The MDM2 Oncogene Overexpression in Chronic Lymphocytic Leukemia and Low-Grade Lymphoma of B-Cell Origin

By Takashi Watanabe, Tomomitsu Hotta, Atsushi Ichikawa, Tomohiro Kinoshita, Hirokazu Nagai, Toshiki Uchida, Takashi Murate, and Hidehiko Saito

The expression of the murine double minute-2 (MDM2) gene, the product of which binds to and inactivates p53, was studied in 60 patients with B-cell chronic lymphocytic leukemia (B-CLL) or non-Hodgkin’s lymphoma (B-NHL). Northern blot analysis showed that the level of MDM2 gene expression was low in normal human B-cells, whereas 17 of the patients (28.3%) with B-CLL or NHL had more than 10-fold higher levels of MDM2 gene expression than that observed in normal B-cells. Immunohistochemical analysis confirmed MDM2 overexpression at the cellular protein level. MDM2 gene overexpression was found more frequently in patients with the low-grade type of lymphoma (56.5%) than in those with intermediate/high-grade types (10.8%) (P<0.001). Moreover, MDM2 overexpression was found significantly more frequently in patients at advanced clinical stages. Simultaneous analysis of p53 gene mutation showed that three patients had both MDM2 gene overexpression and p53 gene mutation. The results of the present study suggest that MDM2 gene overexpression may play an important role in the tumorigenicity and/or disease progression of CLL and low-grade lymphomas of B-cell origin.

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A cellular phosphoprotein (apparent molecular mass, 90 kD), capable of forming specific complexes with p53, has recently been characterized and identified as a product of the murine double minute-2 (MDM2) gene. This gene was originally identified and cloned on the basis of its amplification in a highly tumorigenic derivative of NIH-3T3 cells. Although the precise biochemical nature of the product remains to be determined, the deduced amino acid sequence contains a putative nuclear localization signal, as well as two zinc finger proteins, which suggests that it is a DNA binding protein. This gene also contains an acidic domain often found in transactivators and is evolutionarily conserved, as is the p53 gene. These findings suggest that this gene provides a fundamental cellular function. On the other hand, an excess of MDM2 protein can abrogate transcriptional activation by the transactivated wild-type p53. Hence, the MDM2 product has been implicated as a functionally negative regulator of p53, the overexpression of which may mimic the effects brought about by mutational inactivation of the p53 gene. High levels of the MDM2 gene product can also bind and inactivate the p53 protein in ways similar to those shown by some DNA viral gene products, such as simian virus 40 T antigen, adenovirus E1B, and human papil- loma virus E6. However, the MDM2 protein can bind to both mutant- and wild-type p53 proteins. Thus, MDM2 gene overexpression may provide a mechanism of p53 inactivation that differs from p53 mutations in human cancers. The human homologue of the murine MDM2 gene has been cloned, and more than one third of 47 human sarcomas examined were found to exhibit amplification of the MDM2 gene.

Overexpression of this gene has been shown to confer tumorigenic properties on transfected murine cells, but thus far overexpression of human MDM2 gene has been reported only in sarcoma and breast carcinoma cell lines. Mutations of the p53 gene have been reported frequently in several types of cancers. However, in hematologic disorders, this mutation appears to be limited to blastic crisis of chronic myelogenous leukemia, some acute lymphocytic leukemias and lymphomas, and a few cases of myelodysplastic syndrome. The human MDM2 gene is localized to chromosome 12q13-14, chromosome 12 appears to be altered in many patients with sarcoma and also in some with chronic lymphocytic leukemia of B-cell origin (B-CLL) (21% to 62%) or low-grade lymphoma (8% to 25%). We speculated that MDM2 gene alteration and/or overexpression may play a role in the tumorigenicity of B-cell lymphoproliferative disorders. In the present study, we performed Southern blot analysis in patients with B-CLL and B-cell non-Hodgkin’s lymphoma (B-NHL) to examine whether this gene was amplified or rearranged, and we performed Northern blot analysis and immunohistochemical analysis to examine the expression of the gene. We simultaneously analyzed p53 gene alterations of the same specimens by polymerase chain reaction–mediated single-strand conformation polymorphism (PCR-SSCP) and by a direct sequencing technique.

