Altered p16^{NK4} and Retinoblastoma Protein Status in Non-Small Cell Lung Cancer: Potential Synergistic Effect with Altered p53 Protein on Proliferative Activity

Ichiro Kinoshita, Hirotoshi Dosaka-Akita, Takayuki Mishina, Kenji Akie, Motoi Nishi, Hiromitsu Hiroumi, Fumihiro Hommura, and Yoshikazu Kawakami

First Department of Medicine, Hokkaido University School of Medicine [I. K., H. D.-A., T. M., K. A., H. H., F. H., Y. K.], and Department of Public Health, Sapporo Medical University School of Medicine [M. N.], Sapporo 060, Japan

Abstract

P16^{NK4} protein (p16) and retinoblastoma protein (pRB), like p53 protein, are important tumor suppressors that regulate the cell cycle. We immunohistochemically examined fresh-frozen specimens of 114 resected non-small cell lung cancers (NSCLCs) for loss of p16 and pRB expression, together with aberrant accumulation of p53 protein and the proliferative activity determined by the Ki-67 index. Three pRB-positive tumors were uninterpretable for p16 status. Of the remaining 111 tumors, 30 (27%) lacked p16 expression, and 10 (9%) lost pRB expression. No tumors showed coincident loss of both proteins, supporting the hypothesis that they function in a single pathway. Of 25 tumors, including 4 p16-negative tumors, examined by Southern blot analysis, only 2 p16-negative tumors were considered to have reduced gene dosage consistent with possible homozygous deletion of the CDKN2 gene encoding p16, suggesting that immunohistochemistry is a sensitive and suitable method to screen for p16 alteration. Loss of p16 expression did not correlate with any clinical factors or p53 status, whereas loss of pRB expression correlated with heavy smoking (P = 0.03 by Fisher’s exact test and P = 0.01 by the multivariate logistic regression analysis). Proliferative activity was considerably higher in p53-positive tumors than in p53-negative tumors (P < 0.001). Loss of p16 or pRB expression was associated with a further increase in proliferative activity in the p53-negative tumors (P = 0.009) but not with proliferative activity in the p53-negative tumors. These results suggest that alteration of the p16/pRB pathway is relatively frequently involved in the development and progression of NSCLCs and that its effect on the proliferative activity is potentially synergistic with altered p53 protein.

Introduction

The CDKN2 and the RB^{2} tumor suppressor genes play a pivotal role in the regulation of the cell cycle at the G_{1} checkpoint. In normal cells, active hypophosphorylated pRB prevents cells from transition across the G_{1} checkpoint by sequestering transcription factors such as E2F, which is essential for DNA replication, but inactivation of pRB by CDK-mediated phosphorylation allows cells to progress to the S phase through the G_{1} checkpoint (1). p16^{NK4} protein (p16), the product of the CDKN2 gene, which has been recently found to be deleted in a variety of tumor cells (2, 3), inhibits CDK4- and CDK6-mediated phosphorylation of pRB (4, 5). Therefore, p16 and pRB are suggested to function in a single regulatory pathway of the cell cycle (4, 6), which is supported by observation of an inverse correlation between alteration of both proteins in several types of tumors (7–13).

In NSCLCs, whereas the RB gene is shown to be inactivated in 6–32% of the tumors (14–18), the frequency of alteration involving the CDKN2 gene is controversial. Of NSCLC cell lines, homozygous deletion of the CDKN2 gene is observed in 20–50% and point mutation in 6–21% (2, 3, 10–12, 19–22). Recent reports showed DNA hypermethylation with CDKN2 gene silencing in about 30% of NSCLC cell lines (12). For primary NSCLCs, although there is a study reporting 30% of intragenic mutations of the CDKN2 gene (23), most studies showed that the abnormalities of the CDKN2 gene are rare (10, 21, 22, 24) and are largely restricted to metastatic sites (21, 22). Nevertheless, the rare occurrence of CDKN2 alterations in primary NSCLCs may be underestimated, not only because epigenetic alterations, including DNA hypermethylation, has not been investigated thus far, but also because homozygous deletion may be obscured by contamination due to normal stromal cells (13). Moreover, in NSCLCs, little is known about the clinical and cell biological implications of the inactivation of the p16/pRB pathway and its association with the status of p53 protein (25), another tumor suppressor working at the G_{1} checkpoint (26).

