RAPID COMMUNICATION

Detection of Homozygous Deletions of the Cyclin-Dependent Kinase 4 Inhibitor (p16) Gene in Acute Lymphoblastic Leukemia and Association With Adverse Prognostic Features

By Marco Fizzotti, Giuseppe Cimino, Simona Pisegna, Giuliana Alimena, Cristina Quarterone, Franco Mandelli, Pier Giuseppe Pellicci, and Francesco Lo Coco

A recently described putative tumor suppressor gene, the cyclin-dependent kinase 4 inhibitor (p16), has been shown to be altered by deletions and/or point mutations in various human cancers. To assess the incidence and clinico-biologic correlations of p16 homozygous deletion in hematopoietic tumors, we studied a panel of 244 DNA samples representative of distinct acute (99 cases) and chronic (57 cases) leukemia subtypes, myelodysplastic (22 cases) and myeloproliferative (15 cases) syndromes, and lymphomas (51 cases). A 361-bp probe complementary to the p16 exon 2 gene sequences was generated by polymerase chain reaction and used in Southern blot hybridization against these tumor DNAs. Homozygous deletions of p16 (p16del/p16del) were detected in 10 of 58 (17%) cases of acute lymphoblastic leukemia (ALL) of either B or T lineage and in no other tumors. Single-strand conformation polymorphism analysis of p16 exons 1 and 2 was also performed in 40 of the 58 ALL cases and in 16 lymphomas. In no cases were point mutations detected. The comparison of clinical features at presentation in p16del/p16del and in p16germ ALL cases showed a greater leukemic cell mass (P = .001) and higher white blood cell counts (P = .01) in the former group. Two ALL cases in which diagnostic and relapse DNA samples were available showed p16del/p16del in both specimens. We conclude that homozygous p16 gene deletions characterize a subset of ALL with features of aggressive disease.

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In EUKARYOTES, various proteins with positive or negative regulatory activity participate in the control of the cell cycle.1 The cyclin-dependent kinase 4 (CDK4) inhibitor gene encodes a 16-kD protein (p16) with function of negative regulation of cell cycle progression.2

Recently, deletions and mutations in the p16 gene have been reported in a variety of human cancers, including leukemia, suggesting that p16 is a novel tumor-suppressor gene.3'4 Thus, like other previously characterized anti-oncogenes (e.g., p53, mias), it would be expected that its encoded protein, and these alterations might represent important steps during tumorigenesis.

Whereas preliminary studies reported p16 deletions and mutations in a striking proportion of tumor cell lines,5'6 other investigators have found that such alterations are much less frequent in primary cancers.5'6 As concerns hematopoietic neoplasia, scarce information is available on the incidence and tumor subtype distribution of p16 abnormalities.7 In addition, nothing is known about their clinical significance.

We analyzed the frequency of p16 homozygous deletion in a wide panel of pathologic DNAs obtained from patients with various types of leukemia, lymphoma, and myelodysplastic syndromes.

Our results indicate that p16 homozygous deletions are found in a significant fraction of acute lymphoblastic leukemias (ALLs). These cases are characterized by other clinically relevant genetic alterations, aggressive disease features, and poor clinical outcome.

MATERIALS AND METHODS

DNA samples. Two hundred forty-four samples stored in our DNA bank were used for this study. This panel included various types of hematopoietic diseases diagnosed and treated at a single institution (Hematology, Department of Human Biopathology of the University "La Sapienza" of Rome) during the period from 1991 to 1994. As shown in Table 1, this series included ALL (58 cases); acute myeloid leukemia (AML, 41 cases, representative of all main French-American-British [FAB] subtypes); chronic myeloid leukemia (CML) in chronic (33 cases) or blast phase (10 cases); chronic lymphoid leukemia (14 cases); myelodysplastic syndromes (22 cases, including refractory anemia with or without excess of blasts [16 cases] and refractory anemia with excess of blasts in transformation [6 cases]); Philadelphia chromosome-negative myeloproliferative syndromes (MPS; including polycythemia vera [5 cases], essential thrombocytopenia [7 cases], and myelofibrosis [3 cases]); non-Hodgkin's lymphomas (43 cases, representative of low- and high-grade tumors); and Hodgkin's disease (HD; 8 cases). Selection of leukemia and lymphoma samples was based on the presence of at least 70% blast infiltration. In non-Hodgkin's lymphomas, the intensity of rearranged Ig heavy chain gene bands observed in previous experiments indicated the presence of almost pure clonal populations in all the selected biopsies (data not shown).