MATERIALS AND METHODS

Patient characteristics. We analyzed 60 consecutive patients, 17 with B-CLL/small-cell lymphocytic lymphoma (SCL) and 43 with B-cell lymphoma other than SCL (follicular type, n = 8; diffuse type, n = 35), who were admitted to our hospital between April 1986 and March 1993, including nine patients with CLL who were treated in our affiliated hospitals, and whose RNA was available for Northern blot analysis. The diagnosis of CLL was based on the criteria proposed by the International Workshop on CLL, and three of the 17 B-CLL patients were histologically diagnosed as having SCL at lymph node biopsy. Histologic and surface marker studies using an immunohistochemical technique showed B-cell phenotype in all examined patients. The characteristics of these patients are listed in Table 1. Fifty of 60 patients were untreated, but 10 patients had received a variety of prior treatments. Patients with CLL were classified according to a revised prognostic staging system as proposed by the International Workshop on CLL, which encompasses features of both the Rai and the Binet systems.
Table 1. Characteristics of 60 Patients With B-CLL or B-NHL

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>51</td>
</tr>
<tr>
<td>Median</td>
<td>15-86</td>
</tr>
<tr>
<td>Range</td>
<td>40/20</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>50/10</td>
</tr>
<tr>
<td>Clinical stage of CLL</td>
<td></td>
</tr>
<tr>
<td>A (0)</td>
<td>4</td>
</tr>
<tr>
<td>B (I)</td>
<td>1</td>
</tr>
<tr>
<td>C (II)</td>
<td>3</td>
</tr>
<tr>
<td>D (III)</td>
<td>4</td>
</tr>
<tr>
<td>E (IV)</td>
<td>5</td>
</tr>
<tr>
<td>Clinical stage of NHL (except SCL)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>9</td>
</tr>
<tr>
<td>III</td>
<td>11</td>
</tr>
<tr>
<td>IV</td>
<td>23</td>
</tr>
<tr>
<td>Histopathology for NHL (modified Working Formulation)</td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td></td>
</tr>
<tr>
<td>Small lymphocytic (including CLL)*</td>
<td>17</td>
</tr>
<tr>
<td>Follicular small cleaved</td>
<td>4</td>
</tr>
<tr>
<td>Follicular mixed</td>
<td>2</td>
</tr>
<tr>
<td>Intermediate/High grade</td>
<td></td>
</tr>
<tr>
<td>Follicular large</td>
<td>2</td>
</tr>
<tr>
<td>Diffuse small cleaved</td>
<td>7</td>
</tr>
<tr>
<td>Diffuse mixed</td>
<td>4</td>
</tr>
<tr>
<td>Diffuse large</td>
<td>23</td>
</tr>
<tr>
<td>Diffuse large, immunoblastic</td>
<td>1</td>
</tr>
</tbody>
</table>

The staging system of the International Workshop on CLL was applied to 17 patients with CLL/SCL. Roman numerals in parentheses represent the Rai classification.

* The histopathologic category of small lymphocytic consisted of three small lymphocytic lymphomas and 14 CLLs diagnosed according to the clinical criteria.

Working Formulation Classification was applied for histologic classification of the lymphomas. DNA and RNA samples were extracted from peripheral blood lymphocytes (PBL), lymph nodes (LN), extranodal tumors, or pleural effusions filled with lymphoma cells. Mononuclear cells isolated by ficoll-paque (Pharmacia, Piscataway, NJ) gradient centrifugation were substituted for PBL. LN and extranodal tumors were obtained at biopsy performed for diagnosis after informed consent was obtained.

Control specimens. As normal controls, we used DNA and RNA obtained from the PBL of healthy volunteers, from the spleen excised from a patient with gastric cancer, and from the LN of patients with reactive lymphangitis. The excised spleen was depleted of monocytes/macrophages by incubation at 37°C in 1% silica particles (KAC-2; JIMRO, Takasaki, Japan), and subsequently mononuclear cells were isolated by ficoll-paque gradient centrifugation. B cells were then enriched by depleting T cells through rosetting with sheep erythrocytes (Kyokuto, Tokyo, Japan), yielding splenic B cells. Then enriched B cells were then enriched by depleting T cells through rosetting with sheep erythrocytes (Kyokuto, Tokyo, Japan), yielding splenic B cells.

