Overexpression of the MDM2 Gene by Childhood Acute Lymphoblastic Leukemia Cells Expressing the Wild-Type p53 Gene

By Muxiang Zhou, Andrew M. Yeager, Stephen D. Smith, and Harry W. Findley

The wild-type (wt) p53 tumor suppressor gene is commonly inactivated in human malignancies, either by mutations or by loss of expression. An additional proposed mechanism for inactivation of wt-p53 is amplification of the murine double minute 2 (MDM2) gene and overexpression of the MDM2 protein, which binds to p53 and eliminates its tumor suppressor function. To investigate a potential role for MDM2 in the inactivation of wt-p53 in pediatric acute lymphoblastic leukemia (ALL), we examined the expression of MDM2 and p53, as well as the occurrence of p53 mutations and possible amplification of the MDM2 gene, in 19 pediatric ALL cell lines and one pediatric acute myelogenous leukemia (AML) line. Although we did not find significant amplification of the MDM2 gene in any of the leukemic lines, we detected overexpression of MDM2 in all 10 lines that expressed wt-p53.

I N A C T I V A T I O N O F T H E p53 gene has been implicated in the pathogenesis of a wide variety of human malignan-
cies, and can occur by point mutation, gene deletion, or loss of expression due to intronic mutations. However, these mechanisms for inactivation of p53 appear to be relatively infrequent in leukemia. In childhood acute lymphoblastic leukemia (ALL), point mutations have been detected in some patients (16% to 28%) at relapse and in cell lines established from patients at relapse; the incidence of p53 point mutations appears to be lower at diagnosis. Loss of p53 expression as a result of gene deletion or intronic mutations leading to defects in posttranscriptional processing has been detected in some leukemic cell lines5,17 but does not appear to be a common mechanism for inactivation of p53 in leukemia. Thus, most cases of ALL, both at diagnosis and in relapse, appear to express normal p53.

The human homolog of the murine double minute 2 (MDM2) gene has been shown to regulate p53 expression. An additional proposed mechanism for inactivation of p53 appears to be related to amplification of the MDM2 gene and overexpression of the MDM2 protein, which binds to p53 and eliminates its tumor suppressor function. Recently, Bueso-Ramos et al24 have reported overexpression of MDM2 in some cases of adult leukemia, including ALL.

In this study, we examined MDM2 expression and amplification as well as p53 expression and mutations in childhood acute leukemia. Amplification of MDM2 was not detected by Southern blot analysis in any of the 20 lines studied. However, overexpression of MDM2 was observed in all lines that expressed wild-type p53 (wt-p53) and not in lines that lacked p53 expression or expressed mutant p53. These results suggest that overexpression of MDM2 may be important in the etiology or progression of those cases of pediatric acute leukemia that express wt-p53.

MATERIALS AND METHODS

Cell lines and primary leukemic cells. Nineteen cell lines established from children with ALL and one line (EU-4) established from a child with acute myelogenous leukemia (AML) were studied. Nine of these lines (EU-1 through EU-9) were established at Emory University (Atlanta, GA), and nine (UoC-B1, UoC-B3, UoC-B4, UoC-B11, SUP-B2, SUP-B7, SUP-B13, SUP-B15, and KT) were established by one of the investigators (S.D.S., University of Chicago Medical Center, Chicago, IL). The REH ALL line was obtained from C. Rosenfeld (INSERM, Villejuif, France), and CCRF-CEM was obtained from the American Type Culture Collection (ATCC; Rockville, MD). Namalva, a B-cell lymphoma cell line known to have a p53 mutation, was provided by J. Ambrus (Washington University, St. Louis, MO) as a control. The phenotypes of these cell lines are summarized in Table 1 and in previous publications.

Bone marrow samples were obtained from patients during routine diagnostic procedures after receiving informed consent. Leukemic cells were enriched by Ficoll-Hypaque (1,077 g/mL) centrifugation, and aliquots were either cultured to establish the cell lines or cryopreserved in 10% dimethyl sulfoxide (DMSO). The diagnosis and classification of the leukemias were based on immunophenotypic, cytochemical, and morphologic criteria of the Pediatric Oncology Group and the French-American-British system.

