Abnormalities of the Retinoblastoma Gene in the Pathogenesis of Acute Leukemia

By H.G. Ahuja, P.S. Jat, A. Foti, M. Bar-Eli, and M.J. Cline

The retinoblastoma-susceptibility (Rb) gene is an anti-oncogene that is frequently altered in retinoblastomas, sarcomas, and some epithelial tumors. We examined the structure of the Rb gene by Southern blotting in 215 cases of leukemias and lymphomas of diverse phenotype and in 15 leukemic cell lines. In selected cases Rb protein expression was examined with specific monoclonal antibodies. Structural abnormalities of the Rb gene with absent protein expression were frequent in all types of human acute leukemia, but were particularly common (27% incidence) in M, and M, myeloid leukemia with monocytic differentiation and in Philadelphia chromosome (Ph)-positive leukemia of lymphoid phenotype (11% to 29% incidence). Changes in Rb were observed early in the transition to acute leukemia in cases of myelodysplastic syndrome and in the accelerated phase of chronic myelocytic leukemia in transition to blast crisis. In one case, molecular changes in Rb could be correlated with leukemia remission and relapse. We conclude that the Rb anti-oncogene is commonly involved in the evolution of human acute leukemias, particularly in those of a monocytic phenotype and in lymphoid leukemia in which there is an antecedent alteration of the Ph chromosome.

GENES PRESUMED to be important in the pathogenesis of malignant diseases are thought to act by two general mechanisms. The first involves structural alterations of proto-oncogenes to generate oncogenes that act dominantly to induce a tumor phenotype. The second involves loss or inactivation of one or more genes of the class of “tumor suppressor” genes or “anti-oncogenes.” Three prototypic human tumor suppressor genes have been well characterized and include: (1) a gene on the short arm of chromosome 11 that predisposes to Wilms’ tumor; (2) the p53 anti-oncogene on the short arm of chromosome 17; and (3) the Rb or retinoblastoma-susceptibility gene on chromosome 13. Several types of human cancers have been shown to be contain cells that have altered forms of one of these anti-oncogenes. Rb gene inactivation by deletions, rearrangements, or point mutations has been observed in retinoblastomas, soft tissue sarcomas, osteosarcomas, lung, breast, and prostate cancers. Further support for an anti-oncogene function of the Rb gene product comes from the observation that introduction and expression of an exogenous Rb gene into retinoblastoma and osteosarcoma cell lines lacking functional Rb genes alters their neoplastic phenotype and suppresses tumorigenicity in nude mice. The Rb gene product has been identified as a nuclear phosphoprotein that may be associated with DNA-binding activity and has been shown to complex with several tumor-inducing proteins, including the SV40 large T antigen, the EIA proteins of adenoviruses, and the E7 proteins of the human papilloma virus type 16. Experimental studies of the synthesis and modification of the Rb protein during cell proliferation have indicated a role for this protein at specific stages of the cell cycle. These observations prompted us to investigate the status of this gene in primary hematopoietic malignancies and neoplastic cell lines to determine whether the Rb locus had a role in the pathogenesis of these tumors.

MATERIALS AND METHODS

Bone marrow, peripheral blood, and lymph node specimens from patients with various forms of hematologic malignancies were collected after obtaining informed consent and kept frozen at −70°C. Diagnoses were made by standard clinical and hematologic criteria. The immunophenotype was determined by terminal deoxynucleotidyl transferase (tdt) activity and reactivity with a set of well-defined lineage-specific monoclonal antibodies (MoAbs). A variety of malignant hematologic cell lines of defined phenotype were also analyzed, including the following: (1) chronic myelocytic leukemia (CML) in blast crisis of myeloid or undifferentiated phenotype: K562, JOSK-M, EM2, BV173, and Cloherty (the gift of J. Goldman, Royal Postgraduate Medical School, London, UK); (2) CML blast crisis of pre-B lymphoid phenotype: NALM-1; (3) Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia (ALL): MR-87; (4) acute myeloid leukemia (AML): KG-1 and HL-60; (5) AML of monocytic or myelomonocytic phenotype: GDM-1, U937, THP-1, CTY-1, and ML-1; (6) ALL (pre-B): NALM-6. Cell lines were grown in RPMI 1640 supplemented with fetal calf serum.