MDM2 probe. The MDM2 probe was produced by PCR, using cDNA from normal human PBL as a template. The DNA was amplified with the following primers: 5'-CTGTGTGTCGGAAAGAT-3' and 5'-CTGCTACTGCTTCTTTCA-3'. The 911-bp MDM2 probe spanned nucleotides -273 to +638 of the published cDNA sequence of the human MDM2 gene. The oligonucleotide primers used were synthesized by the phosphoramid method with a 391 DNA synthesizer (Applied Biosystems, Foster City, CA), and were purified with OPC columns (Applied Biosystems).

Preparation of DNA and RNA. DNA was prepared by the method reported previously. All tissues and cell samples used were homogenized in guanidine isothiocyanate, and total cellular RNA was extracted as described previously. After phenol and chloroform extraction, RNA was precipitated in ethanol for storage.

Southern blotting. Five micrograms of each DNA was digested with several different restriction enzymes such as EcoRI, BamHI, HindIII, and PvuII (Boehringer Mannheim, Mannheim, Germany), and then separated by 1% agarose gel electrophoresis and transferred to nylon membranes (GeneScreen Plus, Biotechnology Systems, Boston, MA) as described previously. The membranes were hybridized to the human MDM2 probe; hybridization was performed as previously described.

Northern blot analysis. Denatured RNA samples (10 μg total cellular RNA per lane) were separated by electrophoresis in 3-(N-Morpholino)propanesulfonic acid formaldehyde 1% agarose gels, transferred to Hybond N membranes (Amersham, Buckinghamshire, UK), and analyzed by Northern blot hybridization. Transfer and hybridization were performed as described previously, except that Quickhyb (Stratagene, La Jolla, CA) was used for hybridization. The quality of the RNA was verified by noting the integrity of the 28S and 18S ribosomal RNA stained by ethidium bromide. Hybridization was performed to the human MDM2 probe, and all blots were boiled and subsequently rehybridized to a β-actin probe, which provided a qualitative and quantitative control for RNA preparations.

Quantification of expression and amplification. To determine the levels of expression and amplification of the MDM2 gene, we exposed the blots to phosphor imaging plates (Imaging plate BAS III, Fuji, Japan), which were subsequently studied with a laser image analyzer (FUJIX BAS2000, Fuji, Japan). We then calculated the intensity of phosphostimulated luminescence (PSL) of the bands on the blots, since PSL is proportional to the absorbed radioactive energy. These methods have been described in detail elsewhere. Concerning gene amplification, the signal of each specimen was compared with that of the control splenic B cells.

Immunohistochemical analysis. Immunohistochemical analysis was performed on cytosin preparations, using the IgG2 mouse monoclonal antibody (MoAb) IF2 (Oncogene Science, Uniondale, NY), which specifically recognizes the terminal epitope of human MDM2 protein. We made the cytoxin preparations with cytosin 2 (Shandon, Pittsburgh, PA), either immediately after sampling or thawing cell suspensions of peripheral blood and LN, which were then washed with 20% fetal calf serum and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen and stored at -80°C. The slides were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C following in 100% ethanol for more than 2 hours at -20°C, and washed in PBS before labeling. After endogenous peroxidase was quenched by a 2-hour incubation in 0.5% hydrogen peroxide, the slides were incubated first with goat normal serum for 1 hour, and finally with the IgG2, mouse IgG2 monoclonal antibody (MoAb) IF2 (OncoGene Science, Uniondale, NY), which specifically recognizes the terminal epitope of human MDM2 protein. We then calculated the intensity of phosphostimulated luminescence (PSL) of the bands on the blots, since PSL is proportional to the absorbed radioactive energy. These methods have been described in detail elsewhere. Concerning gene amplification, the signal of each specimen was compared with that of the control splenic B cells.
RESULTS

MDM2 gene expression in normal B cells. To determine the extent of MDM2 gene expression in normal and reactive lymphocytes, we performed Northern blot analysis of the PBL of healthy volunteers, the splenic B cells, and the reactive B cells separated from the LN of four patients with reactive lymphangitis. These cells showed a single 5.5-kb transcript, in accordance with previous reports, but their bands were all faint. The level of the transcript in splenic B cells was as low as that of the PBL from healthy volunteers. However, levels in reactive lymphangitis LN were 1.2- to 1.8-fold higher than those in PBL or splenic B cells (Fig 1A).

