Correlation of Abnormal RB, p16\textsuperscript{INK4a}, and p53 Expression with 3p Loss of Heterozygosity, Other Genetic Abnormalities, and Clinical Features in 103 Primary Non-Small Cell Lung Cancers\textsuperscript{1}

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ABSTRACT

This study was performed to determine the frequency of inactivation and clinical correlates in non-small cell lung cancer (NSCLC) of three known tumor suppressor genes [TSGs; RB, MTS1/CDKN2 (p16), and p53] and various regions of 3p loss of heterozygosity (LOH) as other major potential TSG sites. Paraffin sections from 103 resected NSCLCs were analyzed for expression of pRB, p16, and p53 by immunohistochemistry, whereas DNA from tumor and normal tissue were tested for LOH at 3p25–26, 3p21, and 3p14. Previously published LOH data for 5q, 11p, 17q, and 18q were also available. Loss of pRB or p16 expression and overexpression of p53 were considered abnormal. The immunohistochemical and LOH data were correlated with a variety of clinical parameters including stage, age, sex, smoking history, and survival. With respect to pRB, p16, and p53, the tumors could be grouped into four categories: normal for all three proteins (21%); abnormal for pRB or p16 and normal for p53 (30%); normal for pRB and p16 and abnormal for p53 (20%); and abnormal in both pathways (28%). Aberrant expression of pRB, p16, p53, and 3p LOH, either individually or in combination, was not associated with survival differences or any other clinical parameters, with the exception that pRB/p16 abnormalities were more common in older patients ($P = 0.0005$). pRB and p16 expression showed a strong inverse correlation ($P = 0.002$), whereas there was no correlation between expression of pRB, p16, and p53. Abnormal expression of any of the three genes inversely correlated with $K$-ras codon 12 mutations ($P = 0.004$), but not with 3p LOH or LOH at other TSG loci. We conclude that resectable NSCLCs show distinct patterns of TSG inactivation, but that no clear clinical correlates exist either alone or in combination for pRB, p16, p53, and 3p abnormalities.

INTRODUCTION

There is increasing evidence that abrogation of cell cycle control is a common and, possibly, obligate feature of malignant neoplasms (1). The mammalian cell cycle is partly controlled by TSGs. Three of these genes, which have been well characterized in lung cancer, include the $RB$, the $p16$, and the $p53$ genes (2–5). The protein products of the former two genes, pRB and p16, are integral components of the late G1 restriction point (1). Hypophosphorylated pRB prevents the cell from entering S phase, and p16 prevents hyperphosphorylation of pRB by inhibiting the CDK4/cyclin D complex. p53 is also involved in controlling the G1 checkpoint, and this effect is mediated by p21\textsuperscript{WAF1} (6). It is well known that p53 has several additional physiological functions, including control of the G2 cell cycle checkpoint and mediation of apoptosis (6).

RB, p16, and p53 have been shown to be frequently altered in NSCLC, which is responsible for a large proportion of cancer deaths worldwide (2–4). RB protein expression is abnormal in 15–30% of NSCLCs, and p16 expression is altered in 30–70% of NSCLCs, whereas p53 overexpression is seen in 35–60% of NSCLCs (7–17). Most studies have focused on one or, at the most, two of these TSGs (Table 1). Kinoshita et al. (11) examined the expression of these three genes in same cohort of frozen sections. To the best of our knowledge, all three genes have not been examined in other studies or in the same cohort of formalin-fixed and paraffin-embedded NSCLCs. In addition to these three well studied genes, the other most frequent genetic abnormality in NSCLC is LOH at one or more sites in chromosome region 3p occurring in over 50% of tumors (reviewed in Ref. 4). The VHL gene at 3p25 is very rarely mutated, whereas the $FHIT$ gene at 3p21 is frequently mutated and probably important in the development of NSCLC.

\textsuperscript{4}The abbreviations used are: TSG, tumor suppressor gene; RB, retinoblastoma; LOH, loss of heterozygosity; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; SSCP, single strand conformation polymorphism; CDK, cyclin-dependent kinase; p16, MTS1/CDKN2/p16\textsuperscript{INK4a}.
gene at 3p14.2 frequently suffers large intronic deletions and splicing abnormalities but very rarely has a true inactivation point mutation (18–20). The other sites (such as at 3p21.3, 3p12, and 3p22–24) do not yet have their TSGs identified. In addition, these 3p LOH abnormalities have not yet been related to 3p12, and 3p22–24; do not yet have their TSGs identified. In addition, these 3p LOH abnormalities have not yet been related to p16, RB, and p53 lesions. Thus, there are relatively few studies that comprehensively describe the expression of p16, pRB, and p53 in lung cancer. Likewise, there are few studies where multiple genetic abnormalities are studied in the same cohort of patients and related to each other and to the clinical data.

