High Incidence of Potential p53 Inactivation in Poor Outcome Childhood Acute Lymphoblastic Leukemia at Diagnosis

By David I. Marks, Benedikt W. Kurz, Michael P. Link, Eliza Ng, Jonathan J. Shuster, Stephen J. Lauer, Isadore Brodsky, and Dale S. Haines

Previous studies have indicated that p53 gene mutations were an uncommon event in acute lymphoblastic leukemia (ALL) in children. In one series of 330 patients, p53 mutations were seen in fewer than 3%. We analyzed bone marrow mononuclear cells derived from 10 children with ALL at diagnosis who subsequently failed to achieve a complete remission or who developed relapse within 6 months of attaining complete remission for p53 gene mutations and mdm-2 overexpression. We found that three children had p53 gene mutations, and four overexpressed mdm-2. Also, experiments comparing relative levels of mdm-2 RNA and protein in these patients demonstrated that mdm-2 overexpression can occur at the transcriptional and posttranscriptional level in primary leukemic cells. Although we were unable to link Waf-1 RNA expression with p53 status in childhood ALL, our data show potential p53 inactivation by multiple mechanisms in a large percentage of these patients and demonstrate that these alterations can be detected at diagnosis. Inactivation of the p53 pathway may, therefore, be important in children with ALL who fail to respond to treatment and may be useful for the early identification of children requiring alternative therapies.

Overexpression of mdm-2 constitutes a potential alternative mechanism of p53 pathway inactivation, but its significance has not been determined in pediatric ALL. Mdm-2 RNA overexpression is seen in about 20% to 30% of ALL. Mdm-2 RNA overexpression is seen in all pediatric ALL cell lines where there was a wild type p53 gene present, but the significance of mdm-2 overexpression in primary leukemic cells at diagnosis remains unclear.

In this study, we examine p53 gene status and mdm-2 expression in the bone marrow mononuclear cells of 10 children with ALL who failed to achieve complete remission or who relapsed shortly after achieving remission (“poor outcome” ALL). Our goal was to determine whether molecular alterations in the p53 pathway through p53 gene mutations or mdm-2 protein overexpression were a frequent event in poor outcome pediatric ALL. We show that there is a high incidence of potential p53 pathway inactivation in this group of patients and that the alterations can be detected at diagnosis. Three patients demonstrated p53 gene mutations, while four other patients overexpressed mdm-2 protein. In addition, we describe two patients with mdm-2 protein over-

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expression who had normal mdm-2 RNA levels, indicating the importance of measuring protein and not just RNA and pointing to a novel posttranscriptional mechanism of mdm-2 overexpression in primary leukemic cells.

MATERIALS AND METHODS

Patients and samples. Patients included in this study were treated on the Pediatric Oncology Group (POG) AIL C #15 Studies for Standard and High Risk Acute Lymphoblastic Leukemia, conducted between January 1991 and November 1994. Informed consent for sample submission was obtained from patients or from their parents. Bone marrow specimens were routinely obtained from all patients at diagnosis entered onto these studies and were sent by overnight courier to the St Jude Children’s Research Hospital. Mononuclear cells from these bone marrow specimens were separated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient sedimentation, washed in phosphate-buffered saline, resuspended in 70% RPMI/20% fetal calf serum/10% dimethyl sulfoxide, and cryopreserved in liquid nitrogen. We identified children on these studies who failed to achieve complete remission or who achieved complete remission, but developed relapse within 6 months of diagnosis and had cryopreserved cells available for study. Cryopreserved cells from these patients were sent by overnight courier in dry ice and were immediately thawed and processed. Lymphocytes from normal donors (obtained with informed consent) were also prepared by Ficoll-Hypaque density gradient sedimentation and were stored as described above.