DNA was isolated by proteinase K digestion and phenol-chloroform extraction and after restriction endonuclease digestion was separated on 0.8% agarose gels and transferred to Zetaprobe (BioRad, Richmond, CA) membranes for Southern blotting as reported previously. Restriction enzymes used in analysis of gene structure included Xba I, EcoRI, HindIII, BamHI, and PstI. The Rb probes used in our analysis were obtained by Kpn I and EcoRI digestion of a plasmid containing a 4.9-kb DNA Rb clone kindly provided by T. Dryja. The resulting 5’ and 3’ Rb fragments were labeled to high specific activity by the random primer labeling method and used to hybridize filters that were washed and exposed at −70°C with Kodak intensifying screens (Eastman Kodak, Rochester, NY).

Immunoblotting of leukocyte lysates was performed by a slight modification of standard methods. Extracts were prepared by thawing frozen cells directly in NP-40 lysis buffer containing aprotinin and phenylmethylsulfonyl fluoride and immunoprecipitated with an antibody to Rb coupled to agarose (Rb[Ab-1]; Oncogene Sci, Manhasset, NY). Purified immunocomplexes were fractionated by electrophoresis on Laemmli gels and transferred to

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**Table 1. Rb Gene Abnormalities in Hematologic Malignancies**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Phenotype</th>
<th>Abnormal Rb Gene Structure</th>
<th>Undetectable Rb Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>CALLA⁺</td>
<td>5 of 22</td>
<td>NT</td>
</tr>
<tr>
<td>ALL</td>
<td>T-cell</td>
<td>2 of 17</td>
<td>2 of 2*</td>
</tr>
<tr>
<td>AML</td>
<td>M₂, M₃, M₄, M₅</td>
<td>1 of 39</td>
<td>1 of 1*</td>
</tr>
<tr>
<td>AML</td>
<td>M₁, M₂</td>
<td>4 of 15</td>
<td>3 of 3*</td>
</tr>
<tr>
<td>MDS</td>
<td>Myeloid</td>
<td>2 of 18</td>
<td>1 of 1*</td>
</tr>
<tr>
<td>CML</td>
<td>Myeloid</td>
<td>1 of 17</td>
<td>NT</td>
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<tr>
<td>Chronic phase</td>
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<tr>
<td>CML</td>
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<td>NT</td>
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<tr>
<td>Accelerated and crisis</td>
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<tr>
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<td>1 of 1*</td>
</tr>
<tr>
<td>Blast crisis</td>
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<td>Ph⁺ ALL</td>
<td>Lymphoid</td>
<td>3 of 11</td>
<td>5 of 7*</td>
</tr>
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<td>CLL/NHL</td>
<td>Lymphoid</td>
<td>1 of 25</td>
<td>1 of 1*</td>
</tr>
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Abbreviation: NT, not tested.

*Rb protein undetected in cells with rearranged or deleted Rb genes.

†Rb protein undetected in cells with normal Rb genes by Southern blotting.

Hybond-C extra membranes (Amersham, Amersham, UK) and visualized by probing with a second Rb antibody (IF-8, generously provided by J. Gannon and D. Lane, Imperial Cancer Research Fund, London, UK) followed by a radiiodine-labeled sheep antimouse antibody. A human cell line, 293, known to contain Rb protein served as a positive control. The same lysates were also immunoblotted with antibody to p53 protein as additional control.

Selected leukemias with Rb abnormalities were also examined for mutations and gross structural rearrangements of the p53 and bcr genes as previously described.

**RESULTS**

Two hundred fifteen clinical cases of diverse hematologic malignancies and 15 cell lines were analyzed for possible alterations of the Rb gene by Southern blotting using DNA isolated from malignant cells and probes from both the 5' and 3' regions of the Rb gene. Sixteen of the cases (Table 1) and nine cell lines were also studied for expression of Rb protein by immunoblotting. Gross structural abnormalities of the Rb gene were observed in 26 cases and 8 of the 15 cell lines. Altered Rb protein production was found in 14 of 16 patients selected because of structural Rb gene abnormalities or disease phenotype and in 5 of 9 cell lines studied. All cases studied by immunoblotting that had structurally altered Rb genes had no detectable protein. Similar observations have been made in other tumor systems. The results are summarized by disease category in the sections that follow.

**ALL.** Among 17 cases of T-cell ALL and 22 cases of common ALL of CALLA-positive, early B-cell phenotype, we found two and five cases, respectively, with novel gene fragments (18% incidence). These fragments were presumed to represent rearrangements of the Rb genes in cases of ALL (Figs 1 and 2) as well as other leukemias. The possibility that they represented restriction fragment length...
polymorphisms (RFLPs) was considered; however, each was unique among more than 300 cases analyzed (220 cases in this study and an additional 95 unreported normal and tumor tissues). Moreover, we found no such RFLPs in an extensive search of the literature. Furthermore, in several cases of ALL and other leukemias we could unequivocally exclude RFLPs by identifying novel bands with multiple restriction enzymes (see Figs 2, 3, 5, and 6). Additionally, several cases with novel bands had no detectable Rb protein by immunoblotting. Consequently, these novel fragments almost certainly represent Rb gene rearrangements.

