RAPID COMMUNICATION

The Human MDM-2 Oncogene Is Overexpressed in Leukemias

By Carlos E. Bueso-Ramos, Yun Yang, Elizabeth deLeon, Patrick McCown, Sanford A. Stass, and Maher Albitar

The human homologue of the mouse double minute 2 (MDM-2) gene codes for a cellular protein that forms a complex with the mutant and wild-type p53 protein and modulates its trans-activation activity. Overexpression of the MDM-2 gene in cells increases their tumorigenic potential and overcomes the growth-suppressive activity of p53. Previous reports have shown that the MDM-2 gene is amplified in approximately one third of human sarcomas. To examine the role of MDM-2 in leukemia, we analyzed MDM-2 gene amplification and mRNA expression in various types of leukemias. We did not detect gene amplification in any of the 48 cases of leukemia that we examined. In contrast, we observed significant MDM-2 mRNA overexpression in 34 of 64 cases (53%). The level of mRNA overexpression in some cases of leukemias was comparable to that observed in some cases of sarcomas, which demonstrate more than 50-fold MDM-2 gene amplification. Furthermore, we divided these cases into different prognostic groups according to their karyotypic abnormalities. MDM-2 overexpression seemed to be associated with unfavorable chromosomal abnormalities. These findings suggest that the expression of the MDM-2 gene is altered in a significant fraction of human leukemias and MDM-2 may play a significant role in leukemogenesis. In addition, these results suggest that mechanisms other than gene amplification may play a significant role in deregulating the MDM-2 expression.

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MATERIALS AND METHODS

Cell lines and patient samples. Bone marrow or peripheral blood specimens were obtained from patients evaluated in the leukemia clinics at The University of Texas M.D. Anderson Cancer Center. Specimens were acquired during routine diagnostic or therapeutic procedures, under approved protocols. Myeloid cell lines

β-actin mRNAs in normal and leukemic patient samples and in leukemic cell lines. β-actin was used as an internal control. No gene amplification was detected by Southern blot analysis in any of the 48 cases of leukemia we studied. However, 34 of 64 (53%) leukemias showed significantly high levels of MDM-2 mRNA expression. MDM-2 overexpression was also detected in some leukemic cell lines (MDA/AML-8, K562, and Raji). Furthermore, in acute myeloid leukemia (AML) patients, although the number of patients was somewhat small, overexpression of MDM-2 mRNA was apparently associated with cytogenetic characteristics that typically confer an unfavorable prognosis. The overexpression of MDM-2 mRNA in these leukemias may play a role in the leukemogenic process and may account for the relatively low incidence of p53 mutations in hematopoietic malignancies.

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(OCI/AML-1, OCI/AML-2, OCI/AML-3, OCI/AML-4, OCI/AML-5, MDA/AML-8, MDA/AML-9, and MDA/AML-10) were a gift of Dr. McCulloch from the University of Toronto, Canada. These cell lines were generated by Dr. McCulloch at M.D. Anderson (MDA) Cancer Center or at the Ontario Cancer Institute (OCI).15-16 K562 (CCL243), Raji (CCL214), and Hela (CCL2) cell lines were obtained from the American Type Culture Collection. Mononuclear cells were separated on Ficoll-Hypaque (Sigma Diagnostic, St Louis, MO) and washed with phosphate-buffered saline. Cells and tissues were cryopreserved for various periods before RNA extraction. The diagnosis and classification of the leukemias were based on morphologic, immunophenotypical, cytochemical, and molecular evaluation at the Hematopathology Section at MDA Cancer Center. Cytogenetic studies were performed as previously described.17

