern for AML cells containing no RB alterations. The positive control cell line K562 is also shown. B, representative Western blot for RB protein expression. Blots were also probed for actin, PCNA, and BCL2 as internal controls to verify the presence of protein in the sample and to monitor for expression. Patients with no RB signal or with a signal less than or equal to normal are scored as 0 and 1 (110 kDa), respectively. Patients with RB signals with evidence of hyperphosphorylated RB (maxphos RB) had scores of 2, 3, or 4 and are considered to have abnormal or AF-RB. Low RB was assumed equivalent to nonfunctional RB because the study by Paggi et al. (28) showed that all patients with low RB had nonfunctional RB. This supports the idea that all patients with low RB have nonfunctional RB. The detection of low RB is likely due to the presence of contaminating lymphocytes and monocytes as shown by the signal generated by the normal PBMC control (Fig. 1B, lanes labeled NL).

Single Cell Immunochemical Analysis. Immunochemical analysis for RB was performed as described previously (20) in a separate laboratory, by an observer who was blinded to the Western blot results on 103 patients, including 11 with low RB, 76 with elevated RB, and 16 with maxphos RB levels as defined by Western blotting. Technical problems with sample quality prevented meaningful analysis in 11 samples.

Statistical Analysis. \( \chi^2 \) tests or Kruskall-Wallis tests compared distributions of prognostic features between RB groups. Survival distributions were estimated by the method of Kaplan and Meier (29), and comparisons were based on log-rank tests (30). Regression methods based on Cox proportional hazards model (31) 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 in the model based on their previous recognition as important prognostic factors (32–34) and evidence of association with survival in this population.

Data Set Analyzed with and without APL Patients. The pathogenesis and therapy of APL are distinct from other forms of AML; therefore, inclusion of APL patients along with other FAB types may obscure the results. Furthermore, an association between low levels of RB and APL was noted by us previously (20), and a low level of RB function was observed in 12 of 22 patients with APL in a study by Paggi et al. (28). The proliferation rate of APL cells is lower than in other cases of AML (35), and expression of RB is very low in cells in G0 or early G1 (36, 37). Therefore, the low levels of RB observed in APL could be normal given the low proliferation rate of APL cells. As presented below, one-third of the patients with low RB protein expression had APL. Because there is an overrepresentation of APL patients in the low RB population and because APL is pathogenically and therapeutically distinct from AML.
we performed the analysis both including and excluding the 21 APL patients. To account for the increased probability of false positives due to performing multiple analyses, the Ps were corrected using the Bonferroni method (38).

**RESULTS**

**RB Levels in AML Patients.** Forty-one AML patients (19.5%) had RB levels less than or equal to that observed in the normal individuals. In many of these cases, the RB level was undetectable, whereas low levels of RB were always observed in the normal samples by Western blot, and this was always the case in the normal PBMCs (39, 40). The level of RB, as measured by densitometry, did not correlate with the percentage of leukemic cells present in the sample after Ficoll separation ($r^2 = 0.102$).

The IHC analysis gave technically interpretable results in 92 of 103 patients analyzed. There was concordance of results between Western blot and immunohistochemistry in 90 of 92 patients studied by both techniques, and only nuclear staining was observed. Two patients were negative by Western blotting but had rare RB-positive cells by IHC analysis. Seven of 10 maxphos RB patients had extremely strong RB nuclear staining.

**Comparison of Clinical Features Previously Identified as Prognostic in AML.** The distribution of selected clinical characteristics for the three groups is shown in Table 1. The distribution of previously identified prognostic features (34, 35) for patients with AML were generally similar regardless of whether patients were divided into groups based on expression level or function. The median age was 54 years (range, 18–87 years). There were no significant differences in age, sex, hemoglobin, platelet count, bilirubin, fibrinogen, percentage of marrow blasts, or performance status between the low RB, elevated RB, and maxphos RB groups. The maxphos RB group had more patients with an antecedent hematological disorder than patients did in the other two groups ($P = 0.001$). Categories of favorable and unfavorable cytogenetic abnormalities were based on the results of earlier studies (32, 34). The distribution of patients by cytogenetic groups was statistically similar when APL cases were excluded.

