RAPID COMMUNICATION

Inactivation of Multiple Tumor-Suppressor Genes Involved in Negative Regulation of the Cell Cycle, MTS1/p16\textsuperscript{INK4A}/CDKN2, MTS2/p15\textsuperscript{INK4B}, p53, and Rb Genes in Primary Lymphoid Malignancies

By Akira Hangaishi, Seishi Ogawa, Nobutaka Imamura, Shuichi Miyawaki, Yasusada Miura, Naokuni Uike, Chihiro Shimazaki, Nobuhiko Emi, Kunihiko Takeyama, Shinshu Hiroawata, Nanuo Kamada, Yutaka Kobayashi, Yoshinobu Takimoto, Teroo Kitani, Keisuke Toyama, Shigeki Ohtake, Yoshih Paka, Ryuzo Ueda, and Hisamaru Hirai

It is now evident that the cell cycle machinery has a variety of elements negatively regulating cell cycle progression. However, among these negative regulators in cell cycle control, only 4 have been shown to be consistently involved in the development of human cancers as tumor suppressors: Rb (Ratinoastoma susceptibility protein), p53, and two recently identified cyclin-dependent kinase inhibitors, p16\textsuperscript{INK4A}/MTS1 and p15\textsuperscript{INK4B}/MTS2. Because there are functional interrelationships among these negative regulators in the cell cycle machinery, it is particularly interesting to investigate the multiplicity of inactivations of these tumor suppressors in human cancers, including leukemias/lymphomas. To address this point, we examined inactivations of these four genes in primary lymphoid malignancies by Southern blot and polymerase chain reaction-single-strand conformation polymorphism analyses. We also analyzed Rb protein expression by Western blot analysis. The p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} genes were homozygously deleted in 45 and 42 of 230 lymphoid tumor specimens, respectively. Inactivations of the Rb and p53 genes were 27 of 91 and 9 of 173 specimens, respectively. Forty-one (46.1%) of 91 samples examined for inactivations of all four tumor suppressors had one or more abnormalities of these four tumor-suppressor genes, indicating that dysregulation of cell cycle control is important for tumor development. Statistical analysis of interrelations among impairments of these four genes indicated that inactivations of the individual tumor-suppressor genes might occur almost independently. In some patients, disruptions of multiple tumor-suppressor genes occurred; 4 cases with p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, and Rb inactivations; 2 cases with p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, and p53 inactivations; and 1 case with Rb and p53 inactivations. It is suggested that disruptions of multiple tumor suppressors in a tumor cell confer an additional growth advantage on the tumor.

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for tumor-suppressor genes located on human chromosome 9p21,25 it has now become clear that they are among the genes most frequently altered in human cancers.26,27 Because these two INK family CDKIs and p53 seem to depend their actions of cell cycle regulation upon intact Rb protein,29 it is particularly interesting how inactivations of these four tumor suppressors occur in human cancers. Although there are several reports as to multiple tumor-suppressor gene disruptions in diverse tumors including leukemia,34-36 inactivations of these four cell cycle-related tumor-suppressor genes have not been fully investigated in a large series of patients with leukemias and/or lymphomas. Therefore, in this study, we examined incidences and correlations of inactivations of these four tumor-suppressor genes in primary lymphoid malignancies by Southern blot, polymerase chain reaction—single-strand conformation polymorphism (PCR-SSCP), and sequencing analyses. We also evaluated loss of Rb protein expression by Western blot analysis.

MATERIALS AND METHODS

Patients and samples. Sixty-four medical centers and hospitals in Japan participated in this collaborative study promoted by the Japan Leukemia Study Group of the Ministry of Health and Welfare, Japan. A total of 230 patients with primary lymphoid malignancies admitted to these collaborating centers and hospitals between April 1988 and April 1995 were included in this study. One hundred seventy-nine of the 230 patients were previously analyzed for deletions of the p16INK4A and/or p15INK4B genes,29 and an additional 51 patients were newly registered and analyzed together in this study. Diagnoses of the patients were acute lymphocytic leukemia (ALL) in 47 patients, chronic lymphocytic leukemia (CLL) in 25 patients, prolymphocytic leukemia (PLL) in 4 patients, non-Hodgkin’s lymphoma (NHL) in 47 patients, multiple myeloma (MM) in 6 patients, and mixed-lymphocytic leukemia (mixed AL) in 10 patients. The preparation of samples and collection of patients’ clinical and cytogenetic information were the same as previously described.38