The clinical significance of alterations in these genes has generated conflicting results. Numerous publications have detailed the prognostic significance of p53 alterations in NSCLC, and many of these have demonstrated that p53 abnormalities (increased expression or mutation) adversely affect patient survival, whereas others have found no such effect [summarized by Graziano (5)]. Likewise, studies on decreased expression of pRB by immunohistochemical analysis in NSCLC found this to be an adverse prognostic indicator, whereas others could not confirm these findings (8–10, 21). Two previous reports on p16 expression in NSCLC found that loss of this protein was associated with shortened overall survival (10, 13). We wanted to know the frequency of the different possible patterns of expression of pRB, p16, and p53 in individual tumors, as well as the relationship of these patterns to other genetic changes such as 3p LOH. Finally, we wished to ask if these patterns affect the clinical behavior of NSCLC.

In this study, we describe immunohistochemical studies of pRB, p16, and p53, as well as additional 3p LOH data on 103 primary, resected NSCLCs that had previously been analyzed for LOH at chromosomal regions 11p, 17q, 18q, 5q, and 3p14.2, as well as for mutations of K-ras and exons 5–8 of the p53 gene by PCR-SSCP (20, 22–25). We assessed the prognostic significance of aberrant gene expression, both singly and in combination, and correlated this with other genetic abnormalities and clinical parameters. Our results show: (a) that the frequency of the different patterns of pRB, p16, and p53 expression are approximately equal; (b) that these expression patterns are usually randomly allocated with respect to other common genetic abnormalities such as 3p LOH; and (c) that the patterns do not provide for prediction of survival or other important clinical parameters.

**MATERIALS AND METHODS**

**Patient Material.** Tumor blocks were obtained from 108 patients with primary NSCLC, at The Prince Charles Hospital (Brisbane, Australia), who had been treated with curative resectional surgery. This cohort of unselected patients had been investigated previously for molecular genetic changes at candidate chromosomal regions (20, 22–25). From this cohort, pathology review (by J. G.) identified 103 cases where adequate material remained for immunohistochemical evaluation. The samples were collected from June 1990 through March 1993, and survival data of ≥5 years were available on most patients. There were 73 males and 30 females, ages 28–81 years (mean, 61 ± 11 years at diagnosis). The patients had an overall 42 ± 31 mean pack-years of smoking (range, 0–150 pack-years). Independent histological examination of the tumors was performed according to 1982 WHO criteria, and pathologically confirmed pTNM stage was assigned in accordance with the International Union Against Cancer. There were 58 stage I, 22 stage II, and 23 stage III tumors. Histological subtypes included 40 squamous cell carcinomas, 44 adenocarcinomas (including 4 with bronchiolo-alveolar characteristics), 11 adenosquamous cancers, 4 large cell cancers, 3 malignant atypical carcinoids, and 1 typical carcinoid. A detailed medical and smoking history was available, and there were 8 never/nonsmokers and 95 smokers with mean pack-years of 41–50. This study is part of an ongoing research project and, thus, data on other molecular markers were available for analysis including ras mutations, p53 mutations; 3p14.2 LOH, 5q LOH, 17q LOH, 18q LOH, and microsatellite alterations (20, 22–25).

**Immunohistochemistry.** One representative block was retrieved for each case, and four micron paraffin sections were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of abnormal pRB, p16, and p53 protein expression as detected by IHC in resected NSCLCs: present series versus published literature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Series (n)</td>
</tr>
<tr>
<td>Kinoshita (n = 111)</td>
<td>10 (9%)</td>
</tr>
<tr>
<td>Kratzke (n = 100)</td>
<td>15 (15%)</td>
</tr>
<tr>
<td>Sakaguchi (n = 61)</td>
<td>23 (38%)</td>
</tr>
<tr>
<td>Tanaka (n = 101)</td>
<td>42 (42%)</td>
</tr>
<tr>
<td>Kashiwabara (n = 82)</td>
<td>28 (34%)</td>
</tr>
<tr>
<td>Taga (n = 115)</td>
<td>ND</td>
</tr>
<tr>
<td>Xu (n = 101)</td>
<td>24 (24%)</td>
</tr>
<tr>
<td>Dosaka-Akita (n = 91)</td>
<td>19 (21%)</td>
</tr>
<tr>
<td>Nishio (n = 208)</td>
<td>42 (20%)</td>
</tr>
<tr>
<td>Harpole (n = 271)</td>
<td>ND</td>
</tr>
<tr>
<td>Dalquen (n = 247)</td>
<td>ND</td>
</tr>
<tr>
<td>Kawasaki (n = 111)</td>
<td>ND</td>
</tr>
<tr>
<td>Present series (n = 103)</td>
<td>14 (14%)</td>
</tr>
<tr>
<td>Totals</td>
<td>217/958 (23%)</td>
</tr>
</tbody>
</table>