### Table 1 Comparison of patient characteristics, response, and outcome for the three groups classified by RB protein expression

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Low RB ($n = 41$)</th>
<th>Elevated RB ($n = 138$)</th>
<th>Maxphos RB ($n = 31$)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age in years</td>
<td>52.5</td>
<td>54</td>
<td>59</td>
<td>0.47</td>
</tr>
<tr>
<td>Antecedent hematological disorder (%)</td>
<td>18–78</td>
<td>19–86</td>
<td>26–87</td>
<td></td>
</tr>
<tr>
<td>With APL</td>
<td>17%</td>
<td>10%</td>
<td>42%</td>
<td>0.001</td>
</tr>
<tr>
<td>Without APL</td>
<td>25%</td>
<td>11%</td>
<td>42%</td>
<td>0.0003</td>
</tr>
<tr>
<td>Zubrod performance status 3–4</td>
<td>10%</td>
<td>9%</td>
<td>19%</td>
<td>0.27</td>
</tr>
<tr>
<td>% achieving remission</td>
<td>71</td>
<td>75</td>
<td>61</td>
<td>0.32</td>
</tr>
<tr>
<td>Median survival (weeks)</td>
<td>48</td>
<td>122</td>
<td>51</td>
<td>0.013</td>
</tr>
<tr>
<td>Median remission duration (weeks)</td>
<td>34</td>
<td>98</td>
<td>51</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Data are shown with APL patients included and excluded. There were no maxphos RB patients with APL.
* Non-APL shown in parentheses.
* NS, not significant.

### Table 2 Correlation between cytogenetic findings and RB group

<table>
<thead>
<tr>
<th>Cytogenetic finding</th>
<th>Low RB, $n = 41$, (%)</th>
<th>Elevated RB, $n = 138$, (%)</th>
<th>Maxphos RB, $n = 31$, (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inversion 16</td>
<td>1 (2)</td>
<td>11 (8)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>2 (5)</td>
<td>7 (5)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>t(15;17)$a$</td>
<td>12 (29)</td>
<td>6 (4)</td>
<td>0</td>
</tr>
<tr>
<td>Diploid</td>
<td>9 (22)</td>
<td>50 (36)</td>
<td>8 (26)</td>
</tr>
<tr>
<td>Insufficient metaphases</td>
<td>0</td>
<td>9 (7)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>5 (12)</td>
<td>20 (14)</td>
<td>4 (13)</td>
</tr>
<tr>
<td>-5, -7</td>
<td>8 (20)</td>
<td>22 (16)</td>
<td>9 (29)</td>
</tr>
<tr>
<td>+8</td>
<td>4 (10)</td>
<td>10 (7)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>11q23</td>
<td>0</td>
<td>3 (2)</td>
<td>2 (7)</td>
</tr>
</tbody>
</table>

* Three patients morphologically defined as APL lacked the t(15;17) on cytogenetic analysis.

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Zubrod performance status code.
Relationship of RB Level to FAB Type. By the FAB classifications, there were 55 M1, 39 M2, 21 M3, 45 M4, 14 M5, 7 M6, 1 M7, 10 M0, 1 undifferentiated, and 15 unclassified patients. Low RB protein expression was observed in all FAB types evaluated but was seen significantly more frequently in patients with APL (13 of 21 patients; \( P = 0.000003 \)) and significantly less frequently in M4 cases (\( P = 0.046 \)). There were no APL cases in the maxphos group.

Relationship of RB Level to Clinical Outcome. The percentage of patients who achieved a complete remission was somewhat lower in patients with no low (29 of 41, 71%) or maxphos (19 of 31, 61%) RB protein expression compared to patients with an elevated RB pattern (103 of 138, 75%), but these differences were not statistically significant (\( P = 0.32 \) by group or \( P = 0.22 \) by function). However, the median survival was significantly shorter in the low RB group compared with the elevated RB group with or without inclusion of the APL patients (48 versus 122 weeks, \( P = 0.05 \), and 34 versus 98 weeks, corrected \( P = 0.008 \), respectively; Table 1, Fig. 2, A and B).