**RNA and DNA extraction.** RNA was extracted by the guanidine hydrochloride method as described previously.18 Briefly, tissues were lysed in a highly concentrated solution of guanidine hydrochloride in the presence of dithiothreitol (DTT). The cell suspension was centrifuged at 3,000 rpm for 10 minutes at 4°C, and the supernatant was transferred to a 15-mL tube and precipitated overnight with 0.5% volume of 100% ethanol. The following day, the solution was centrifuged at 3,000 rpm for 30 minutes at 4°C. The pellet was resuspended again in a highly concentrated solution of guanidine hydrochloride in the presence of DTT and precipitated overnight with 0.5% volume of 100% ethanol. The suspension was centrifuged as on day 2, and the pellet was resuspended in 25 mmol/L EDTA and mixed with an equal volume of 4:1 chloroform:isoamyl alcohol solution. The suspension was then centrifuged for 5 minutes at 1,000 rpm, and the upper layer was transferred to a 15-mL conical tube and precipitated overnight with 3X volume of 4 mol/L sodium acetate. On day 4, the sample was centrifuged and the pellet was resuspended in 1 mL of 80% ethanol, precipitated for 1 hour at -75°C, followed by centrifugation at 14,000 rpm at 4°C for 35 minutes. The RNA pellet was lyophilized and resuspended in 50 to 100 µL of water and stored at -75°C. DNA for Southern blot studies was extracted within 8 hours of collection as described previously.18 Briefly, white blood cells were lysed in 3 mL of aqueous buffer with detergent. Proteins were digested by the addition of proteinase K (Sigma Chemical Co) and incubated at 50°C for 1 hour, followed by sequential organic extraction in phenol/chloroform and chloroform. The purified DNA in the aqueous phase was precipitated by addition of at least 0.1 volume of 3 mol/L sodium acetate and 100% isobutanol. The DNA was dissolved in Tris-EDTA buffer and digested with the appropriate restriction enzyme.

**Southern blot analysis of the MDM-2 gene.** Southern blot analysis was performed as previously described.19 Ten micrograms of digested DNA was loaded into an 0.7% agarose gel containing ethidium bromide. Electrophoresis was performed at 22 V. The electrophoresed DNA was transferred to a nylon membrane by the vacuum transfer technique at 6 to 8 mm Hg for 90 minutes. Next, the membrane was dried in an 80°C oven for 30 minutes and prehybridized in 20 mL of standard prehybridization solution for 2 hours. MDM-2 probe was radioactively labeled to high specific activity by the random primer method (Bethesda Research Laboratories Life Technologies, Inc, Gaithersburg, MD). The final probe concentration was 2 to 2.5 X 10^6 dpm/mL in the hybridization mix. We used a 1.4-kb fragment of the MDM-2 cDNA, which represents almost the entire coding sequence, as a probe. Following hybridization the filters were exposed to Kodak AR film (Eastman Kodak, Rochester, NY) for 1 to 15 days. The filters were then stripped and probed for the heavy-chain immunoglobulin (JH) (Oncor, Gaithersburg, MD). MDM-2 gene amplification was determined by comparing the intensity of the MDM-2 probe signals with those of the JH immunoglobulin probe using the Betagen phosphor image analyzer (Intelligenetics, INC, Mountain View, CA).