Therefore, in the present study, we immunohistochemically examined primary NSCLCs for the loss of p16 and pRB expression, together with aberrant accumulation of p53 protein and the proliferative activity determined by Ki-67 index. We show a relatively frequent loss of p16 in both early and late stages of NSCLCs. The reciprocal loss of p16 and pRB provides further support for the hypothesis that both proteins function in a single pathway. Furthermore, we demonstrate the potential synergistic effect of the altered p16/pRB pathway with altered p53 protein on the proliferative activity.

Materials and Methods

Tissue Specimens. Primary tumor specimens from 114 NSCLCs were obtained by surgery from the Hokkaido University Medical Hospital, Sapporo Minami-Ichijo Hospital, and National Sapporo Minami Hospital during 1990 and 1995. Tumors were snap-frozen in liquid nitrogen and stored at −80°C in OCT compound (Miles, Elkhart, IN) for immunohistochemistry. For 25 specimens, portions of the tumor were also snap-frozen and stored at −80°C for genomic DNA preparation. Adjacent non-frozen blocks were fixed in 10% neutral buffered formalin and embedded in paraffin, and then several sections from each specimen were stained with H&E to observe histopathology and normal contamination. According to the 1981 WHO classification, they were histopathologically diagnosed as adenocarcinoma (n = 74), squamous cell carcinoma (n = 34), and large cell carcinoma (n = 6).