Cytogenetic and fluorescent in situ hybridization analysis. Standard cytogenetic techniques were employed and at least 20 metaphases were examined on each patient where possible. Single color metaphase fluorescent in situ hybridization (FISH) was performed using a mixture of digoxigenin labelled p53 cosmids and labelled 17-alpha satellite probes (Oncor, Gaithersburg, MD). Hybridization, posthybridization washes, and detection with fluorescein isothiocyanate labelled antidigoxigenin antibody were performed according to the manufacturer’s recommended conditions.

DNA extraction, polymerase chain reaction single strand conformational polymorphism analysis and DNA sequencing. High molecular weight DNA was purified from ethanol after a proteinase K digestion and a phenol-chloroform extraction.14 Polymerase chain reactions (PCR) were performed with p53 amplimer primers (exons 4-9, Clontech, Palo Alto, CA) using the manufacturer’s recommended conditions. Labelled PCR products were then denatured, chilled on ice, and immediately loaded onto two gel systems to maximize the potential for observing mobility shifts.15 These were a 6% polyacrylamide/0.5X TBE gel containing 5% glycerol16 and a mutation detection enhancement gel with 0.6X TBE (Hydrolink-MDE, AT Biochem, Malvern, PA). Products were electrophoresed at 25 W for 5 hours (or at 10 W for 14 hours for MDE gels) at room temperature, dried under vacuum, and subjected to autoradiography. Aberrant migrating bands were excised from dried gels, resuspended in water, boiled for 15 minutes, and reamplified with appropriate primer combinations. PCR products were then subcloned into PCRIII (Invitrogen, San Diego, CA). DNA from clones containing inserts was pooled and sequenced with a Sequenase II kit from US Biochemicals (Cleveland, OH) using the p53 amplimer primers.

Northern analysis. RNA was isolated using RNAzol-B (Biotex, Houston, TX), and approximately 10 μg of total cellular RNA was blotted onto Hybond N+ membrane (Amersham, Arlington Heights, IL) after separation on 1% formaldehyde-agarose gels.17 Blots were then hybridized with a random hexamer 32P labelled mdm-2 probe18 or Waf-1 probe (a kind gift from W. El-Dindy, University of Pennsylvania, Philadelphia, PA) in Rapidhyb buffer (Amersham) as per the manufacturer’s instructions. Blots were subsequently washed once at room temperature with 2× SSC/0.1% sodium dodecyl sulfate (SDS) and then twice with 0.2× SSC/0.1% SDS at 65°C. Densitometry was performed with RFLP-scan software (Scanalytics, Laurel, MD), and relative overexpression of mdm-2 and Waf-1 were determined after standardization with 18S RNA.

Western analysis. Western blot analysis with 100 μg of total cellular protein was performed with monoclonal antibodies to mdm-2 (IF2, Oncogene Sciences, San Diego, CA), p53 (DO-1, Oncogene Sciences) and actin (Amersham) as described previously.19 Verification of loading was also evaluated after transfer using Ponceau S (Sigma, St Louis, MO).

RESULTS

Patient characteristics, therapy, and survival. The major clinical and prognostic findings in the 10 patients treated on one of two POG protocols (900518 and 900617) are summarized in Table 1. All individuals responded poorly to therapy and had short event-free survivals (median 39 days). Nine of 10 patients had at least one adverse prognostic feature. The majority were male, and five of 10 were younger than 2 or older than 10 years. Eight of 10 had a white cell count exceeding 50 × 10⁹/L, and four had extramedullary disease. Cytogenetic abnormalities were seen in 70%, but only one patient had involvement of chromosome 17. Two patients had a t(4;11) and one had an atypical t(9;22); none had a t(1;19) rearrangement.