Cloning of the p16 exon 2 probe. A DNA fragment corresponding to the p16 exon 2 was obtained by polymerase chain reaction (PCR) amplification of human normal DNA using the primers 5'-CACCCTGGCTGACATT-3' (sense) and 5'-CTGATCTAGTCGCCTC-3' (antisense) designed to amplify the entire coding region of exon 2 (nt 65 to nt 426) as reported in Kamb et al. The amplification product was then cloned into the pcRII vector and used as probe for Southern blotting experiments.

Southern blot analysis of p16. High molecular weight DNA was extracted from Ficoll-Hypaque isolated mononuclear cells; digested to completion with EcoRI, HindIII, or BglII restriction endonucle-
Table 1. Incidence of Homozygous Deletion of the p16 Gene in Various Hematopoietic Tumors

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Deleted/Tested</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-lineage ALL</td>
<td>8/47</td>
<td>17</td>
</tr>
<tr>
<td>T-lineage ALL</td>
<td>2/11</td>
<td>18</td>
</tr>
<tr>
<td>AML</td>
<td>0/41</td>
<td></td>
</tr>
<tr>
<td>CML-CP</td>
<td>0/33</td>
<td></td>
</tr>
<tr>
<td>CML-BC</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>CLL</td>
<td>0/14</td>
<td></td>
</tr>
<tr>
<td>MDS</td>
<td>0/22</td>
<td></td>
</tr>
<tr>
<td>MPS (Ph' -)</td>
<td>0/15</td>
<td></td>
</tr>
<tr>
<td>NHL</td>
<td>0/43</td>
<td></td>
</tr>
<tr>
<td>HD</td>
<td>0/8</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CP, chronic phase; BC, blastic crisis; CLL, chronic lymphoid leukemia; MPS, myeloproliferative syndromes; Ph', Philadelphia chromosome-negative; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease.

RESULTS

Nucleotide sequence of the cloned 360-bp probe generated by PCR using p16-specific oligomers confirmed that this fragment corresponded to the p16 exon 2 (data not shown). Tumor DNAs showing at Southern analysis hybridizing bands with various control probes and no hybridization signal after probing with p16 were considered to be homozygously deleted (Fig 1).

Homzygous loss of p16 in different hematopoietic tumors. Ten of 244 cases studied were found to be homozygously deleted at the p16 locus (Table 1). All of these cases

Fig 1. Representative Southern blot experiments showing ALL cases with p16 homozygous deletions. The same filters containing Bgl II-digested tumor DNAs were first probed with p16 (A) and dehybridized and rehybridized with the 3' M-BCR probe (exploring the major breakpoint cluster region of the Ph' chromosome). Four cases showed no hybridization (lanes 5 and 12) or very weak hybridizing bands (lanes 3 and 8) after probing with p16 (A), as compared with hybridizing bands detected after probing with 3' M-BCR (B). In cases of lanes 3 and 8 (nos. 2 and 9 in the tables), 70% blasts and 30% normal leukocytes were present in the cell samples used for DNA extraction. Molecular weight markers of 5.1, 4.2, and 2.0 kb are indicated by dashes in (A). The 5.0-kb germline BCR fragment is indicated by a dash in (B).
Fig 2. Representative results of the PCR-SSCP analysis of p16 exon 2 in ALL. Cases no. 3, 6, and 7 showed p16 homozygous deletion at the Southern blot. The faint bands visible in lanes 3 and 7 were most likely caused by the amplification of normal contaminating cells present in the DNA extracted from these tumors. The abnormal migration pattern observed in lane 1 corresponded to an already described polymorphism at nucleotide 436.

were ALL of the B (8 cases) or T (2 cases) lineage. In all samples, at least 70% blasts were present. In none of the AML (41 cases), CML (33 chronic and 10 blastic phase cases), MDS (22 cases), Ph'-negative MPS (15 cases), NHL (43 cases), and HD (8 cases) cases were p16 homozygous deletions detected. Representative Southern blot experiments showing p16 deletions are shown in Fig 1.