Immunohistochemistry for p16, pRB, and p53 Proteins. In a pilot study to choose the immunostaining protocol for p16 protein on frozen materials, we used cytospun p16-positive and -negative cell lines, NCI-H209 and HL60, respectively (8), and tested three rabbit polyclonal anti-p16 primary
proper nuclear staining and weak cytoplasmic staining in the NCI-H209 cells. For additional experiments, therefore, 5-p@m frozen sections of cells. For additional experiments, therefore, 5-p@m frozen sections of (PharMingen) as a primary antibody and methanol as a fixative yielded the buffered formalin. The protocol using anti-entire-human p16 antibody antibodies, one against the entire human p16 protein (PharMingen, San antibodies, one against the entire human p16 protein (PharMingen, San Diego, CA), and the other two against the NH2 terminus or COOH terminus of human p16 proteins (Santa Cruz Biotechnology, Santa Cruz, CA), in various concentrations, and three fixatives: methanol, acetone, and 10% buffered formalin. The protocol using anti-entire-human p16 antibody (PharMingen) as a primary antibody and methanol as a fixative yielded the most nucleus-specific staining with the highest intensity in NCI-H209 cells, whereas all of the combinations of antibodies and fixatives generated proper nuclear staining and weak cytoplasmic staining in the NCI-H209 cells. For additional experiments, therefore, 5-µm frozen sections of NSCLC specimens were fixed in methanol at 4°C for 5 min and were reacted with the anti-entire-human p16 primary antibody (PharMingen) at a 1:200 dilution or with control rabbit immunoglobulin at 4°C overnight. Immunostaining was performed by the biotin-streptavidin immunoperoxidase method with 3,3'-diaminobenzidine as a chromogen (SAB-PO kit; Nichirei, Tokyo, Japan). Methyl green was used for counterstain. Immunohistochemical analyses to detect normal expression of pRB and aberrant accumulation of p53 protein using frozen tissue sections were performed with mouse monoclonal primary antibodies PMG3-245 (PharMingen) and PAb1801 (Oncogene Science, Manhasset, NY), respectively, as described previously (15, 27). Tumors were scored as p16-negative (p16−) if any malignant cells had nuclear staining, whereas surrounding normal stromal cells showed adequate nuclear staining as a positive internal control (13). Tumors were regarded as p16-positive (p16+) if any malignant cells showed nuclear staining. Small lymphocytes, which showed no nuclear staining of p16, were used as a negative internal control (9, 28). These criteria for the evaluation of p16 staining were the same as described previously for pRB staining (14, 16), which were also used to classify pRB-positive (pRB+) and -negative (pRB−) tumors in the present study. Tumors were considered to be p53-positive (p53+) and thus to contain the putative p53 mutations when more than 10% of tumor cells showed nuclear staining, and the others were scored as p53-negative (p53−), as described previously (27). Southern Blot Analysis. Genomic DNA was extracted from 25 NSCLC tumor specimens, Southern blotted after digestion with EcoRI, and hybridized to [α-32P]dCTP-labeled human CDKN2 cDNA (7, 10) and cardiac actin cDNA. The final wash was performed twice in 0.2× SSC, 0.1% SDS at 50°C for 20 min. The blots were then exposed to Kodak XAR-5 film (Kodak, Rochester, NY) and quantitatively analyzed by a densitometer. The autoradiographs were developed at various times to ensure that this analysis was performed in the linear range of the film. The CDKN2 signal intensity was normalized against the cardiac actin signal intensity. Because normal cells were present in tumor specimens, we considered a tumor to have reduced gene dosage consistent with possible homozygous deletion of the CDKN2 gene in the tumor cells when the normalized CDKN2 signal intensity in the tumor sample was reduced to less than 80% of that in the normal lung control, and the reduced rate was similar to the proportion of the tumor cells in the sample, essentially as described previously (10). Additional Southern blot analysis for the GAPDH gene was performed to confirm the appropriateness of the cardiac actin gene as an internal control. Ki-67 Immunostaining and Index Scoring. Frozen tissue sections were immunohistochemically analyzed using Ki-67 monoclonal antibody (Dako, Tokyo, Japan) as described previously (29). Cells showing any nuclear staining were considered to be Ki-67 positive. Ki-67 index (percentage of Ki-67-positive cells) was determined by scoring at least 500 tumor cells in three or more well-preserved areas at X400. Sections with fewer than 500 tumor cells were excluded. Statistical Analysis. The associations between loss of p16 or pRB and categorical variables were analyzed by the χ2 test or Fisher’s exact test as appropriate (30). The associations between loss of p16 or pRB and age were analyzed by Student’s t test. To examine the effect of more than one factor simultaneously on loss of p16 or pRB, the multivariate logistic regression analysis was used (31). The differences of Ki-67 indices between two groups were determined by Mann-Whitney’s U test. Multiple comparisons of Ki-67 indices among groups divided by p53 and p16/pRB status were performed by

### Table 1 p16 and pRB expression in 114 resected NSCLCs

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>p16 expression</th>
<th>pRB expression</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>pRB expression</td>
<td>71</td>
<td>30</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>62.7 ± 9.7</td>
<td>64.6 ± 9.6</td>
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<tr>
<td>Sex</td>
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</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Smoking (pack years)b</td>
<td>23</td>
<td>9</td>
</tr>
<tr>
<td>0–19</td>
<td>56</td>
<td>20</td>
</tr>
<tr>
<td>≥20</td>
<td>56</td>
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</tr>
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<td>7</td>
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<tr>
<td>Squamous</td>
<td>53</td>
<td>19</td>
</tr>
<tr>
<td>Large</td>
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<td>4</td>
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<td>T2–4</td>
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<td>21</td>
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<tr>
<td>pN statusc</td>
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<td>75</td>
<td>28</td>
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<td>N1, N2</td>
<td>6</td>
<td>2</td>
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<td>pM status</td>
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<td>48</td>
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<tr>
<td>III–IV</td>
<td>33</td>
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</tr>
<tr>
<td>Normal</td>
<td>35</td>
<td>11</td>
</tr>
<tr>
<td>Altered</td>
<td>35</td>
<td>11</td>
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</table>

> a Three pRB+ tumors could not be evaluated for p16 status (see text).
> b Smoking history data were not available for three cases.
> c Lymph nodes were not resected for two cases.
the ANOVA factorial with Fisher’s protected least significant difference. The significance level chosen was $P < 0.05$, and all tests were two-sided. All immunohistochemical studies were done without knowledge of the clinical data.