Southern blot analysis. Procedures for Southern blot analysis were essentially identical to those previously described.70 HindIII (for p16INK4A, p15INK4B, and Rb) or EcoRI (for p53) endonucleases were used for DNA digestion. We also used EcoRI and BamHI endonucleases to confirm homozygous deletion of the Rb gene. The same probes were used for detecting the p16INK4A and p15INK4B genes and the AML1 gene, the latter being used for calibrating DNA amounts loaded.29 Filters were subjected to deproofing procedures and rehybridized with different probes for detection of the Rb gene. The 5' and 3' cDNA probes of Rb were kindly provided by Dr Rei Takahashi (Kyoto University, Kyoto, Japan). We amplified the exons 5 through 9 of the p53 gene (see below) and used them collectively as p53 probes. Gene dosage of the p16INK4A, p15INK4B, p53, and Rb genes was determined by visual inspection of autoradiograms compared with AML1 signals used as internal controls. For an objective estimation of gene dosage to classify patients as having homozygous or hemizygous deletions, radioactivities of the interest bands were standardized by using those of AML1 signals as controls. For an objective estimation of gene dosage to classify patients as having homozygous or hemizygous deletions, radioactivities of the interest bands were standardized by using those of AML1 signals as controls.

RESULTS

Structural alterations of the Rb gene and loss of Rb protein. A total of 189 samples were analyzed by Southern blot analysis using HindIII endonuclease and two Rb cDNA probes. Structural alterations of the Rb gene were observed in 4 cases: 2 with ALL (patients no. 77 and 219; Fig 1, lanes 9 and 2), 1 with NHL (patient no. 246; data not shown), and 1 with mixed AL (patient no. 75; Fig 1, lane 6; Table 1). Patient no. 246 showed a rearrangement. Patients no. 75, 77, and 219 had homozygous deletions in the 3' portion of the Rb gene (Fig 1). Gene alterations of these four patients were confirmed by Southern blot analysis using EcoRI and BamHI endonucleases (data not shown). Expression of Rb protein was also evaluated, because loss of Rb protein was reported to frequently occur with structurally intact Rb gene in Southern blot analysis.39,41 We examined 89 patients whose mononuclear cell samples were available and contained more than 70% of tumor cells. Representative blots were shown in Fig 2. Twenty-five of the 89 samples had low or remarkably low levels of Rb protein expression with normal expression levels of Hsp70 expression (Table 1). In total, inactivations of the Rb gene were found in 27 patients (4 with structural alteration of the Rb gene, 25 with loss of Rb protein, and 2 with both structural alteration and loss of Rb protein). Loss of Rb expression seemed more frequent in ATL and mixed AL, (5 of 10 and 2 of 4, respectively) than in the others (9 of 27 ALL, 5 of 21 CLL, 4 of 23 NHL, and 0 of 4 PLL), but statistically, it was not significant.

Mutations and structural alterations of the p53 gene. In
Southern blot and PCR-SSCP analyses of the p53 gene, aberrations of the gene were detected in 15 of 173 (8.7%) patients, i.e., 10 of 101 patients with ALL (9.9%), 2 of 21 patients with CLL (9.5%), 2 of 4 patients with PLL (50%), 1 of 27 NHL (3.7%), and none of 14 ATL, 1 MM, and 5 mixed AL (Table 1). Among these 15 patients, 13 patients showed polymorphic bands in PCR-SSCP analysis (Table 2). PCR products showing altered migrating bands were subcloned into the plasmid vectors and at least 4 independent clones were sequenced in both directions (Fig 3). Results were summarized in Table 2. Ten patients had missense mutations, 2 had a silent mutation, 1 had a 3-base insertion, and the other 2 had a 13-bp deletion or a 2-bp deletion, both of which caused a frameshift of the coding sequence. Patients no. 269 and 338 had both a small deletion and a nucleotide substitution on one allele and a large deletion on another allele.

Inactivations of the p53 gene were found in nine cases with aberrations of both alleles, one allele mutation, and another allele deletion. Two patients lost one allele of the p53 gene and the other allele remained intact. In four patients, a point mutation of the gene was detected in one allele and the other allele was normal. There were no cases with homozygous loss of the gene.