P53 gene status. Single strand conformational polymorphism (SSCP) has been used extensively to detect mutations within the p53 gene and has been shown to be >90% effective when analyzing PCR products between 100 and 300 bp long (reviewed in reference 20). We, therefore, employed this type of analysis to identify if any of the 10 poor outcome patients possessed mutations within the “hot spot” regions (exons 4 to 9) of p53 at diagnosis. Three patients (1, 6, and 10) had mobility shifts involving exons 8, 6, and 5 respectively (Fig 1). Patients 6 and 10 appeared to possess wild type alleles in the analyzed cell population, while patient 1 had only mutant p53 alleles. DNA sequencing showed that patient 1 had a pro, arg insertion at AA282. This is within a “hot spot” region of p53 and is in an evolutionarily conserved domain, which is one of the most frequent sites of missense mutations. The second (patient 6) had an asp to gyl substitution at AA207, which is not in an evolutionarily conserved site, but is close to a region of p53 that is recognized by a mutant conformation specific p53 monoclonal antibody.10 It is noteworthy that this patient, who did not respond to induction chemotherapy and developed progressive disease at 22 days, had no adverse clinical prognostic factors. The only patient (number 10) to have loss of heterozygosity for the p53 gene by karyotype analysis (Table 1) had a single nucleotide deletion (−C) at codon 179 resulting in a frameshift mutation. Karyotype analysis, but not SSCP, suggested loss of the other functional allele in malignant cells from patient 10. FISH analysis was performed to determine the p53 gene status in the malignant cell population from this patient (Fig 2) and demonstrated that this patient had two populations of cells. Approximately 50% of metaphases examined contained two chromosome 17s and two
Table 1. Initial Characteristics, Prognostic Features and Outcome of Childhood ALL Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>WCC (&lt;10^9/L)</th>
<th>Karyotype*</th>
<th>R</th>
<th>EMD</th>
<th>EFS (d)</th>
<th>p53 Gene Status</th>
<th>mdm-2 Overexpression</th>
<th>Waf-1 Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>783</td>
<td>46,XY,+X,der(4)(t4;11)(11;22)(q21;q23)q25;q11), der(11)(t4;11)(11;22)(q21;q23)q25;q11),inv(16) (q21q23),-22</td>
<td>CR</td>
<td>S</td>
<td>84</td>
<td>LOH and Pro, Arg insertion at codon 282</td>
<td>—</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>172</td>
<td>46,XY,add(9)(p21)(5)q7,XY,+10(46,XY)(11)</td>
<td>PR</td>
<td>L, S</td>
<td>253</td>
<td>Wt mdm-2 protein</td>
<td>5.0</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>205</td>
<td>52,XY,+X,+Y,del(13)(q12q21)x2,21,21</td>
<td>CR</td>
<td>—</td>
<td>169</td>
<td>Wt mdm-2 protein and RNA</td>
<td>—</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>6</td>
<td>46,XX,del(7)(q31)</td>
<td>NR</td>
<td>—</td>
<td>36</td>
<td>Wt mdm-2 protein</td>
<td>26.3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>254</td>
<td>47,XY,+5</td>
<td>NR</td>
<td>—</td>
<td>27</td>
<td>Wt mdm-2 protein and RNA</td>
<td>—</td>
<td>22.0</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>7</td>
<td>46,XX</td>
<td>NR</td>
<td>—</td>
<td>22</td>
<td>Asp → Gly at codon 207</td>
<td>—</td>
<td>35.7</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>103</td>
<td>45,XY,t(3;13;9)(p21;q34;p22),t(4;11)(q21;q23), der(6)(t6;12)(q25;q13),dic(12;12)(q13;p11)</td>
<td>CR</td>
<td>L</td>
<td>173</td>
<td>Wt</td>
<td>—</td>
<td>2.0</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>61</td>
<td>46,XY</td>
<td>NR</td>
<td>—</td>
<td>22</td>
<td>Wt</td>
<td>—</td>
<td>33.0</td>
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<tr>
<td>9</td>
<td>10</td>
<td>95</td>
<td>46,XY</td>
<td>NR</td>
<td>—</td>
<td>41</td>
<td>Wt</td>
<td>—</td>
<td>117.0</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>259</td>
<td>44,XX,del(12)(p11),der(17)(t17;22)(p11;q11), add(19)(p13),-21,-22(11)44,XY, der(9)(t9;22)(p21;q11),del(12)(p11),-17, +2(19)(p13),-21,-22(4)46,XY</td>
<td>NR</td>
<td>N</td>
<td>25</td>
<td>LOH and frame-shift at codon 179</td>
<td>—</td>
<td>60.0</td>
</tr>
</tbody>
</table>