The ALL cases with abnormal Rb genes ranged in age from 6 to 35 years with white blood cell (WBC) counts of 12 to $42 \times 10^9/L$ and high percentages of blasts (88% to 95%) in the blood. The CALLA-positive cases were of ALL-L1 phenotype and none had rearrangement of the $bcr$ locus. No Rb abnormalities were detected in the pre-B cell ALL line NALM-6, which produced Rb protein (see Fig 7).

*Acute myelocytic leukemia and myelodysplastic syndromes (MDS).* Fifty-four cases of AML were studied and abnormalities of the Rb gene were found in five and were possibly present in a sixth case. These included rearrangements and deletions of parts of the Rb gene (Fig 3) that disrupted protein production (see Table 1 and Fig 7). Four of these five cases had an M5 or M6 phenotype, ie, had features of monocytic differentiation. In all, the incidence of altered Rb gene structure in M5 and M6 disease was 4 of 15 (27%). Consequently, we examined five AML cell lines of monocytic or myelomonocytic phenotype. Four had rearranged...
Rb genes and only one, the U937 line, was normal. KG-1, a line of poorly differentiated AML cells, also had rearranged Rb genes. Rb gene alterations in the GDM-1 myelomonocytic cell line are shown in Fig 3.

The MDS are a heterogeneous group of clonal hematologic disorders, some of which frequently precede the development of overt AML. Two of 18 cases of MDS had gross alterations of the Rb gene. One alteration was similar but not identical to that of the GDM-1 cell line and the other was a partial deletion of the large HindIII fragment of the gene detected with the 5' Rb probe (Fig 3). One case was a chronic myelomonocytic leukemia (CMML) variant of MDS with elevated WBC count (58 × 10^9/L) and 10% blasts in the marrow. The other was a RAEB-T variant of MDS with leukopenia and 24% blasts in the marrow. Thus, both cases had characteristics of a preleukemia of myeloid or myelomonocytic phenotype.

Myeloid blast crisis of CML. CML usually evolves from a chronic leukemia through an accelerated phase of disease into an acute leukemia of either myeloid or lymphoid phenotype. We have previously shown that development of myeloid transformation is frequently associated with abnormalities of the p53 anti-oncogene. 23,30,35 We re-examined 42 cases of myeloid blast transformation or accelerated-phase CML previously studied 23,30 and found abnormalities of the Rb gene in six cases (Figs 4 and 5), an incidence of 14%. Five were typical cases with a transition to blastic crisis after an antecedent chronic phase, but the sixth case (Fig 5) was unusual. The patient was a 13-year-old girl who presented with a clinical picture of AML but with the Ph' chromosome present in blast cells. Her disease remitted with chemotherapy, but when it relapsed it was again the picture of an acute leukemia. A typical chronic phase of CML was never observed, and this case may represent an example of the
Fig 4. Myeloid blast crisis of CML. (A) DNAs from 10 patients with myeloid blast crisis restricted with Xba I and tested with 5' Rb probe. Lane b shows a complete deletion of two Rb fragments (arrows) in the WBCs of a 63-year-old man with early myeloid blast crisis. The WBC count was 26,000/mm³ with 34% blasts in the marrow and a deletion of chromosome 21 in addition to the Ph' chromosome. (B) Three cases tested with EcoRI and the 5' Rb probe. Lane c shows a complete loss of several fragments of the gene (arrows). This 68-year-old man had well-advanced myeloid blast crisis with 200,000 WBCs/mm³ and 51% blasts. Trisomy 12 and 13 were present in addition to the Ph' chromosome. (C) Three cases tested with HindIII and 5' Rb probe. Lane c is DNA from a case in accelerated phase (WBC count 143 × 10⁹/L and 13% blasts) showing a relative decrease in intensity of the 1.2-kb band and a novel low molecular weight band. (D) DNA from a case in accelerated phase of CML digested with HindIII and hybridized with 3' Rb probe showing a novel band (arrow).

Four myeloid blast crisis cell lines were also studied (K562, JOSK, EM-2, and Cloherty) and none had overt rearrangement of the Rb gene. EM-2 and Cloherty both had detectable Rb protein but none was detected in JOSK lysates.