Deletions of the p16\(^{\text{INK4A}}\) and p15\(^{\text{INK4B}}\) genes. Deletion analysis of the p16\(^{\text{INK4A}}\) and p15\(^{\text{INK4B}}\) genes in 179 of 230 patients was previously published.\(^3\) The results of the deletion analysis including additional 51 patients were summarized in Table 1. To be brief, 45 and 42 of 230 patients had homozygous deletions and/or rearrangement of the p16\(^{\text{INK4A}}\) and p15\(^{\text{INK4B}}\) genes, respectively (Table 1). Hemizygous deletions or rearrangements were found in 22 patients for the p16\(^{\text{INK4A}}\) gene and in 20 patients for the p15\(^{\text{INK4B}}\) gene. Homozygous loss of the p16\(^{\text{INK4A}}\) and p15\(^{\text{INK4B}}\) genes occurred in 32 and 29 of 117 cases with ALL, respectively. In the other type of lymphoid tumors, homozygous deletions of the p16\(^{\text{INK4A}}\) and p15\(^{\text{INK4B}}\) genes were found in the same cases: 5 of 21 cases with ATL, 2 of 25 patients with CLL, 6 of 47

### Table 1. p16, p15, p53, and Rb Aberrations in Lymphoid Malignancies

<table>
<thead>
<tr>
<th>Disease</th>
<th>Deletion of the p16 Gene</th>
<th>Deletion of the p15 Gene</th>
<th>Deletion of the Rb Gene</th>
<th>Loss of the Rb Protein</th>
<th>Aberration of the p53 Gene</th>
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<td>43 (29)/117</td>
<td>2/98</td>
<td>9/27</td>
<td>10 (5)/101</td>
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<td>6 (5)/21</td>
<td>0/19</td>
<td>5/10</td>
<td>0/14</td>
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<td>2 (2)/25</td>
<td>0/21</td>
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<td>2 (1)/21</td>
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<tr>
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<td>0 (0)/4</td>
<td>0/4</td>
<td>0/4</td>
<td>2 (2)/4</td>
</tr>
<tr>
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<td>8 (6)/47</td>
<td>1/35</td>
<td>4/23</td>
<td>1 (1)/27</td>
</tr>
<tr>
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<td>0 (0)/8</td>
<td>0/5</td>
<td>0/0</td>
<td>0/1</td>
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<tr>
<td>Mixed AL</td>
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<td>3 (0)/10</td>
<td>1/7</td>
<td>2/4</td>
<td>0/5</td>
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<td>Total</td>
<td>67 (45)/230</td>
<td>62 (42)/230</td>
<td>4/189</td>
<td>25/89</td>
<td>15 (9)/173</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate homozygous deletions of p16 or p15, or mutation in one allele and loss of another allele of the p53 gene.
cases with NHL, and 0 of 6 MM and 10 mixed AL cases. Forty-one of 45 patients with homozygous deletions of the p16\textsuperscript{INK4A} gene also had those of the p15\textsuperscript{INK4B} gene, and 41 of 42 patients with homozygous deletions of the p15\textsuperscript{INK4B} gene had those of the p16\textsuperscript{INK4A} gene. Mutations of exons 1 and 2 of the p16\textsuperscript{INK4A} were also studied in 45 patients, including 11 with hemizygous deletions of the gene by PCR-SSCP and direct sequencing analyses, but we found none.\textsuperscript{38}

Interrelations among inactivations of the p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, Rb, and p53 genes in primary lymphoid malignancies. Patients with aberrations of p16, p15, Rb, or p53 were summarized in Table 3. To analyze interrelations among inactivations of the four tumor suppressors, the registered 230 patients were divided into four subgroups, in every one of which a particular combination of the four genes was completely evaluated. All 230 patients were examined for deletions of both the p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} genes. Strongly expected from a short distance between the two genes in chromosome 9p21, both genes were simultaneously deleted in most of the cases (41 of 230 cases). Thus, in the following analysis, we treated both genes (p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B}) as a group. Ninety-one patients could be evaluated as to the status of the p16\textsuperscript{INK4A} (and p15\textsuperscript{INK4B}) genes, and the Rb gene (Table 4). Four patients (no. 77, 132, 219, and 254) had inactivations of all three genes. All four patients had homozygous deletions of both the p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} genes (Fig 4). Two of the four patients (no. 219 and 77) lost the Rb gene homozygously (Fig 1, lanes 2 and 9). The other two patients