Abbreviations: WCC, white cell count; R, response; CR, complete response; PR, partial response; NR, no response; EMD, extramedullary disease; S, spleen; L, liver; CNS, central nervous system; N, Nodes; EFS, event free survival; Wt, wild-type; LOH, loss of heterozygosity.

* Patients 2 and 10 possessed multiple malignant clones in the analyzed population.
† The relative levels of Waf-1 RNA for each patient were determined by densitometric analysis after normalization for loading differences with 18S RNA.

p53 alleles, while the remaining metaphases had only one p53 gene copy. Therefore, it is likely that patients 1 and 10 have loss of p53 function via loss of heterozygosity and p53 gene mutations in a significant percentage of malignant cells. However, because patient 6 has retained one wild type p53 allele and does not have a mutation in an evolutionarily conserved domain of p53, it remains uncertain if p53 is inactivated in this individual.

Mdm-2 status. Similar to p53 gene mutations, overexpression of the mdm-2 oncogene has the potential to inhibit the tumor suppressing function of p53 (reviewed in reference 10). Mdm-2 RNA overexpression appears to be common in hematological malignancies and has been found in 20% to 50% of cases of AML and ALL. Figure 3 shows a Northern blot of the 10 pediatric ALL patients using RNA from samples obtained at diagnosis. Of 10 patients analyzed by northern blots, two overexpressed mdm-2 RNA more than 10-fold compared with bone marrow cells from a normal donor.

Recent work in choriocarcinoma cell lines and in primary leukemic cells from Sezary syndrome patients showed that mdm-2 proteins can be overexpressed in the absence of increased levels of mdm-2 transcripts. This underscores the need to analyze relative levels of mdm-2 proteins to fully evaluate mdm-2 overexpression in human cancers. Similar to control bone marrow cells, mdm-2 protein was barely detectable or not detectable in six of 10 patients. However, four poor outcome patients had cells with elevated levels of mdm-2 protein (Fig 4). Interestingly, two of these four patients had normal levels of mdm-2 RNA. Also, multiple mechanisms of overexpression may play a role in patients with elevated levels of mdm-2 RNA as the extent of mdm-2 protein overexpression was much greater than the extent of mdm-2 RNA overexpression. All of the patients with mdm-2 protein overexpression had wild type p53 genes, but only four of the seven patients with wild type p53 had mdm-2 overexpression.

Measurement of Waf-1 RNA levels. It is not certain which p53 gene mutations or what degree of mdm-2 overexpression is necessary to cause p53 pathway inactivation in vivo. Waf-1 is a gene that is transcriptionally upregulated...
Fig 2. FISH analysis of metaphases from patient 10. Single color metaphase FISH was performed using a combination of digoxigenin labeled p53 cosmids and labelled 17-alpha satellite probes. Chromosomes were identified by PI staining (not shown). (A) shows centromeric and p53 signals on the two normal chromosome 17s. (B), which is of a karyotype of monosomy 17, shows only one set of centromeric and p53 signals. Patient 10 possessed >95% blasts in the marrow at diagnosis. Because approximately 50% of the analyzed metaphases had one set of signals, it is likely that a large percentage of malignant cells from this individual has one copy of the p53 gene.