Northern blot analysis. Total cellular RNA was extracted from cell lines by the guanidine thiocyanate (GCTC)-cesium chloride method, as previously described. Total RNA (10 µg per lane) was electrophoresed using a 1% agarose/6% formaldehyde gel and then transferred to a nylon filter. The p53 and MDM2 cDNA probes
were diluted 1/20 in 95% formamide (containing 20 mmol/L EDTA, block (Minicycler; MJ Research Inc, Watertown, ME). PCR products (55°C), and extension (72°C) were performed in an automated heat-

were provided by D. Phillips (Centers for Disease Control, Atlanta, GA); the sequences of the primers have been previously described.35

Southern blot analysis. Genomic DNA was prepared from individual cell lines and normal peripheral blood lymphocytes (PBLs) as a control.35 Cells were washed twice with phosphate-buffered saline (PBS), resuspended in lysis buffer [0.5% sodium dodecyl sulfate (SDS), 100 mmol/L NaCl, 10 mmol/L Tris HCl, 25 mmol/L EDTA, pH 8] containing 0.2 mg/mL proteinase K, and incubated for 14 hours at 50°C. DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol), precipitated with 100% ethanol, and resolubilized in 10 mmol/L Tris HCl/1 mmol/L EDTA, pH 7.5. Purified DNA (5 μg) was digested with restriction endonucleases, fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized to random labeled cDNA probes.

Single-strand conformation polymorphism (SSCP) analysis. The p53 exons 5 to 8, which are commonly mutated in tumor cells, were analyzed for point mutation by SSCP.36,37 Oligonucleotide primers used for the initial polymerase chain reaction (PCR) amplification were provided by D. Phillips (Centers for Disease Control, Atlanta, GA); the sequences of the primers have been previously described.35 Briefly, genomic DNA (100 ng) was subjected to PCR amplification using 10 pmol of each primer, 2.5 μmol/L dNTPs, 1 μCi of alpha-32P-dCTP (New England Nuclear, Boston, MA; specific activity, 3,000 Ci/mmol), and 0.5 U of Taq polymerase in a buffer containing 10 mmol/L Tris HCl (pH 8.8), 50 mmol/L KCl, 1 mmol/L MgCl2, and 0.01% gelatin. Thirty cycles of denaturation (94°C), annealing (55°C), and extension (75°C), and extension (72°C) were performed in an automated heat-block (Minicycler; MJ Research Inc, Watertown, ME). PCR products were diluted 1/20 in 95% formamide (containing 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heated to 90°C for 3 minutes, chilled on ice, and immediately loaded (3 μL) onto a 6% acrylamide/TBE gel containing 5% (vol/vol) glycerol. Gels were run at 8 W for 12 to 15 hours at room temperature. Autoradiography was performed for 6 to 24 hours.

Direct sequencing of PCR products. PCR products that demonstrated an aberrant migration pattern in the SSCP autoradiogram relative to the wild-type gene were analyzed for nucleotide sequence. Before sequencing, the targeted DNA was amplified with 1 μg of genomic DNA and 50 pmol of each primer in a final volume of 100 μL. The conditions and number of cycles of PCR were as described for the SSCP analysis, omitting the 32P-dCTP. The PCR products were separated by electrophoresis in an agarose gel and purified using the Ultrafree-MC filter unit (Millipore Corp, Bedford, MA). Purified cDNA was sequenced using a modification of the dideoxy chain-termination procedure38 (Double-Stranded DNA Cycle Sequencing System; Life Technologies Inc, Gaithersburg, MD) following the manufacturer's recommendations.

RESULTS

Southern blot analysis of the MDM2 gene. Genomic DNA prepared from individual cell lines was digested with restriction enzyme and electrophoresed on agarose gels. After digestion with EcoRI, five fragments detected in normal PBLs were observed in all 20 acute leukemia cell lines and the control Namalva line. No significant gene amplification was detected in any of these cell lines (Fig 1).

SSCP analysis of the p53 gene. The results of the SSCP analysis for the p53 gene are summarized in Table 1, and representative results are shown in Fig 2. Three ALL cell lines (EU-2, EU-7, and CCRF-CEM), like the Namalva line, showed an abnormal migration pattern for PCR-amplified

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>T-Cell</th>
<th>B-Cell</th>
<th>Myeloid</th>
<th>p53</th>
<th>MDM2</th>
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<tr>
<td>Phenotype</td>
<td>HLA-DR</td>
<td>CD2</td>
<td>CD7</td>
<td>CD10</td>
<td>CD19</td>
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<tr>
<td>EU-1: R, EPB (855)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>EU-5: R, EPB (196)</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>EU-7: R, T (91-17)</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>EU-8: R, EPB (91-06)</td>
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<td>+</td>
<td>+</td>
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<td>EU-9: R, T (93-10)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>SUP-B7: D, EB7</td>
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<td>+</td>
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<td>-</td>
<td>+</td>
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<tr>
<td>CCRF-CEM: R, T</td>
<td>-</td>
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Abbreviations: R, relapse; D, diagnosis; EPB, early pre-B-ALL; PB, pre-B-ALL; T, T-ALL; WT, wild type; MUT, mutant.

* Amount of mRNA compared with that in PHA-activated normal PBL as one "+" (see Fig 4).
fragments derived from exon 7. Cell line EU-6 showed an abnormal migration pattern in exon 8. The overall frequency of p53 mutation among the childhood leukemia cell lines studied was 20% (4 of 20); however, p53 mutations seemed to be more frequent in T-cell ALL (T-ALL; 40%, two of five) than in B-cell precursor (BCP) ALL (13%, 2 of 14). All 20 lines had normal PCR-SSCP patterns for exons 5 and 6 of the p53 gene (data not shown).