Overexpression of the MDM2 gene in neoplastic B cells. To our knowledge, no detailed data on the expression of the MDM2 gene in human lymphoid cells have been reported to date. We first performed a preliminary study in which we examined the levels of MDM2 RNA in several human cell lines; we found that the MDM2 gene was overexpressed in MT-2 cells, a T-cell line derived from human cord leukocytes by cocultivation with T cells infected with human T-cell leukemia virus type I (HTLV-I). The level of MT-2 RNA expression was 15- to 20-fold higher than that in normal human B cells (Fig 1B). We therefore used this cell line as a positive control.

We next investigated whether the MDM2 gene was expressed in neoplastic B cells of NHL (Fig 1B) and CLL (Fig 2). The expression of this gene in the 60 patients varied; 17 of 60 patients with NHL or CLL (28.3%) were found to overexpress the gene at levels more than 10-fold higher than that observed in normal splenic B cells, and 19 showed twofold to fivefold higher expression than that in normal B cells.

Amplification or rearrangement of the MDM2 gene. To examine whether overexpression of the MDM2 gene resulted from its amplification or its alteration, we analyzed the genomic DNA of 60 patients by Southern blot analysis. However, none of the patients who showed MDM2 gene overexpression appeared to have either amplification or rearrangement of the MDM2 gene.

MDM2 expression at the cellular protein level. To evaluate MDM2 expression at the cellular protein level, we first examined proteins derived from MT-2, the cell line which was shown to overexpress the MDM2 gene by Northern blot analysis. A strong exclusively nuclear signal was observed in MT-2 cells immunostained with the IF-2 MoAb (Fig 3A). The nuclear localization of MDM2 is consistent with previous studies of mouse cells and the previous report that human MDM2 contains a nuclear localization signal. This MoAb was then used to stain the specimens in which MDM2 RNA was analyzed. Those specimens that showed levels of MDM2 RNA lower than 10 times those of normal B cells showed no or very weak staining of lymphoma cells (Fig 3B). We examined almost all specimens in which levels of MDM2 RNA were more than 10-fold higher than those of normal splenic B cells, and found that leukemic or lymphoma cells with strong atypical nuclei (ie, irregular shaped or accompanied with clear nucleoli) stained strongly in all cases (Fig 3C).
groups of the Working Formulation classification. Overexpression of the MDM2 gene was defined as levels more than 10-fold higher than those in normal human B cells; of 17 patients with MDM2 gene overexpression, eight were classified as B-CLL/SCL (47.1%) and nine were B-NHL non-SCL (20.9%); five follicular type (62.5%) and four diffuse type (11.4%). We examined the relationship between MDM2 overexpression and the subtypes of mature B-cell neoplasm;
**Table 2. Overexpression of the MDM2 Gene in Relation to Subgroups of the Modified Working Formulation Classification**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>MDM2 Gene Overexpression*</th>
<th>Low Grade</th>
<th>Intermediate/High Grade</th>
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<tbody>
<tr>
<td>+</td>
<td>13 (56.5%)†</td>
<td>4 (10.8%)</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>10</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>37</td>
<td></td>
</tr>
</tbody>
</table>

* "+" for MDM2 gene overexpression indicates levels more than 10-fold higher than those in normal splenic B cells; "−" indicates levels of MDM2 expression lower than 10 times those observed in normal splenic B cells.

† MDM2 overexpression was found in 13 of 23 patients with low-grade type (56.5%) and in four of 37 patients with intermediate/high-grade type of lymphoma (10.8%). This difference was highly significant (P = .001).

**DISCUSSION**

The widespread expression of the MDM2 gene in many murine and human tissues was investigated, and it was shown that the level of expression appeared to be different in different tissues. However, human lymphoid tissues were not examined in these previous studies. The present study showed that human splenic B cells, as well as normal PBL, expressed low levels of MDM2. In addition, the level of expression was slightly less than twofold enhanced in reactive B cells relative to that in normal splenic B cells.