a The references for the work cited in this table are given in Refs. 8–14, 16, 17, and 33–35.
b ND, not done.

* ND, not done.
prepared and used within 72 h for IHC (see below) or stored at 4°C. Murine monoclonal anti-RB antibody 3C8 was purchased from QED (San Diego, CA; Ref. 26). Anti-p16 polyclonal and monoclonal (clone G175–405) antibodies were obtained from PharMingen (San Diego, CA; Ref. 27). Monoclonal anti-p53 antibody DO7 was obtained from Novocastra via Vector Laboratories (Burlingame, CA). Nonspecific mouse IgG or rabbit serum, respectively, were used as negative controls. The detection reactions used the Vectastain Elite ABC kit from Vector Laboratories. Standard immunohistochemical assays were performed to demonstrate the presence of pRB and p16 in our archival tissues. These assays have been described in detail.

Fig. 1 Normal and abnormal immunohistochemical expression patterns of pRB, p16, and p53. A, pRB (+) poorly differentiated squamous cell carcinoma; strong pRB nuclear reactivity in most tumor cells. B, pRB (−) poorly differentiated adenocarcinoma; the tumor (top right) is completely pRB negative, with preserved nuclear staining in nonneoplastic epithelium and stroma. C, p16 (+) poorly differentiated squamous cell carcinoma; strong p16 nuclear and weaker cytoplasmic reactivity in tumor cells. D, p16 (−) large cell carcinoma; the tumor (right) is completely negative for p16, whereas adjacent stromal cells (arrows) show distinct p16 nuclear staining. E, p33(−) adenocarcinoma; the adenocarcinoma contains scattered cells with weak p53 nuclear staining (arrows), which we consider normal. F, p53(+) large cell carcinoma; there is strong p53 nuclear staining in almost all tumor cells. A–F, original magnifications, ×400.
elsewhere (26–28). For detection of pRB, deparaffinized sections were incubated with anti-pRB antibody 3C8 at 2 μg/ml for 2 h after an antigen retrieval step in 0.01 M citrate buffer (95–100°C). The detection reaction followed the Vector Laboratories-recommended protocol. For detection of p16, sections of all cases were incubated with the p16 polyclonal antiserum (negative controls, preimmune rabbit serum) at a 1:400 dilution at 4°C overnight, without prior antigen retrieval. A subset of cases was also reacted with the anti-p16 monoclonal antibody at 2 μg/ml at room temperature overnight after an antigen retrieval step. For both p16 staining procedures, the detection reaction was modified from the Vectastain Elite ABC protocol: biotinylated secondary antibody at 1:250 for 15 min at 42°C; streptavidin-biotinylated enzyme complex at 1:250 for 15 min at 42°C. For detection of p53, sections were incubated in an automated stainer (BioTek; Ventana Systems, Tucson, AZ), after antigen retrieval in hot citrate buffer. The tissues were reacted with antibody DO7 at a 1:500 dilution for 25 min. The detection reaction followed the recommended Vectastain Elite protocol. Diaminobenzidine and hematoxylin counterstain were used for color development in all assays. Negative controls were stained under identical conditions. The following external positive controls were used: mesothelioma cell line NCI-H2373 for pRB; lung cancer cell line NCI-H2009; and a small cell lung carcinoma for p16. In addition, nonneoplastic stromal cells served as internal positive controls for pRB and p16 in every tumor section. For p53, lung cancer cell line NCI-H441 (containing a p53 R158L missense mutation) and a small cell lung carcinoma, which markedly overexpressed p53, were used. Paraffin-embedded pellets of the cell lines were sectioned and handled as the other material.