Sequence analysis. The p53 mutations in childhood ALL cell lines detected by SSCP analysis were further examined by direct sequencing of PCR-amplified exons. Figure 3 shows the representative results of p53 mutations in ALL cell lines. Cell line EU-7 had a point mutation (G to A) in exon 7 at codon 248. At this codon, a band corresponding to the normal nucleotide was still present, consistent with the SSCP analysis finding of a PCR band with normal mobility (Fig 2). A similar mutation was found in the EU-2 cell line (data not shown). Cell line EU-6 had a point mutation (G to T) in exon 8 at codon 273 and lacked the wild-type sequence. This confirmed the SSCP analysis result that a band with normal mobility was not found (Fig 2).

Expression of p53 and MDM2 in ALL cell lines. Both p53 and MDM2 mRNA were detected by Northern blot analysis using both 32P-dCTP-labeled probes in the same reaction. In 5 of the 19 ALL cell lines and in the AML line EU-4, p53 mRNA was not detected. The remaining 14 ALL cell lines and Namalva showed a normal p53 transcript (2.8-kb band) as compared with phytohemagglutinin (PHA)-activated normal PBLs (Fig 4). Based on these results, the cell lines could be divided into three groups: wt-p53-positive, mutant-p53-positive, and p53-negative.

MDM2 mRNA (5.5-kb band) was overexpressed (compared with both normal bone marrow mononuclear cells and normal PHA-stimulated PBLs, which were essentially identical) in all wt-p53-positive lines (Fig 4). In contrast, no MDM2 overexpression was found in any cell line that did not express wt-p53. Instead, all but two of the p53-negative lines lacked any detectable expression of MDM2; the excep-
tions were SUP-B2 (ALL) and EU-4 (AML), which showed low expression of this gene. Similarly, most of the mutant-p53-positive lines showed low MDM2 expression, comparable with that seen with PBLs.

Expression of p53 and MDM2 by primary leukemic cells. To determine whether the patterns of MDM2 and p53 expression observed with these cell lines were also characteristic of the primary leukemic cells from which these lines were derived, we analyzed previously cryopreserved samples of

leukemic bone marrow cells from seven patients from whom cell lines had been established. Total RNA was extracted from cryopreserved relapse cells after thawing, and expression of the MDM2 and p53 genes was analyzed by Northern blotting as described. Similar results were obtained from both primary leukemic cells and derived cell lines (Fig 5).

Fig 3. Sequence analysis of p53 mutation in ALL cell lines. Sequences from exon 7 and 8 are shown for cell lines EU-7 (91-17) and EU-6 (920), respectively. Arrows point to bands corresponding to mutated base pairs.

DISCUSSION

In this study, we detected point mutations in the p53 gene in exons 5 to 8 in four ALL lines (two of five in T-ALL and 2 of 14 in BCP-ALL). These exons contain 98% of all p53 mutations detected in human malignancies. Additionally, five of the 19 ALL lines and one AML line did not express p53 mRNA. No structural abnormalities in the p53 gene were detected in these p53-negative lines, nor were point mutations in genomic DNA detected in exons 5 to 8. The remaining 10 lines expressed wt-p53 as detected by Northern blotting, SSCP analysis, and immunoprecipitation. Importantly, all 10 lines that expressed wt-p53 also overexpressed the MDM2 gene. Conversely, no overexpression of the MDM2 gene was detected in the 10 lines that either expressed mutant p53 or did not express p53. No amplification of the MDM2 gene was detected in either the wt-p53-negative lines or the wt-p53-negative lines.

Experimental evidence suggests that the p53 and MDM2

Fig 4. Northern blot analysis of MDM2 and p53 mRNA from ALL cell lines. Total RNA was extracted with the GITC cesium chloride centrifugation method. Total RNA (10 μg per lane) was electrophoresed on a 1% agarose/6% formaldehyde gel. The p53 and MDM2 probes were prepared by randomized labeling using cDNAs provided by B. Vogelstein (Johns Hopkins Oncology Center). Membranes were hybridized with both probes in the same reaction and then washed and rehybridized with beta-actin as a control for equal loading.
genes interact with each other to regulate cell growth. The p53 protein is a transcription-activating factor that can bind to specific nucleotide sequences and activate adjacent genes.\(^{40-42}\) The first intron of the MDM2 gene contains a p53 consensus DNA-binding site that provides the presumptive mechanism for the regulation of MDM2 by wt-p53.\(^{43}\) Mutant forms of p53 lack a transcriptional activating function in several experimental systems and, in particular, are unable to activate expression of MDM2.\(^{44-46}\) However, we have found that some leukemic cell lines expressing mutant-p53 (including line EU-6 with a homozygous mut/mut phenotype) show low levels of MDM2 expression, similar to that seen in normal bone marrow mononuclear cells and activated PBLs. Furthermore, two lines that did not express detectable p53 showed low-level expression of MDM2, indicating that some mutant forms of p53 (including line EU-6 with a homozygous mut/mut phenotype) show low levels of MDM2 expression, similar to that seen in normal bone marrow mononuclear cells and activated PBLs. Furthermore, two lines that did not express detectable p53 showed low-level expression of MDM2, indicating that some mutant forms of p53 retain transcriptional activating ability for MDM2 or, alternatively, that a p53-independent pathway exists that can give basal levels of MDM2 expression.