**cDNA synthesis and PCR.** Approximately 2 µg of cellular RNA in 12.5 µL of ultrafiltered water, 0.2 µg of β-actin, and 0.2 µg of MDM-2 primers complementary to the 3' end of the β-actin and MDM-2 mRNAs were added to a tube containing an equal volume of 2X reverse transcriptase buffer (final concentration 180 µg/mL of actinomycin D, 100 mmol/L NaCl, 20 mmol/L DTT, 100 mmol/L Tris, pH 8.4, 10 mmol/L MgCl₂, and 400 mmol/L dNTP each), heated to 95°C for 3 minutes, and then immediately cooled on wet ice. Twenty units of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc, St Petersburg, FL) was added to the reaction mixture and incubated at 42°C for 1 hour. The synthesized cDNA was extracted with an equal volume of phenol/chloroform and precipitated from 0.1 mol/L NaOAc solution with 2.5 volume of ethanol. PCR was performed as described by Albitar et al.11,12 The 5' primers of both β-actin and MDM-2 were end-labeled with 32P to the same specific activity. The end-labeling was performed...
Fig 2. Verification of the identity of the RT/PCR products. (A) Autoradiograph showing the amplification products of MDM-2 and/or β-actin as well as the products of their restriction enzyme digestion. The RT/PCR products were run as undigested (U), digested with HindIII (H), or Rsa I (R). The expected size MDM-2 HindIII and Rsa I digestion products are 159 and 249 bp, respectively. The 123-bp fragment seen on MDM-2/β-actin sample in the Rsa I digestion resulted from the β-actin digestion. The β-actin product was not digested by the HindIII enzyme. The fragment migrating at approximately 150 bp (R) was caused by partial digestion of the β-actin. Lane (M) is 32P end-labeled pBR322/Msp I size marker. (B) A schematic of the MDM-2 mRNA and its amplification and digestion is shown. The MDM-2 mRNA is shown on the top. Positions and orientations of the primers used in the RT/PCR reaction are shown as small arrows, and the expected size of the amplified and digested fragments is shown. The asterisk indicates the 32P end-labeling of the primer.

with [gamma-32P]ATP (5,000 Ci/mmol; American Corp, Arlington Heights, IL) as described previously. To 25 μL of cDNA solution, we added 25 μL of master mixture containing 5 μL of 10× PCR buffer, 0.2 μg of 2 MDM-2, 0.2 μg of 2 β-actin primers, and an equal amount of labeled 5' MDM-2 and labeled 5' β-actin, 1.5 mmol/L of each of the deoxynucleotides, and 2 U of Taq polymerase. The sequence of the β-actin primers has been previously described. The 3' MDM-2 primer used for reverse transcription and PCR is 5'-TGAAGTTTCTCTTCCTGAAG-3'; and the 5' primer is 5'-TTATTAAAGTCTGTTGGTCA-3'. Oligonucleotides were synthesized using a DNA synthesizer from Applied Biosystems (Foster City, CA). Oligonucleotides were fully deprotected after synthesis and purified using high-pressure liquid chromatography. Samples were amplified through 30 consecutive cycles. Each amplification cycle consisted of a denaturation step at 94°C for 1 minute, primer annealing at 57°C for 1 minute, and extension at 74°C for 4 minutes. Cycles were preceded by incubation for 5 minutes at 95°C to ensure full denaturation of the target DNA, and were followed by an extra 7 minutes of incubation at 74°C after the final cycle to ensure full extension of product. The PCR reactions were performed on a DNA thermal cycler (Perkin Elmer-Cetus Instruments, Norwalk, CT). The amplified fragment of MDM-2 was 335 bp, and the β-actin–amplified fragment was approximately 240 bp.

Analysis of amplified samples. In all cases the completed reac-
ions were resolved by electrophoresing a 10-mL aliquot of each amplification reaction on a gel containing 8% polyacrylamide and 8 mol/L urea. The gel was then transferred to Whatman 3M paper (Schleicher and Schuell, Inc, Keene, NH), covered with Saran wrap (Reynolds Corp, Richmond, VA), and exposed to Kodak AR film at -70°C. The relative density of the amplification product bands was counted using a 603 Betascope phosphor image analyzer.

For restriction enzyme analysis of the PCR product, the amplified cDNA was extracted with phenol/chloroform and precipitated with ethanol before restriction enzyme digestion. Restriction enzyme digestion of the amplified cDNA was performed with HindIII and RsaI (New England Biolabs, Beverly, MA), according to the manufacturer’s suggested conditions.

**Northern blot analysis.** RNA was separated by electrophoresis in a MOPS-formaldehyde gel and transferred to nylon filter. Transfer and hybridization were performed as described previously.20 RNA was hybridized to the same MDM-2 probe that was used for Southern blot analysis. The same blot was stripped and rehybridized with β-actin probe. (B) RT/PCR analysis of the same RNA samples.