Patients with maxphos RB expression also had a significantly shorter survival compared to patients with elevated RB (median, 51 versus 122 weeks; \( P = 0.007 \); Fig. 2A). Similarly, patients with NF-RB (elevated) versus AF-RB (low and maxphos RB) had significantly longer survival regardless of inclusion or exclusion of APL patients (median survival, 122 versus 48 weeks, \( P = 0.004 \), with APL; 98 versus 35 weeks, corrected \( P = 0.002 \) without APL; Fig. 2, C and D). In addition, patients with NF-RB had a significantly longer remission duration compared to patients with AF-RB (median remission duration, not reached versus 57 weeks, \( P = 0.05 \) with APL included; not reached versus 35 weeks, corrected \( P = 0.02 \) with APL excluded; Fig. 3).

To determine whether the prognostic importance of RB expression was dependent on cytogenetic findings, the effect of RB expression on survival was analyzed separately for patients with favorable, intermediate and poor prognosis cytogenetics. Among patients with intermediate (diploid or insufficient metaphases karyotypes) or poor prognosis cytogenetics (\(-5, -7, +8, 11q23, Ph^1\), and miscellaneous abnormalities), both low and maxphos RB patients had significantly shorter survival than patients with normal RB function (Fig. 4) when analyzed individually (corrected \( P = 0.05 \) and \( P = 0.04 \), respectively) or...
DISCUSSION

This prospective study confirms that altered RB function due to either absence or hyperphosphorylation of RB protein is associated with significantly shorter survival in newly diagnosed patients with AML. Both the no/low RB and maxphos RB patterns were independent predictors of outcome in regression analyses. RB status was also an independent predictor of outcome when considered in terms of presumed function (no/low and maxphos RB cases considered to have nonfunctional RB and elevated RB considered to have normal RB function).

RB level was not predictive of outcome among patients with favorable cytogenetics but was a significant prognostic factor for patients with intermediate and poor prognosis cytogenetics. Among patients with poor prognosis cytogenetics, those with an NF-RB pattern had a 30% long-term survival, compared with 8% for those with an AF-RB pattern (data not shown). Therefore, detection of altered RB expression in patients with intermediate and poor prognosis cytogenetics can be used as grounds upon which to allocate these patients to alternative treatment that might improve the overall outcome of therapy. Because remission rates are only slightly inferior for AF-RB patients, one strategy would be to use conventional agents to achieve remission and then allocate AF-RB patients to immediate allogeneic or autologous transplant instead of traditional consolidation. Clinical trials to formally test this strategy are currently in preparation at this institution.

In our previous retrospective study, patients with no/low RB protein expression had an inferior response to induction therapy (20). Whereas patients with low and maxphos RB had lower CR rates in this prospective study, the differences were not statistically significant. The median survival observed in all groups was also significantly shorter in the prior study (20) compared to this study. This may reflect improvements in therapy with the recent inclusions of idarubicin, fludarabine, and granulocyte colony-stimulating factor into induction regimens. When patients treated since 1991 were compared with those treated from 1984 to 1990 (the accrual period for the retrospective study), the overall remission rates were 67 and 57%, respectively, and the median survival was 51 and 34 weeks, respectively. These differences, as well as the fact that the prior study included a higher proportion of
Table 3  Summary of results of proportional hazards regression models (Ps)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Survival</th>
<th>Remission duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model 1*</td>
<td>Model 2</td>
</tr>
<tr>
<td>Population in model</td>
<td>All patients</td>
<td>Non-APL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RB function*</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>cytogenetics</td>
<td>0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td>Antecedent hematological disorder</td>
<td>0.015</td>
<td>0.01</td>
</tr>
<tr>
<td>Performance status</td>
<td>0.002</td>
<td>0.0007</td>
</tr>
<tr>
<td>Age</td>
<td>0.02</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Model 1 was repeated with RB function split into separate variables for low RB (P = 0.01) and maxphos RB (P = 0.02). Other Ps were as above except cytogenetics (P = 0.00004).