Other studies have identified overexpression of MDM2 in some sarcomas.\(^{26}\) A recent study detected overexpression in 27% of the tumors studied; however, in most of these cases, overexpression was due to amplification of the gene.\(^{47}\) Interestingly, 11% of these cases showed overexpression of both MDM2 and p53 genes; overexpression of p53 protein was detected by immunohistochemistry using a monoclonal antibody (Ab-2; Oncogene Science, Uniondale, NY) that recognizes both wild-type and mutant forms of p53. Furthermore, these cases were associated with a significantly worse prognosis compared with cases without overexpression of either MDM2 or p53. Although the number of cases overexpressing wt-p53 as opposed to mut-p53 protein was not reported, mutant-p53 protein is typically present at greater concentrations than wt-p53 protein due to its longer half-life.\(^{1}\) Based on our findings, we would not expect to see overexpression of MDM2 in cases expressing mut-p53. Although we did not detect any ALL samples that clearly overexpressed wt-p53 at the mRNA level, it is possible that overexpression may be detected at the protein level, as in the above sarcomas. Further studies are required to address the possible occurrence and clinical significance of simultaneous MDM2/p53 overexpression in leukemia.

Recently, Bueso-Ramos et al\(^{24}\) found that MDM2 mRNA was overexpressed but not amplified in 53% of adult leukemias, including three of seven (42%) cases of ALL. These investigators did not report whether overexpression of MDM2 was correlated with expression of wt-p53 in these leukemias. The present study confirms and extends these findings by showing a strict correlation between overexpression of MDM2 and expression of wt-p53 in childhood leukemic cells.

Although all specimens expressing the heterozygous wild-type (wt/wt)-p53 genotype also overexpressed MDM2, two cell lines (EU-2 and EU-7) expressing both wild-type and mutant genes lacked MDM2 overexpression. This suggests that the mut-p53 protein in these cases was able to abrogate the inhibitory function of the wild-type protein and permit cell proliferation in the absence of MDM2 overexpression. Both of these lines had a codon-248 mutation in exon 7, which has been associated with mutations exhibiting dominant-negative suppression of wild-type function.\(^{48}\)

The mechanism by which MDM2 is overexpressed in leukemias in the absence of gene amplification is not known. The MDM2 gene contains a cis-acting DNA element capable of binding wt-p53 protein, which acts as a transcriptional regulator.\(^{43,49}\) In normal cells, increased expression of MDM2 is induced after DNA damage, such as that due to irradiation, in direct response to induction of wt-p53, and MDM2 expression is dependent on the presence of wt-p53 protein.\(^{49}\) In contrast, we have detected low levels of MDM2 expression in cells expressing either mut-p53 or entirely lacking p53 expression. This suggests that other trans- or cis-acting transcriptional factors may contribute to regulation of MDM2 expression, and derangements in these factors as well as in the p53 regulatory pathway may account for overexpression.

Our findings are consistent with the idea that overexpression of MDM2 may abrogate the transactivating and growth-inhibitory functions of the wt-p53 protein in tumor
cells expressing this gene, whereby permitting uncontrolled growth. Inactivation of p53 either by mutation or by binding to other proteins appears to be an important step in the development of human tumors. Recent results indicate that human MDM2 can inhibit the transactivating function of p53 by binding to the acidic activation domain of p53. Furthermore, the MDM2 protein itself has similarities to several transcriptional regulatory proteins, including two putative metal-binding motifs and a highly acidic domain, which may permit MDM2 to regulate directly transcription of certain genes involved in oncogenesis. Although the full regulatory features of the MDM2 protein remain to be elucidated, the ability of overexpressed MDM2 to overcome the growth inhibitory activity of the wt-p53 protein is particularly important given the large percentage of childhood ALL cases that express wt-p53; indeed, the p53-suppressive effects of the MDM2 protein may in part explain the low incidence of p53 mutations in this type of leukemia.

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Overexpression of the MDM2 gene by childhood acute lymphoblastic leukemia cells expressing the wild-type p53 gene

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