Lymphoid blast crisis (LBC) of CML and Ph'-positive ALL. LBC of CML and Ph'-positive ALL share the features of fusion of the c-ABL oncogene with bcr gene sequences and the clinical manifestations of an acute lymphocytic leukemia, which is usually of early B phenotype. In LBC there is a clear antecedent chronic myelocytic phase, whereas in
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9.4
6.6
23.1

Fig 5. DNA from an unusual case diagnosed as either myeloid blast crisis of CML or Ph' positive AML (see text) digested with BamHI (A and B) and EcoRI (C). Panels A and C are hybridized with the 3' Rb probe and panel B with sequences near the Wilms' tumor locus on chromosome 11. The patient's DNA is in lane b of panels A and B and in lane a of panel C, and controls are in the remaining lanes. Arrows indicate altered fragments. At the time of study the patient had 55,000 WBCs/mm³ of blood with 38% blast cells. When her disease subsequently remitted, Rb gene abnormalities were no longer detectable.

Ph'-positive ALL the cases present de novo as acute lymphocytic leukemia. Some cases of Ph' ALL which do not remit cannot be unequivocally distinguished from LBC, and we therefore grouped together 21 cases of LBC and Ph'-positive ALL in this study. We identified Rb abnormalities in 1 of 9 cases of apparent LBC and in 3 of 11 cases of Ph'-positive ALL (Fig 6). Interestingly, Rb protein was often undetectable in Ph'-positive ALL cases without gross structural abnormalities of the Rb gene (Fig 7), suggesting that many cases may have more subtle genetic alterations such as mutations. Thus, five of seven cases of Ph'-positive ALL with normal genes by Southern blotting had no detectable Rb protein. In addition, two Ph'-positive cases were studied. NALM-1, which is clearly lymphoid had extensive deletions in the Rb gene, and BV173, which is poorly differentiated, appeared to be heterozygous with both a germline pattern and a novel band (Fig 6) and synthesized some immunoreactive Rb protein (Fig 7).

Chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma (NHL). Abnormalities of the Rb gene were found in 1 case among 25 CLL and NHL cases studied. This was in an aggressive early B-cell lymphoma whose cells showed a novel EcoRI fragment with the 5' probe and had no detectable Rb protein by immunoblotting (data not shown).

DISCUSSION

Our data indicate that abnormalities of the Rb anti-oncogene are common in human acute leukemias occurring in frequencies of more than 10% to over 30% in diverse tumors. Abnormalities of Rb appear to be more common in the acute leukemias than in the indolent leukemias and lymphomas. It is likely that the Rb gene plays a role in the pathogenesis of these malignancies and that inactivation of Rb protein is associated with disease progression rather than initiation. The occurrence of Rb gene alterations early in the evolution of blast crisis of CML and in preleukemia, and the disappearance of Rb gene abnormalities from hematopoietic cells with disease remission provide evidence in this regard. Alterations of the p53 gene, the other well-characterized anti-oncogene, also appear to be associated with progression of malignancy, at least in the case of colon cancer and CML. However, unlike the case with p53 the structural alterations of Rb in leukemias appear usually to result in gene inactivation and disruption of protein synthesis, whereas a high percentage of tumors have a mutated form of the p53 gene that generates an aberrant protein. Consequently, both alleles of Rb must be inactivated to promote tumorigenesis, whereas a single allele of p53 with a mutation may exert a dominant effect.

Several molecular abnormalities have been found in
Fig 6. Lymphoid blast crisis and Ph' + ALL. Panels A and B are, respectively, lymphoid blast crisis cell line NALM-1 and Ph'-positive undifferentiated cell line BV173 digested with the enzymes shown and hybridized with the 3' Rb probe. NALM-1 in lane b of the HindIII digest and lane d of the BamHI digest is extensively deleted in an area of the gene that is a "hot spot" for alterations. BV173 in lane a of the XbaI digest and lane b of the BglII digest shows a novel band (arrow) in addition to the germline pattern. A normal Rb pattern is seen in the other lanes containing DNA from patients and control subjects. (C) DNAs from lymphoid blast crisis or Ph'-positive ALL cases digested with EcoRI and hybridized with the 3' Rb probe. Lane e from a lymphoid blast crisis case with a WBC count of $111 \times 10^9$/L and 69% blast cells shows a novel band of less than 1 kb (arrow). No Rb protein was detectable in patient's cells. (D) DNAs from cases digested with HindIII and hybridized with 5' Rb probe. Lane d from a Ph'-positive ALL case with WBC count of $54 \times 10^9$/L and 9% blast cells shows a novel band of about 2 kb (arrow).
human hematologic malignancies, including alterations in N-RAS, c-ABL, c-MYC, bcl-1 and -2 genes, tcl-1, -2, and -3 genes, and the p53 gene.37-39 In this study, we found that the Rb anti-oncogene is also structurally altered in many acute leukemias of diverse phenotype. Previously, alterations of the Rb gene were described in only rare cases: one each of ALL, T-cell ALL, CMML, and CLL.40-41 It is worth considering how these new observations regarding the Rb gene fit into current concepts of molecular alterations in the pathogenesis of various human leukemias.