**Results**

To determine the loss of p16 and pRB expression in tumor cells with the surrounding stromal cells as a normal internal control in NSCLCs, we subjected fresh-frozen tissues from 114 resected NSCLCs to immunohistochemistry for these proteins. Three pRB+ tumors were uninterpretable for p16 expression due to lack of nuclear staining of p16 in all tumor and stromal cells. Of the remaining 111 tumors, 30 (27%) lacked p16 expression (p16−), and 10 (9%) lost pRB expression (pRB−; Table 1). No tumors showed coincident loss of p16 and pRB expression. The inverse correlation between loss of both proteins was statistically significant ($P = 0.04$). Typical p16+/pRB− and p16+/pRB+ tumors are shown in Fig. 1. All pRB− tumors had strong and homogeneous nuclear staining of p16 in most tumor cells (Fig. 1A), although 23 of 71 pRB+/p16+ tumors showed weak and heterogeneous nuclear p16 staining.

To investigate to what extent the loss of p16 protein reflected the homozygous deletion of the CDKN2 gene, we examined 25 tumors, including 4 p16− tumors, by Southern blot analysis. Two of the 4 p16-tumors were considered to have reduced gene dosage consistent with possible homozygous deletion of the CDKN2 gene (samples 1 and 5; Fig. 2), whereas none of the 21 p16+ tumors showed such reduced gene dosage.

After completion of immunohistochemical analysis of p16 and pRB, status of these proteins was statistically analyzed to correlate it with clinical and clinicopathological characteristics (Tables 1 and 2). Loss of p16 expression did not correlate with any factors, whereas loss of pRB expression correlated with heavy smoking (pack years $\geq 20$; $P = 0.03$ by Fisher’s exact test and $P = 0.01$ by the multivariate logistic regression analysis). Aberrant accumulation of p53 protein, observed in 47 (41%) tumors of the 114 tumors, did not correlate with the loss of p16 or pRB (Table 1).

Ki-67 antibody recognizes a nuclear non-histone protein expressed throughout the cell cycle but absent in G0 (32). Therefore, the proportion of Ki-67-labeled tumor cells (Ki-67 index) provides a measure of the proliferative activity. To elucidate the cell biological significance of alterations of p16 and pRB in combination with p53, we determined the Ki-67 index in the same tumor specimens as analyzed for p16, pRB, and p53 proteins. The results are summarized in Table 3. The Ki-67 indices were assessed in 105 cases and ranged from 1 to 92% (median, 13.4%; mean, 20.9%). Increased values of Ki-67 indices strongly correlated with the altered expression of p53 protein ($P < 0.001$) but did not correlate with p16 or pRB status. Because p16 and pRB are suggested to function in a single regulatory pathway of the cell cycle (4, 6), we divided tumors into two groups: the tumors retaining both p16 and pRB (p16+ and pRB+; p16/pRB-normal) and the tumors lacking either p16 or pRB (p16− or pRB−; p16/pRB-altered). The difference in Ki-67 indices between the two groups of tumors was not statistically significant. When p53+ and p53− tumors were analyzed separately, however, loss of p16 or pRB expression correlated with higher Ki-67 indices in the p53+ tumors ($P = 0.009$) but not in the p53− tumors. This correlation was also shown to be significant in a multiple comparison analysis; that is, p53+ and p16/pRB-altered tumors had significantly higher Ki-67 indices than p53+ and

Fig. 1. Immunostaining patterns for p16 (A and C) and pRB (B and D) in primary NSCLCs. Representative p16+/pRB− squamous cell carcinoma shows strong and homogeneous nuclear staining of p16 (A) and no nuclear staining of pRB (B) in tumor cells. Representative p16−/pRB+ squamous cell carcinoma displays no nuclear staining of p16 (C) and intense nuclear staining of pRB (D) in tumor cells. Note that admixed stromal cells show distinct nuclear staining (arrows) of p16 and pRB in all specimens, which provide positive internal controls for both proteins. A–D, $\times 400$. 