![Fig 2. Western blot analysis of the Rb protein. Rb protein is observed as broad bands, around 105 kD, representing diffuse levels of phosphorylation. The lower panels show expression of Hsp70 as a control for the amounts and integrity of protein. Lane 1, HL60 (positive control); lane 2, Hep3B (negative control); lanes 3 through 10, patients no. 317, 327, 219, 132, 162, 336, 251, and 254, respectively.](image)
INACTIVATION OF MULTIPLE TUMOR SUPPRESSOR GENE

had intact Rb genes in Southern blot analysis, but did not show Rb protein expression (patients no. 132 and 254; Fig 2, lanes 6 and 10). In addition, another patient (no. 75) had homozygous deletion of the Rb gene and hemizygous deletions of both the p16\(^{INK4A}\) and p15\(^{INK4B}\) genes (data not shown). Thirty-eight of 91 patients had inactivations of one or both of the p16\(^{INK4A}\) and Rb genes (Table 4).

One hundred and seventy-one patients could be analyzed for deletions of the p16\(^{INK4A}\) (and p15\(^{INK4B}\)) gene and for deletions and/or mutations of the p53 gene (Table 5). In this subset of patients, 2 ALL patients both had homozygous deletion of the p16\(^{INK4A}\) (and p15\(^{INK4B}\)) gene and mutations of the p53 gene on one allele and loss of the other p53 allele (patients no. 29 and 489; Table 5 and Figs 3 and 4). Among 91 samples in which inactivations of both p53 and Rb genes were examined, one ALL case (patient no. 336) had loss of Rb protein expression (Fig 2, lane 8) and a p53 mutation with loss of the other allele (Table 2 and Fig 3). Of the 91 patients, 31 (34.1%) had p53 and/or Rb inactivations (Table 6). In this study, 91 patients of primary lymphoid malignancies were evaluated for all four genes, and 41 (45.1%) showed one or more inactivations of these three (four) tumor-suppressor genes, the p16\(^{INK4A}\) (and p15\(^{INK4B}\)), p53, and Rb genes (Table 3). We could not find any cases with abnormalities of all three (four) genes. Cochran-Mantel-Haenszel Statistics stratified by the status of p53 shows no obvious interrelations exist among inactivations of the p16 and Rb genes (\(P = .718\)). There are also no correlation between p16 and p53 (\(P = .307\)) and between Rb and p53 (\(P = .608\)).

DISCUSSION

We examined the incidences and manners of inactivations of the four tumor-suppressor genes in a set of patients with primary lymphoid malignancies to clarify roles of inactivation of negative regulators of G1 phase in the cell cycle. Inactivation of each one of these four tumor-suppressor genes was commonly observed in lymphoid tumors.\(^6,16,42-47,48\) The incidences of inactivations of individual tumor suppressor genes were comparable to those previously reported, except that the incidence of Rb inactivation in our analysis was higher than ever reported.\(^49\) Homozygous deletions of the p16\(^{INK4A}\) and p15\(^{INK4B}\) genes and loss of Rb protein expression are among the most frequently observed abnormalities in lymphoid tumors (45/230 [19.6%], 42/230 [18.3%], and 25/89 [28.1%], respectively; Table 1). Up to 45% of the patients evaluated for all four genes had inactivations of at least one of these genes, indicating that unlocking the brakes in the G1 checkpoint may be important for development of lymphoid tumors.

Disruptions of multiple tumor suppressors were observed in 7 of 230 patients, suggesting that inactivations of multiple tumor-suppressor functions in a cell are thought to confer some additional growth advantages upon the tumor cells. A number of other reports also showed multiple mutations of the tumor-suppressor genes in a variety of tumors. p16\(^{INK4A}\) gene aberrations with mutations of the p53 genes were observed in esophageal cancers,\(^50\) bladder cancers,\(^51\) and pancreatic adenocarcinomas.\(^52\) Alterations of the Rb and p53 genes were also reported to occur frequently in various types of human cancers.\(^54,55\) Williams et al\(^{56}\) reported that mice deficient for both Rb and p53 showed a faster rate of tumor development and a shorter survival rate than mice deficient only in the Rb or p53 gene. Mutations in the Rb and p53 genes can cooperate in the transformation of certain cell types in the mouse. Although p53 is implicated in cell cycle regulation through induction of a potent G1 CDKI, p21\(^{Cip1}\), it is also known to have roles in DNA repair and in apoptosis.\(^23,24,55\) Thus, inactivation of the p53 gene in addi-
<table>
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<th>Patient No.</th>
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<th>Gene Deletion</th>
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INACTIVATION OF MULTIPLE TUMOR SUPPRESSOR

Table 3 (Cont’d). Patients With Aberrations of p16, p15, Rb, or p53

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The number of mitoses is in parentheses.