SSCP analysis of p16 exons 1 and 2. Forty ALL cases, including 6 of the 10 found deleted at Southern blot, and 12 of the 43 NHL cases were also analyzed by PCR-SSCP of p16 exons 1 and 2. In none of these cases were point mutations detected. Of the 6 deleted ALL cases, 4 did not show PCR bands at the SSCP and 2 showed faint PCR products most likely caused by the amplification of residual normal cells present in the analyzed samples. Figure 2 shows the results of exon 2 PCR-SSCP analysis in some representative cases.

I~nmu,lophenot~pic and genetic features ofp16 -/- ALLs. Table 2 shows the results of immunophenotypic, cytogenetic, and molecular characterization of ALL cases with p16 homozygous deletion. The techniques used for these studies had been reported elsewhere. Of the 8 patients with B-lineage ALL, 6 had a pre-pre B CALLA+ phenotype (TdT+, HLA/DR+, CD19+, CD10+, Cyt.Ig- and 2 showed less differentiated early B features (TdT+, HLA/DR+, CD19+, Cyt.Ig-). The 2 other cases presented thymic T-cell features (see Table 2). These phenotypes did not show significant variation from the phenotypes observed in the whole ALL population analyzed in this study.

As to genetic analyses, 6 of 7 p16-/- cases with available karyotype had nonrandom chromosome translocations (Table 2). In particular, a t(9;22) (q34;q11) was found in cases no. 1, 3, and 9; a t(4;11) (q21;q23) in cases no. 4 and 10; and complex numerical and structural changes including a t(8;11) (q13;p15) and a t(3;5) (q21;q31) were present in case no. 2. Molecular study of t(9;22)- and t(4;11)-positive cases by reverse transcription-PCR (RT-PCR) with specific oligoprimers showed the presence in leukemic cells of the corresponding BCR/ABL and ALL1/AF4 hybrid fusion genes (Table 2).

Sequential analysis of p16 at diagnosis and relapse. Diagnostic and relapse DNA specimens were available in 2 cases (nos. 4 and 7) with p16 -/- . In both cases, p16 homozygous loss was present either at diagnosis or at relapse. In patient no. 4, who had a t(4;11), DNA rearrangement of the ALL-1 gene locus was detected together with p16 -/- in both samples (Fig 3).
Fig 3. p16 homozygous deletion (A) and rearrangement of the ALL-1 gene at chromosome 11q23 (B) in diagnostic and relapse leukemic DNA samples of 1 patient (no. 4 of Tables 2 and 3). C, control placental DNA; lane 1, diagnosis; lane 2, relapse. (A) HindIII-digested DNAs were hybridized with the 361-bp p16 exon II probe. (B) Same DNAs digested with BglII and hybridized with the ALL-1 gene-specific B859 probe. Molecular weight markers of 23, 9.4, and 6.5 kb are indicated in (A). Identical ALL-1 gene rearranged bands detected at diagnosis and relapse are marked with an arrow. Dashes in (B) indicate 5.0 and 3.0 germline ALL-1 bands.