**RESULTS**

**Lack of MDM-2 gene amplification in leukemia.** Using Southern blot analysis, we analyzed 48 cases of leukemia and three cases of reactive lymphocytosis for MDM-2 gene amplification (Fig 1). No significant gene amplification was detected in any of the 51 cases examined. This includes 22 cases of chronic lymphocytic leukemia (CLL), 3 cases of hairy cell leukemia (HCL), 6 cases of acute lymphoblastic leukemia (ALL), 15 cases of AML, 2 cases of myelodysplastic syndrome (MDS), and 3 cases of reactive lymphocytosis. The copy number of the MDM-2 gene was compared with immunoglobulin copy number. In some cases of CLL, the signals of the MDM-2 probe, when compared with those of the JH probe, showed a minor increase consistent with trisomy 12, which is a relatively common chromosomal abnormality in CLL.21 One case of CLL showed rearrangement in MDM-2 after digestion with HindIII (Fig 1A) and PvuII (not shown). Chromosomal studies of this case showed no visible chromosome 12 abnormality, but other clonal chromosomal abnormalities were observed, including 45, X, Y, t(9q;18p). This case showed MDM-2 mRNA overexpressed, and further analysis of this case is in progress.

The MDM-2 mRNA is overexpressed in leukemias. The relative level of MDM-2 mRNA in various leukemic samples was established by RT/PCR and coamplification of the MDM-2 and the β-actin mRNAs. We first synthesized MDM-2 and β-actin cDNAs by RT using 3' antisense...
MDM-2 IN LEUKEMIAS

Table 1. Overexpression of MDM-2 mRNA in Various Types of Leukemia

<table>
<thead>
<tr>
<th>Leukemia</th>
<th>No. of Patients</th>
<th>Overexpression</th>
<th>Normal Expression</th>
<th>Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL</td>
<td>12</td>
<td>+ 5 ++ 0</td>
<td>3</td>
<td>9/12 (73)</td>
</tr>
<tr>
<td>HCL</td>
<td>2</td>
<td>0 0 0</td>
<td>2</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>AML</td>
<td>32</td>
<td>9 6 2</td>
<td>15</td>
<td>17/32 (53)</td>
</tr>
<tr>
<td>ALL</td>
<td>7</td>
<td>3 0 0</td>
<td>4</td>
<td>3/7 (42)</td>
</tr>
<tr>
<td>CML</td>
<td>4</td>
<td>0 0 0</td>
<td>4</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>MDS</td>
<td>7</td>
<td>2 2 1</td>
<td>2</td>
<td>5/7 (71)</td>
</tr>
</tbody>
</table>

The level of MDM-2 overexpression is indicated by one plus sign (+) for low, two (+++) for moderate, and three (++++) for high overexpression in relation to β-actin.

Using this multiplex RT/PCR assay, we analyzed MDM-2 expression as compared with the β-actin in several normal tissues including peripheral blood, bone marrow, soft tissue, epithelial tissue, kidney, and liver. All normal tissues showed a low level of MDM-2 mRNA expression. The MDM-2 amplification products of most normal samples were less than 10% of the β-actin amplification products, and none of them was more than 16%. We considered a ratio of MDM-2:β-actin of 20% to 40% as a small increase (+), 41% to 80% as a moderate increase (++), and greater than 80% as a high increase (+++). We analyzed the level of MDM-2 expression in several cell lines (see Materials and Methods), only MDA/AML-8, K562, and Raji cell lines showed significant levels of MDM-2 mRNA overexpression (Fig 4). Table 1 and Figs 4 and 5 summarize our findings in various types of leukemias. Fifty-three percent of all leukemic samples showed overexpression of the MDM-2. In CLL, there were low levels of overexpression in 73% of the cases, whereas no overexpression was detected in two cases of HCL. Most of these CLL cases were in advanced stages (Binet classification) (seven patients stage C, two patients stage A, and three unknown). Fifty-three percent of AML cases showed overexpression of MDM-2 mRNA. Interestingly, no overexpression was detected in any of the four cases of CML or any of the four cases of the acute progranulocytic leukemia (Table 2).