The levels of gene expression in the specimens from NHL and CLL patients were various. As there were no previous basic data concerning B cells, it remains unknown how much expression of the MDM2 gene in B cells is morbid state. It has only been demonstrated that the MDM2 gene induced tumorigenicity in MDM2 transfectants when overexpressed at levels 10- to 15-fold higher than those in NIH-3T3 cells before transfection. Therefore, we speculated that at least those B cells that had levels more than 10-fold higher than those of control B cells might be tumorigenic, and thus defined overexpression of the MDM2 gene tentatively as levels in excess of 10 times those in normal human B cells. If significant proportions of normal cells were present in the specimens, the levels of MDM2 RNA may be underestimated, so MDM2 might play a role in some of the 19 patients who showed twofold to fivefold higher MDM2 expression than normal B cells. A strong exclusively nuclear immunopositive signal was observed with the MoAb IF2 in leukemic or lymphoma cells in all cases in which levels of MDM2 RNA were more than 10-fold higher than those of normal splenic B cells. However, strong staining of lymphoma cells was not found in those cases in which levels of MDM2 transcript were lower than 10 times those of normal B cells. From the results of immunohistochemical analysis, levels of MDM2 RNA correlated well with levels of MDM2 protein. Thus, we hypothesized that overexpressed MDM2 protein may bind to p53, and consequently play a role in the pathogenesis and or disease progression of patients who overexpress the MDM2 RNA via inactivation of p53.

Figure 3C shows that most leukemic cells are MDM2-positive; however, it shows that a fair number of cells do not stain with the anti-MDM2 antibody. According to a morphologic examination, it may be true that some leukemic cells were very weak or negative in MDM2 expression. We can provide two reasons for these negative cells. First, the previous report showed that MDM2 levels increased in the late G1-phase of the cell cycle, and so, if malignant cells have the diversity in their cell cycles, all malignant cells may not have to be strongly stained with the antibody. Second, we
found leukemic cells with strong atypical nuclei stained with the IF-2 MoAb. The tendency that nuclei of tumor cells with strong atypia were prone to be stained was seen in other lymphoma specimens in which MDM2 RNA was overexpressed. In accordance with these observations, we found that cells that stained for strongly positive for MDM2 might tend to appear and increase in CLL and low-grade lymphoma at advanced stages.

Why MDM2 gene overexpression was more frequently observed in the low-grade type of B-NHL (including CLL) than in the intermediate-/high-grade types is unknown. While a previous report has shown that the MDM2 gene was amplified five-fold to 50-fold in sarcomas, none of our 60 patients showed sufficient amplification or rearrangement to explain the more than 10-fold increase in expression of the MDM2 gene. We speculate that the observed abnormal expression of the MDM2 gene may result from aberrations in some as yet undefined regions that control the expression, or from aberrations in some undefined protein that regulates the expression of the MDM2 gene.

MDM2 overexpression was detected more frequently in patients in advanced clinical stages of these diseases (stage C of CLL and stage IV of NHL). Our data are consistent with previous reports that showed p53 alterations were found more frequently in patients at advanced clinical stage of the disease, and p53 and MDM2 presumably affect the same pathway in B-cell neoplasms. The proportion of neoplastic B cells in the PBL of patients with B-CLL was 70% to 90%, and there were no differences between groups A/B versus C. Thus, it was clear that the difference in expression did not result from the proportion of tumor cells in PBL. Five of 17 patients who showed MDM2 overexpression had received previous treatments: all four patients with diffuse lymphoma and one of eight with CLL. In these cases, we were not able to exclude the influence of chemotherapy, which could cause DNA damage and subsequent alterations in the pathway of p53 to maintain genomic stability.

As the result of high levels of MDM2 gene expression is known to be functionally synonymous with mutant-type p53 protein, and the p53 mutation is rarely observed in hematologic malignancies, we expected to find MDM2 overexpression in patients who lacked p53 alterations. A previous study showed that none of five sarcomas examined with MDM2 amplification had p53 gene mutations. However, in the present study, of 17 patients in whom we found MDM2 overexpression, 14 had the wild-type and three the mutant-type p53 gene. Since different mutant alleles of p53 have different biologic properties, it is possible that MDM2 overexpression could also confer an added selective advantage on tumors with weaker mutant alleles, which do not bind to the cellular heat-shock protein, hsc70, and which are less efficient at forming transformed foci in culture. Indeed, one of three patients with both MDM2 overexpression and mutant-type p53 had a point mutation at codon 248, which was reported as a weaker mutant, as well as those for such residues as 273 and 281.

The results of the present study showed that the MDM2 gene was overexpressed in patients with some particular types of B-cell malignancies. It appears that MDM2 gene overexpression may play an important role in tumorigenicity and/or in disease progression in this type of neoplasia. However, the precise molecular mechanism underlying this overexpression is still unclear. In addition, whether MDM2 overexpression is a prognostic factor is now being investigated. Further studies will be needed before the clinical implications of these findings become clear.

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