Clinical features and treatment outcome of p16 −/− ALL. Hematologic and clinical features at presentation and treatment outcome of the 10 patients with p16 −/− are shown in Table 3. Four patients had a white blood cell count (WBC) of greater than 100 × 10⁹/L at presentation. Nine of 10 had massive tumor burden with lymph nodes, liver, and spleen involvement. Patient no. 5 presented with central nervous system (CNS) and orbital tumor masses in addition to hepatosplenomegaly and lymph nodes involvement. Four patients died of their disease for early relapse or resistant leukemia (cases no. 1, 4, 7, and 10) and 1 patient (no. 8) died at 11 days after bone marrow transplantation (BMT) because of pulmonary hemorrhage. Patient no. 6 presented with hyperleukocytosis and could not start treatment because of renal failure. Case no. 5, which showed features of aggressive disease, underwent allogeneic BMT in first complete remis-

Table 3. Clinical Features at Presentation and Treatment Outcome of p16 −/− ALL Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Source</th>
<th>% Blasts</th>
<th>WBC (×10⁹/L)</th>
<th>Organ Involvement</th>
<th>Treatment Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14/M</td>
<td>PB/90</td>
<td></td>
<td>58.000</td>
<td>Yes*</td>
<td>Resistant/dead</td>
</tr>
<tr>
<td>2</td>
<td>43/M</td>
<td>PB/70</td>
<td></td>
<td>32.000</td>
<td>Yes*</td>
<td>CR 10+ mo</td>
</tr>
<tr>
<td>3</td>
<td>15/F</td>
<td>PB/95</td>
<td></td>
<td>42.000</td>
<td>No</td>
<td>CR 36+ mo</td>
</tr>
<tr>
<td>4</td>
<td>64/F</td>
<td>PB/98</td>
<td></td>
<td>239.000</td>
<td>Yes*</td>
<td>Relapsed/dead</td>
</tr>
<tr>
<td>5</td>
<td>12/F</td>
<td>BM/80</td>
<td></td>
<td>10.000</td>
<td>Yes†</td>
<td>I CR/allo-BMT 2+ mo</td>
</tr>
<tr>
<td>6</td>
<td>15/M</td>
<td>PB/97</td>
<td></td>
<td>120.000</td>
<td>Yes*</td>
<td>Untreated (renal failure)</td>
</tr>
<tr>
<td>7</td>
<td>7/M</td>
<td>BM/95</td>
<td></td>
<td>108.000</td>
<td>Yes*</td>
<td>Relapsed/dead</td>
</tr>
<tr>
<td>8</td>
<td>29/M</td>
<td>BM/89</td>
<td></td>
<td>10.000</td>
<td>Yes*</td>
<td>I CR/allo-BMT dead at 11 d</td>
</tr>
<tr>
<td>9†</td>
<td>54/M</td>
<td>BM/70</td>
<td></td>
<td>14.000</td>
<td>Yes*</td>
<td>Relapsed/ICR 1+ mo</td>
</tr>
<tr>
<td>10</td>
<td>16/M</td>
<td>PB/99</td>
<td></td>
<td>215.000</td>
<td>Yes*</td>
<td>Relapsed/dead</td>
</tr>
</tbody>
</table>

Abbreviations: BM, bone marrow; PB, peripheral blood.
† Liver, spleen, and lymph nodes involvement.
† Liver, spleen, lymph nodes, CNS, and orbital (retro-ocular) tumor involvement.
§ Studied at relapse.
sion (CR) and is actually in CR 2 months after BMT. Finally, patients no. 2 and 3 are in first CR at 10 and 36 months, respectively, and patient no. 9, who was studied at relapse, is in second CR at 1 month after reinduction treatment. Chemotherapy protocols used in childhood and adult ALL have been reported elsewhere.21,22

Comparison of prognostic features in p16 −/− and p16 +/+ ALL. The analysis of prognostically relevant parameters in ALL according to p16 gene status showed that patients with p16 homozygous deletion had significantly higher WBC counts and greater leukemic cell mass compared with p16 germline cases. No differences were found in the two groups with respect to age, sex, and immunophenotype (Table 4).