Overexpression of MDM-2 mRNA in AML is associated with unfavorable chromosomal changes. When we divided the AML cases into three prognostic groups according to their chromosomal abnormalities, favorable, unfavorable, and standard (Table 3), we found that overexpression of MDM-2 was more closely associated with the unfavorable prognostic group. Favorable chromosomal changes include AML cases with chromosomal abnormalities such as t(8;21), t(15;17), inv (16), and t(9;11). The unfavorable group included −5, −7, +8, and other complex karyotypes. The standard risk group included patients with normal karyotype. Interestingly, 50% of the standard prognostic group demonstrated overexpression of MDM-2 mRNA. Work is in progress to evaluate whether MDM-2 overexpression in this group has any prognostic value.

Relatively high level of MDM-2 mRNA is expressed in leukemias. To evaluate the relative abundance of the MDM-2 mRNA, we compared the relative expression of MDM-2 mRNA in these leukemic samples with that of sar-

Fig 5. MDM-2 mRNA expression as detected by RT/PCR as compared with the β-actin mRNA. Representative samples of leukemia and myelodysplasia are shown.
coma samples that show a high level of gene amplification (Fig 6). MDM-2 mRNA expression in some cases of sarcoma that showed striking 50- to 100-fold gene amplification was not that different from the level of expression in some cases of leukemias.

**DISCUSSION**

Mutational inactivation of p53, a tumor suppressor gene, is relatively infrequent in hematologic neoplasms. However, intragenic mutation of p53 is not the only mechanism by which this gene is inactivated. Abnormalities in other cellular proteins that interact with the p53 protein and coregulate its function may play a significant role in its inactivation and in the progression of the neoplastic process. In contrast with viral oncoproteins, little is known about the cellular co-regulators of p53. Recent reports suggest that the MDM-2 oncoprotein is one of these cellular co-regulators. MDM-2 forms a complex with the mutant and wild-type p53 protein and suppresses its trans-activation function. The MDM-2 gene is amplified and overexpressed in a significant number of sarcomas, which suggests that it plays a role in the tumorigenic process of sarcomas.

We examined leukemic samples for gene amplification. All 48 cases that we examined showed no evidence of gene amplification (Fig 1). We investigated the levels of MDM-2 mRNA expression in various types of leukemias. We used the highly sensitive RT/PCR technique and co-amplified the MDM-2 and β-actin mRNAs. The use of a PCR-based technique enabled us to analyze small fragments of tissue. β-actin, used as an internal control, allowed us to compare the level of MDM-2 mRNA expression between various samples. The specificity of the amplification products was confirmed by restriction enzyme analysis of the amplification products (Fig 2). The quantitative accuracy of the co-amplification was confirmed by Northern analysis (Fig 3).

In addition, previous work by us and other investigators has demonstrated that the relative ratio of the amplification products using this coamplification technique reflects the exact relative ratio of the mRNAs.

Using RT/PCR, we detected MDM-2 mRNA overexpression in a significant number (34 of 64, 53%) of acute leukemias. As shown in Table 1, MDM-2 overexpression was detected in 9 of 12 cases of CLL (75%), 17 of 32 cases of AML (53%), 3 of 7 cases of ALL (42%), and 5 of 7 cases of MDS (71%). A few cases of chronic myeloid leukemia (CML) (four cases) and HCL (two cases) that we analyzed showed no evidence of MDM-2 overexpression. Table 2 shows the MDM-2 expression profile in AML and MDS cases as subclassified according to the French, American, and British classification. Interestingly, none of the four cases of APL showed overexpression of MDM-2 mRNA. To evaluate whether MDM-2 overexpression has any prognostic value, we subdivided the AML cases according to three cytogenetic prognostic groups: favorable, unfavorable, and standard. Apparently, overexpression was associated with unfavorable chromosomal abnormalities (Table 3). Of interest, we found that 50% of the standard group showed overexpression. Further follow-up of these patients may help us determine whether MDM-2 overexpression in this group may confer a different prognosis. The number of our AML cases was relatively small, and further studies are necessary to confirm the prognostic value of MDM-2 overexpression in various types of leukemias.