5559
Table 3 Ki-67 indices in NSCLC groups divided by p16, pRB, and p53 status

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Median</th>
<th>Mean</th>
<th>Range</th>
<th>SD</th>
<th>P</th>
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<tbody>
<tr>
<td>p16+</td>
<td>75</td>
<td>13.4</td>
<td>19.1</td>
<td>1.2-82.8</td>
<td>18.9</td>
<td>0.57</td>
</tr>
<tr>
<td>p16−</td>
<td>30</td>
<td>14.5</td>
<td>25.4</td>
<td>1.0-92.0</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>pRB+</td>
<td>95</td>
<td>12.6</td>
<td>20.8</td>
<td>1.0-92.0</td>
<td>22.1</td>
<td>0.50</td>
</tr>
<tr>
<td>pRB−</td>
<td>10</td>
<td>15.9</td>
<td>22.1</td>
<td>1.6-57.2</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td>p16+ and pRB+</td>
<td>65</td>
<td>12.6</td>
<td>18.6</td>
<td>1.2-82.8</td>
<td>19.0</td>
<td>0.35</td>
</tr>
<tr>
<td>p16− or pRB−</td>
<td>40</td>
<td>15.9</td>
<td>24.6</td>
<td>1.0-92.0</td>
<td>25.1</td>
<td></td>
</tr>
<tr>
<td>p53−</td>
<td>62</td>
<td>8.4</td>
<td>15.7</td>
<td>1.0-78.8</td>
<td>19.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>p53+</td>
<td>43</td>
<td>19.0</td>
<td>28.4</td>
<td>3.0-92.0</td>
<td>22.6</td>
<td></td>
</tr>
</tbody>
</table>

when p53−
| p16+ and pRB+ | 40  | 13.4   | 19.1  | 1.2-82.8| 18.9| 0.57  |
| p16− or pRB−  | 22  | 8.6    | 16.3  | 1.2-74.0| 18.6| 0.53  |
when p53+
| p16+ and pRB+ | 25  | 15.2   | 22.4  | 3.0-82.8| 19.5| 0.009 |
| p16− or pRB−  | 18  | 18.6   | 24.4  | 12.0-92.0| 24.4|       |

a Tumors retaining both p16 and pRB.

b Tumors lacking either p16 or pRB.

Discussion

The present study showed a relatively frequent loss of p16 expression in primary NSCLCs. The reciprocal loss of p16 and pRB, consistent with previous studies (7–13), provided further support for the plausible hypothesis that both proteins function in a single pathway (4, 6). The invariably strong nuclear p16 staining in pRB− tumor cells also supported a negative feedback model between p16 and pRB, i.e., pRB inactivation leading to increased p16 expression (4, 33).

Furthermore, we demonstrated, for the first time, the association of loss of p16 or pRB expression with increased proliferative activity in p53+ tumors of NSCLCs.

By immunohistochemical analysis, we could determine the loss of p16 expression in tumor cells, using the surrounding stromal cells retaining p16 expression as a normal internal control. Thus, this analysis may more sensitively detect the inactivation of the CDKN2 gene than molecular genetic analyses using homogenates of tumor specimens for three major reasons: (a) homozygous deletion, the principal mechanism of CDKN2 inactivation, may be obscured by contamination by stromal cells when genetic techniques, especially those based on PCR, are used (13); (b) epigenetic changes such as DNA hypermethylation, another common mechanism of CDKN2 inactivation (12), may not be detected by DNA-based analysis (13); and (c) both homozygous deletion and DNA hypermethylation of the CDKN2 gene result in loss of p16 expression. As expected, the present immunohistochemical study showed loss of p16 in 27% (30 of 111) of primary NSCLCs, although previous genetic studies detected the abnormality of the CDKN2 gene in 0–7% of such tumors.
Southern blot analysis in this study suggested reduced gene dosage consistent with possible homozygous deletion of the CDKN2 gene in two of four p16+ tumors analyzed, which agrees with the observations that one-half of NSCLC cell lines lacking p16 expression show homozygous deletion of the CDKN2 gene and that most of the rest sustain DNA hypermethylation (12). It is also true that Southern blot analysis may have lower sensitivity and specificity for detecting homozygous deletions in tumor specimens due to contaminating normal cells and aneuploidy of tumor cells (13). Again, we emphasize that the immunohistochemical detection of p16 is a sensitive and suitable method to screen for p16 alterations resulting from both homozygous deletion and DNA hypermethylation.