With the exception of L3 ALL, abnormalities of the p53 pathway appear to be uncommon in pediatric ALL, occurring in less than 3% of all patients. This finding seems to suggest that abnormalities of the p53 pathway are unlikely to be a significant prognostic factor in this disease. Alternatively, p53 may be important prognostically, but inactivation may occur through mechanisms other than p53 gene mutation. This study of 10 poor outcome children with ALL demonstrates that p53 inactivation may be a consistent and common molecular abnormality at diagnosis in pediatric ALL patients who fail to enter complete remission or relapse soon after achieving remission. Abnormalities of the p53 pathway through p53 gene mutations (three patients) or mdm-2 overexpression (four patients) were seen in seven of 10 individuals. It is likely that these alterations may be restricted to poor outcome patients. This is based on studies by others demonstrating that p53 gene mutations are rare in unselected cases of pediatric ALL, but are much more common in samples taken from patients at the time of relapse. Also, in an initial screen of 25 good outcome patients, we were unable to identify any patients who overexpressed mdm-2 (Marks, Kurz and Haines, unpublished data). Confirmation that p53 pathway alterations are important in the response of this disease to chemotherapy will require a study that compares poor outcome ALL to a group of good outcome patients who respond well to therapy and remain in long-term continuous remission.

It is becoming apparent that p53 mutations may have variable effects on cellular proliferation. Some p53 mutants...
may have dominant negative activity,
while for other mutations, complete p53 pathway inactivation may require alteration of both alleles. Two of the patients demonstrated both loss of heterozygosity for p53 and a p53 gene mutation, while one patient with a p53 mutation appeared to have retained one wild-type allele. It is possible that the p53 pathway is functional in this last individual. Another major uncertainty when assessing p53 pathway function is what degree of mdm-2 overexpression is necessary to inhibit p53 activity. These observations highlight the importance of assessing p53 pathway function in vivo. It may be possible to use downstream transcriptional targets of p53 as a method of assessing p53 function. Unfortunately, our data show no statistically significant difference between the levels of Waf-1 RNA (a gene that is upregulated by wild type and not mutant p53) in individuals with p53 pathway abnormalities compared with those with wild type p53 alleles and normal mdm-2 levels. This was not a surprising finding as other investigators have shown that Waf-1 may be regulated by p53 independent mechanisms in leukemic cells. Other p53 transcriptional targets need to be analyzed in these patients to determine if this type of analysis will prove to be a valid way of assessing p53 function in primary tumor cells.

In keeping with previous studies of cell lines derived from pediatric ALL patients, all individuals with mdm-2 overexpression had wild type p53 alleles. Also, the same study showed that all lines that had wild type alleles overexpressed mdm-2. We were unable to demonstrate either mdm-2 RNA or protein overexpression in three patients with wild type p53 alleles. It is, therefore, unlikely that mdm-2 overexpression in leukemic cells with wild type p53 is simply due to the increased proliferative potential of these cells in vivo.

This study also shows that mdm-2 overexpression can occur in the absence of increased mdm-2 RNA in primary leukemic cells. Two patients had elevated levels of both mdm-2 RNA and mdm-2 protein, while two patients had only elevated levels of mdm-2 protein. This finding emphasizes the importance of measuring mdm-2 protein and not only mdm-2 RNA when evaluating the incidence of mdm-2 overexpression in hematological malignancies. It also demonstrates that mdm-2 overexpression can occur at both transcriptional and posttranscriptional levels. It is supported by our data showing that the relative levels of mdm-2 protein...
overexpression is greater than the relative levels of mdm-2 RNA overexpression in patients with increased mdm-2 RNA. Recent work has proposed that the posttranscriptional mechanism of enhanced translation can cause mdm-2 overexpression in a human choriocarcinoma cell line.\textsuperscript{11} Studies measuring translation efficiency of mdm-2 RNA and mdm-2 protein stability are currently being performed to determine the mechanism of mdm-2 overexpression in leukemic cells. We have recently documented mdm-2 protein overexpression in the absence of mdm-2 RNA overexpression in Sezary syndrome.\textsuperscript{21} These mechanistic studies will be important for elucidating the regulation of mdm-2 expression in normal lymphocytes and for determining the cause of altered expression in leukemia.

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