NS, not significant.

high-risk patients, likely accounts for the variances in response rates and survival.

Two other groups have analyzed RB expression as a prognostic factor in AML. One study found no difference in outcome using a functional assay (28). However, the investigators did not discuss the details in relation to the high percentage of the nonfunctional RB patients that had APL, which could have markedly influenced the results. Preliminary data from the British Medical Research Council (MRC-12) trial found that 23 of 77 patients had “altered or absent RB,” and those patients had a median survival of 28 weeks versus 54 weeks for patients with normal RB, although this difference did not reach statistical significance (41). Neither the method used to quantify RB nor the distributions of FAB types were reported. Also, patients with maxphos RB may have been included in the “normal RB” group, thereby shortening the median survival of that group. In addition, only patients less than 60 years of age were eligible for the MRC-12 trial whereas older patients (which have a higher incidence of unfavorable cytogenetics) were included in this study. Thus, differences in results reported in the limited reports published thus far may be secondary to differences in methods and patient populations. In support of the biological importance of RB, a recent study reported that lower RB mRNA expression was a strong independent unfavorable prognostic factor for survival in childhood ALL (42).

In this study, immunohistochemistry was used as a validation test in a subset of patients. Results were concordant in 98% of patients, with very weak staining noted in two patients who had low levels of RB on Western blot. Because the results were concordant, no separate analysis of the prognostic impact of positive IHC staining was performed. It is interesting to note that extremely strong staining by IHC correlate with maxphos RB by Western blot.

Although these samples contained mixtures of leukemic cells and nonleukemic lymphocytes and monocytes, we believe that these contaminating cells do not compromise the data or conclusions. Samples from normal individuals, which would contain predominantly lymphocytes and some monocytes after Ficoll separation, always show a faint nonphosphorylated band; hence, increased or phosphorylated RB is not seen in normal peripheral blood lymphocytes. Samples from 12 patients with chronic myelomonocytic leukemia, with over 90% monocytes after Ficoll separation, all show absent or low RB, suggesting that differentiated monocytes do not express significant amounts of RB either. Furthermore, there was no correlation between the percentage of leukemic cells present after Ficoll separation and the level of RB expression. There were patients with nearly 100% leukemic cells and extremely low RB and patients with very high levels of RB and low percentages of leukemic cells in the mix. Therefore, because the normal lymphocytes and monocytes do not produce significant amounts of RB, the RB signal observed must have derived from the malignant cells in the population studied. We have compared RB protein expression in blood to that of marrow, obtained simultaneously from 25 patients, and we observed no differences suggesting that the results would be similar if the samples were derived from marrow instead of blood.

Finally, our hypothesis that AML patients with no/low or maxphos RB patterns have altered (nonfunctional) RB expression, which in turn is associated with poor prognosis, is consistent with models indicating that defects in pathways controlling cell proliferation are critical to the pathogenesis of leukemia. Of particular interest is the finding that individuals with a maxphos RB pattern in their leukemia cells have an equally poor outcome as those with no/low RB protein expression. This is the first report of such a relationship, and we hypothesize the hyperphosphorylated RB state results from aberrant expression of an upstream regulator of RB phosphorylation leading to increased RB phosphorylation (inactivation). Perhaps directly related to this hypothesis, there recently have been two reports that indicate that patients whose bladder cancer shows overexpression of RB nuclear protein have a decrease in overall survival and a progression rate as high as patients with absent RB protein expression in their tumors (43, 44). Although RB phosphorylation patterns were not measured in these tumors (43, 44), it was postulated that the overexpression of RB was the result of increased RB phosphorylation (inactivation), similar to the findings of our present study of AML cases. Moreover, the RB overexpression in bladder cancers now appears to be the result of p16 functional loss (45), consistent with our hypothesis.

5 Unpublished data.
mentioned above that an upstream modulator of RB expression may be responsible for the hyperphosphorylated (maxphos) RB pattern seen in AML. Therefore, our findings on hyperphosphorylated RB and its clinical relevance may have implications for many other tumor types in addition to AML.

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