The frequency of p16-negative cases in this study was lower than those in two recent immunohistochemical studies, reported as 47% (8 of 17; Ref. 13) and 49% (30 of 61; Ref. 34) of NSCLCs with the same method of scoring p16 expression. This discrepancy may be attributed to the difference of fixation and preservation of materials; we used fresh rapidly frozen tissues and fixed them in methanol, although the two previous studies used archival formalin-fixed paraffin-embedded tissues. p16 is considered to be highly susceptible to damage and to degenerate, depending on the condition of the fixation and/or preservation of archival tissues (13). Therefore, some of our 23 (21%) tumors with heterogeneous and weak staining of p16 might be evaluated falsely negatively for the p16 expression should formalin-fixed paraffin-embedded tissues be used. Moreover, one of the two immunohistochemical studies did not mention the nuclear staining of normal stromal cells as a positive internal control in p16-negative tumor specimens (34).

Our observation of the loss of p16 expression in the early clinical stage including T1/N0 tumors is intriguing. Nakagawa et al. (22) showed that all homozygous deletions and point mutations of the CDKN2 gene are observed in NSCLC cell lines or tumors from patients with advanced stage disease. Okamoto et al. (21) reported that homozygous deletion and insertions are detected only in metastatic NSCLC tumors. These genetic studies suggest that structural alterations of the CDKN2 gene are associated with progression of NSCLCs. Two immunohistochemical studies (34), including the present study, however, showed no associations between loss of p16 and clinical stage in primary NSCLCs, suggesting that the loss of p16 may be a relatively early event in the development and progression of a subset of NSCLCs. A possible explanation for this discrepancy between the genetic and immunohistochemical studies is that the loss of p16 in the early stage of primary NSCLC results primarily from epigenetic changes such as DNA hypermethylation (35). This hypothesis is supported by the observation that transcriptional inactivation without genetic alteration occurs in NSCLC cell lines, even from patients with the early stage (22).

We demonstrated the possible correlation of the loss of pRB with heavy smoking in NSCLC, although previous studies have not shown such correlation (15, 17). The clinical significance of pRB alteration remains to be determined in larger studies of NSCLC.

This is the first study demonstrating the association of proliferative activity with alterations of p16 and pRB in combination with p53 protein status by measuring the Ki-67 index in primary NSCLCs. The considerably higher proliferative activity in p53+ tumors than in p53− tumors was consistent with previous studies (36, 37). Loss of p16 or pRB expression was associated with a further increase in the proliferative activity in the p53+ tumors, but there was no significant change in the p53− tumors, suggesting that derangement of the p16/pRB pathway might synergistically increase cell proliferation with altered p53 protein. The present finding was consistent with experimental observations that deregulated growth can be inhibited by the introduction of either the RB (38) or p53 gene (39) in SAOS-2 cells, devoid of normal pRB and p53 protein.

In conclusion, the present study suggests that alteration of the p16/pRB pathway is relatively frequently involved in the development and progression of NSCLCs and that its effect on the proliferative activity may be synergistic with altered p53 protein. In the future, it will be necessary to determine whether the status of these proteins can be a biomarker for the clinical outcome and a target of therapy in NSCLCs. Alteration of both p16/pRB and p53 pathways may be linked to poor prognosis because of its association with extremely high proliferative activity (29, 36, 37).

Acknowledgments

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References


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