**AML.** Three consistent molecular abnormalities have been observed in AML. The most frequent is a mutation in the first exon of the N-RAS gene occurring in about 25% of cases.42 Another 25% of cases of AML of undifferentiated phenotype have loss of chromosome 11p sequences from the area that is presumed to contain the presumptive Wilms’ tumor anti-oncogene.43 Altered p53 gene expression is found in about 50% of AML cases, but the molecular basis of this alteration is not known. The present study suggests that Rb gene alterations are another independent molecular abnormality in AML. Rb gene involvement in the monocytic variants of AML appears to occur in at least 25% of cases. The gene was also found to be rearranged in a case of “preleukemia” with monocytic features and four of five monocytic AML cell lines that grossly altered Rb genes. Rb gene abnormalities may be an early event in AML because they were identified in two cases of MDS in transition to acute leukemia.

**Acute lymphoblastic leukemia (ALL).** Acute lymphocytic leukemias of the common pre-B cell as well as the rarer T-cell phenotypes have alterations of N-RAS in 10% to 25% of cases and less frequently have rearranged c-MYC, tcl, PRI, and E2A genes.39 Our data suggest that the Rb gene may also be involved in both the T-cell and the common CALLA-positive ALL variants, with gross structural alterations found in more than 15% of cases. However, it is the association of Rb with Ph' positive disease that is most intriguing.

**CML and Ph' ALL.** CML was the first malignant disease shown to have a consistent chromosomal abnormality and was one of the first demonstrated to have a consistent molecular lesion in the fusion of the c-ABL oncogene to bcr sequences. This fusion is pathogenetic in the chronic phase of disease, but other molecular events apparently dictate the evolution of disease to an accelerated phase or blast transformation.20-22 In myeloid blast transformation abnormalities of the p53 gene occur in approximately 60% of cases with only rare cases involving N-RAS gene activation. Now we have observed that some myeloid blast crisis cases have structural alterations of the Rb gene. The frequency of Rb alterations is probably not much greater than 10% of all cases. Rb gene alterations were found only once among 17 chronic-phase cases. Additionally, several patients with CML had abnormalities of Rb gene structure first detected in accelerated phase, which is an early stage in the evolution of the blastic crisis of CML. These observations suggest that the acquisition of Rb gene abnormalities like those of p53 may be important in the progression of certain cases of CML to myeloid blast crisis; however, more chronic-phase cases will have to be studied to be certain of this point.

Until now, the molecular events involved in lymphoid evolution of CML have eluded detection and no case has been described with the common defects in the p53 gene.
found in myeloid transformation. Our data indicate that some cases of lymphoid transformation of CML, as well as the related Ph'-positive ALL, have abnormalities of the Rb gene. The fact that many Ph'-ALL cases had diminished Rb protein even when the gene was apparently intact, and that two Ph'-positive CML lymphoid blast cell lines also had altered Rb genes, suggests that involvement of this gene is frequent in this disease phenotype and may be involved in disease progression once the primary abnormality in the c-ABL oncogene is established in a clone of malignant lymphoid cells.

The present data support the concept that alterations of the Rb gene are frequent in the evolution of diverse types of human leukemias and some lymphomas. It is probably safe to conclude that we are underestimating the frequency of Rb gene alterations in hematologic malignancy because our Southern blotting analysis would not have detected the mutations and other subtle alterations of the gene that have been found in other tumors such as retinoblastomas, breast cancer, and sarcomas. It is already clear from our more limited studies of Rb immunoblotting that defects in protein synthesis are frequent even when there is no gross structural change in the gene. Because structural rearrangements of the Rb anti-oncogene are seen in hematologic tumors of diverse phenotype it would seem that the Rb effect is not lineage specific, with the possible exception of a prominent role in leukemias with an element of monocytic differentiation and in Ph'-positive ALL. Our observations on alterations of gene structure and expression are consistent with the commonly held concept that it is the loss of normal Rb function which is important in tumor evolution. The suppression of tumorigenicity by the introduction of a functionally normal Rb gene offers the possibility of a novel therapeutic approach to these leukemias in the future.

REFERENCES


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