**Table 2. Overexpression of MDM-2 mRNA in Various Subtypes of AML and MDS**

<table>
<thead>
<tr>
<th>Leukemia</th>
<th>No. of Patients</th>
<th>Overexpression</th>
<th>Normal Expression</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>2</td>
<td>1 ++ 0</td>
<td>1</td>
<td>1/2</td>
</tr>
<tr>
<td>M1</td>
<td>13</td>
<td>3 3 1</td>
<td>6</td>
<td>7/13</td>
</tr>
<tr>
<td>M2</td>
<td>3</td>
<td>1 ++ 0</td>
<td>1</td>
<td>2/3</td>
</tr>
<tr>
<td>M3</td>
<td>4</td>
<td>0 0 0</td>
<td>3</td>
<td>1/4</td>
</tr>
<tr>
<td>M4</td>
<td>4</td>
<td>1 0 0</td>
<td>3</td>
<td>1/4</td>
</tr>
<tr>
<td>M5</td>
<td>1</td>
<td>1 0 0</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>4</td>
<td>2 1 0</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>1</td>
<td>0 0 1</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>CMML</td>
<td>2</td>
<td>0 1 1</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>RAEB</td>
<td>5</td>
<td>2 1 0</td>
<td>2/2</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CMML, chronic myelomonocytic leukemia; RAEB, refractory anemia with excess blasts.

**Table 3. AML Chromosomal Abnormality Groups and MDM-2 mRNA Expression**

<table>
<thead>
<tr>
<th>Cytogenetic Abnormality</th>
<th>Normal MDM-2 Expression (%)</th>
<th>MDM-2 Overexpression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAVORABLE (5 patients)</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>UNFAVORABLE (15 patients)</td>
<td>27</td>
<td>73</td>
</tr>
<tr>
<td>STANDARD (12 patients)</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

The correlation between cytogenetic abnormality and the level of MDM-2 mRNA expression was significant as determined by two-sided Fisher's exact test ($P = .016$).
must point out that most of our CLL patients had advanced-stage disease; therefore, the high frequency of overexpression (73%) in these patients may reflect more aggressive disease.

To examine the level of MDM-2 overexpression, we compared the level of MDM-2 mRNA in some leukemia samples with that in samples of sarcomas that showed significant MDM-2 gene amplification. To our surprise the level of MDM-2 expression in some leukemia samples was comparable to that in some samples of sarcomas that show 50-fold MDM-2 gene amplification. Because we did not detect gene amplification in leukemias, the mechanisms of MDM-2 overexpression in this group of tumors seems to be caused by alteration in either cis- or trans-acting factors that regulate MDM-2 expression.

In summary, we demonstrate that the MDM-2 oncprotein is deregulated in leukemias and mechanisms other than gene amplification are responsible for this overexpression. In addition, our data suggest that MDM-2 overexpression may indicate more aggressive disease. The demonstration by Finlay7 that MDM-2 protein can overcome the growth suppressive activity of p53 may explain the relatively low incidence of p53 mutation in hematologic neoplasms. However, because MDM-2 forms a complex with the mutant p53 in addition to the wild-type, it is possible that MDM-2 propagates the altered function of the mutated p53 protein, thus contributing to the tumorigenic process. Further studies to explore the basis for this overexpression may shed some light on the steps involved in the leukemogenic process.

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