Abbreviations: +/-, no deletion; +/-, hemizygous deletion; -/-, homzygous deletion; R/-, or R+, gene rearrangement in one allele with or without deletion of the other allele, respectively; ND, not done, data not informed or no mitoses obtained; W, wild-type; M/-, normal allele and mutation in another allele; M homzygous loss of one allele and mutation in another allele; M/R, gene rearrangement in one allele and mutation in another allele; PB, peripheral blood; BM, bone marrow; LN, lymph node; PE, pleural effusion.
Inactivations of both p16<sup>INK4A</sup> and Rb genes have not been reported to date in hematologic malignancies. Moreover, several investigators observed complementary distribution of inactivations of both genes; only either of the two was inactivated in a various tumor. We have presented here four cases with inactivations of both p16<sup>INK4A</sup> and Rb proteins. Because several lines of evidences suggest that functional Rb protein is essential for cell cycle inhibition by cyclin-dependent kinases (CDKs) through the retinoblastoma protein. Because several lines of evidences suggest that functional Rb protein is essential for cell cycle inhibition by cyclin-dependent kinases (CDKs) through the retinoblastoma protein. Because several lines of evidences suggest that functional Rb protein is essential for cell cycle inhibition by cyclin-dependent kinases (CDKs) through the retinoblastoma protein. Because several lines of evidences suggest that functional Rb protein is essential for cell cycle inhibition by cyclin-dependent kinases (CDKs) through the retinoblastoma protein. Because several lines of evidences suggest that functional Rb protein is essential for cell cycle inhibition by cyclin-dependent kinases (CDKs) through the retinoblastoma protein. Because several lines of evidences suggest that functional Rb protein is essential for cell cycle inhibition by cyclin-dependent kinases (CDKs) through the retinoblastoma protein. Because several lines of evidences suggest that functional Rb protein is essential for cell cycle inhibition by cyclin-dependent kinases (CDKs) through the retinoblastoma protein.

Haenszel statistics stratified by the status of p53 show that no obvious interrelations exist among inactivations of the p16 and Rb genes in primary lymphoid malignancies. Inactivation of p53 gene do not correlate with either inactivation of the p16 or Rb genes, indicating that p53 participates in an independent pathway such as regulation of the G1 checkpoint in response to DNA damage. So it is presumed that, although multiple inactivations of these tumor suppressors might not always be necessary for tumor development per se, but advantageous for clonal growth of a tumor. The Rb mutation might facilitate loss of the Rb gene.

In summary, inactivations of multiple cell cycle-related tumor suppressors seem to independently occur according to the probable frequency. Of course, there seems some exceptions to this rule; inactivation of the p16<sup>INK4A</sup> gene is expected to precede loss of Rb function, and deletion of the p16<sup>INK4A</sup> gene is often accompanied by p15<sup>INK4B</sup> gene deletion. In addition, we could not rule out a possibility that minor interactions might still exist between inactivations of one gene and another, because the scale of our study was not large enough to detect such small effects. Even if a mutation of one gene doubles the mutation rate of another gene, the expected increase in the number of patient with double mutations would be remarkably low in our study, which would not be likely to be statistically significant in our study. In addition, some investigators suggested that mutations of the p53 gene might possibly facilitate loss of the Rb gene. Further studies involving more patients would be required to address this point.
tumor-suppressor genes may occur in patients with lymphoid tumors and result in predominant growth of the clones with the multiple defects of the genes. However, it is not likely that inactivation of one tumor-suppressor gene affects inactivations of the others. Multiple defects seem to occur almost incidentally. Inactivations of the tumor-suppressor genes involved in negative regulation of G1 transition were commonly observed in lymphoid malignancies, supporting the notion that G1 transition is one of the critical check points of cell cycle regulations, dysregulation of which potentially could contribute to development of lymphoid tumors.

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Inactivation of multiple tumor-suppressor genes involved in negative regulation of the cell cycle, MTS1/p16INK4A/CDKN2, MTS2/p15INK4B, p53, and Rb genes in primary lymphoid malignancies

A Hangaishi, S Ogawa, N Imamura, S Miyawaki, Y Miura, N Uike, C Shimazaki, N Emi, K Takeyama, S Hirošawa, N Kamada, Y Kobayashi, Y Takemoto, T Kitani, K Toyama, S Ohtake, Y Yazaki, R Ueda and H Hirai

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