DISCUSSION

Several antioncogenes have been found altered in hematologic malignancies,5-15 frequently correlating with progression from indolent to aggressive disease.10,23,24 Differently from other genetic abnormalities characterizing human leukemia, such as hybrid fusion genes,25 lesions affecting tumor-suppressor genes are usually less consistent and poorly specific, ie, they are found at low frequency and in various tumor subsets.8,10,25

The recently described alterations of the p16 gene (also known as MTS or multiple tumor-suppressor gene) are particularly relevant for at least two reasons: (1) their high frequency in cell lines derived from different tumors5,7 and (2) the direct involvement of p16 in cell cycle regulation.5

In the present study, we report homozygous deletions of p16 in a sizable fraction of ALL cases. By contrast, it appears from our PCR-SSCP analysis that this gene is rarely affected by point mutations in this leukemic subset and in non-Hodgkin’s lymphoma. Given the difficulty of detecting hemizygous deletions in samples contaminated by variable fractions of normal cells, we did not perform this analysis in our study. On the other hand, the absence of point mutations in the tumors analyzed suggests that loss of one allele and inactivation of the other one by point mutation does not represent a common inactivation mechanism in this gene.

Because this series is retrospective and made up with stored DNAs, the hereby reported frequency of p16 deletion in ALL (17% of cases) might not reflect the exact incidence of such lesion in this tumor subset. However, our data are in agreement with the results recently published by Ogawa et al.1 While confirming the findings of these investigators in ALL by analyzing a larger series of cases, we report here the absence of p16 −/− in other hematopoietic tumors and in myelodysplastic syndromes. Therefore, homozygous p16 deletions seem to be associated with a specific subset in hematologic neoplasia. Furthermore, we provide a preliminary biologic and clinical characterization of p16 −/− ALLs.

Seven cases had a karyotypic characterization. Of these, 5 showed alterations usually associated with ALL within acute leukemias, ie, t(9;22) and t(4;11), and 1 showed a t(3;5). This latter abnormality is mostly detected in myelodysplastic syndromes, therapy-related leukemia, and CML blast crisis.26,27 Notably, all of these genetic changes are associated with unfavorable prognosis.26-28

Clinical features at presentation such as hyperleukocytosis and/or massive tumor burden were present in all but 1 (no. 3) p16 −/− case and correlated with poor clinical outcome. More significantly, we found a statistically significant association between high blood cell counts and leukemic mass in p16 −/− ALL cases as compared with p16 +/+ ALLs (Table 4). Given the different population studied (adults and children) and the low number of patients receiving homogeneous therapy, a comparative analysis of treatment outcome in ALL according to p16 status was not possible in the present study.

Several lines of evidence suggest that this alteration may have a role in ALL progression rather than in tumor initiation. In fact, in none of the 7 cases with available karyotype was the p16 −/− lesion detected as an isolated abnormality. Furthermore, t(9;22), t(4;11), and t(3;5) are all known to be primary abnormalities with pathogenetic relevance,23 probably conferring to their target cells genomic instability and making them prone to acquire additional aberrations. In this context, it is noteworthy that, in the 2 cases here analyzed at diagnosis and at relapse, p16 deletion was already detectable at diagnosis, but the time elapsed between diagnosis and relapse was extremely short; this finding suggests that multiple genetic derangements of leukemic cells at diagnosis might have favored a very aggressive clinical course. In case no. 4, indeed, rearrangement of the ALL-1 locus at 11q23 and p16 homozygous deletion were seen at diagnosis and at relapse (Fig 2); relapse occurred at 2 months after first remission.

In conclusion, we provide here preliminary evidence that alterations in the p16 gene are detectable in a sizable fraction of ALL and correlate with adverse prognostic features in these patients. Further studies analyzing the impact of this lesion on survival in larger series of patients are needed to establish the clinical relevance of p16 deletion in ALL.

REFERENCES

Detection of homozygous deletions of the cyclin-dependent kinase 4 inhibitor (p16) gene in acute lymphoblastic leukemia and association with adverse prognostic features

M Fizzotti, G Cimino, S Pisegna, G Alimena, C Quartarone, F Mandelli, PG Pelicci and F Lo Coco