**Evaluation of Immunohistochemical Stains.** Each case was scored for pRB and p16 reactivity using previously published criteria (27). Briefly, sections were examined for evidence of nuclear staining above any cytoplasmic background; cytoplasmic staining itself was disregarded. If there was nuclear staining in a diffuse or mosaic distribution throughout the tumor, it was considered normal (positive) for the respective protein. If the neoplastic nuclei failed to stain while admixed nonneoplastic cells reacted positively, the lesion was scored as abnormal (negative). p53 was scored as abnormal when >15% of cells showed nuclear staining. We arrived at the 15% cutoff point by determining that all lung tumors with <15% p53 immunohistochemically positive cells had a normal p53 SSCP pattern, suggestive of wild type p53. In addition, we noticed that in the morphologically normal lung in some cases, as many as 5–10% of epithelial or mesenchymal cells, showed weak to moderate nuclear p53 reactivity under the staining conditions described above.

**Genomic PCR and 3p LOH Analysis.** Genomic DNA from tumors and normal tissue of the 103 Australian cases of primary, resected NSCLCs were obtained as described previously (25). LOH at 3p was determined with microsatellite markers D3S1038 (3p25–26), D3S1029 (3p21), D3S1234, D3S1312, D3S1300, D3S1312, D3S1313, and D3S4103 (3p14; Ref. 20) using "IP-labeled, ‘touchdown’ PCR under standard conditions (29). Detailed survival analysis for LOH of markers on 5q, 11p, 17q, and 18q have been reported (22–25), and LOH from all of these loci along with the 3p LOH data were analyzed in conjunction with the IHC data.

**Statistical Analysis.** Statistical analysis of correlations between variables was performed using either χ² test for categorical data or t test for means. Survival curves were calculated by the Kaplan-Meier method and compared by log rank analysis using SPSS for Windows.

**RESULTS**

**Immunohistochemical Staining Results for p16, pRB, and p53.** We performed immunohistochemical stains for pRB, p16, and p53 on our cohort of 103 resected, primary NSCLCs (Fig. 1). The abnormality rates are consistent with a summary of other large published studies (Table 1). In particular, our pRB and p16 abnormality rates are almost identical to those of Kratzke et al. (10) in a cohort of lung cancer patients from Minnesota. Although some tumors stained normally for pRB and p16 (Fig. 1, A and C), in most pRB or p16 abnormal cases the tumors were diffusely negative, with preserved immunoreactivity in admixed nonneoplastic cells (Fig. 1, B and D). In rare cases, loss of protein expression was confined to one or more areas within the cancer, suggestive of intratumor heterogeneity. In the 50 tumors overexpressing p53, moderate to strong nuclear staining was found in 25–95% of cells (Fig. 1F). Overexpression of p53 was strongly correlated with an abnormal p53 SSCP pattern and p53 mutations (P = 0.00001) we had detected in a prior study (25). This indicated that IHC is a suitable, albeit imperfect means to detect p53 mutations. The remaining NSCLCs showed no p53 staining or generally weak reactivity in <15% of cells (Fig. 1E).

In aggregate, 58% of NSCLCs lost expression of either p16 or pRB, the two tumor suppressor proteins controlling the late G1 checkpoint. p16 loss of expression was more frequent than loss of pRB expression, similar to other studies in NSCLC (summarized in Table 1). In this series, as in many others, there was an inverse correlation between loss of pRB expression and loss of p16 expression (P = 0.00187); only one tumor was abnormal for both. In contrast, no correlation was observed for loss of p16 expression and overexpression of p53 (P = 0.746), indicating that these two events occur independently in the formation of NSCLC. On the basis of the expression pattern of the three genes, the tumors could be grouped into four categories

| Table 2 Immunohistochemical staining patterns of pRB, p16, and p53 in 103 NSCLCs |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| pRB*a           | p16*a           | p53*a           | n               | Group (n)      |
| +               | +               | Normal          | 22              | A (22)         |
| −               | +               | Normal          | 6               | B (31)         |
| +               | −               | Normal          | 25              | B (31)         |
| −               | −               | Normal          | 0               | B (31)         |
| +               | +               | Abnormal        | 21              | C (21)         |
| −               | +               | Abnormal        | 7               | C (21)         |
| +               | −               | Abnormal        | 21              | D (29)         |
| −               | −               | Abnormal        | 1               | D (29)         |

*a pRB and p16 were scored as positive (+) nuclear staining (normal) or no (−) nuclear staining (abnormal). p53 was scored as normal (no staining) or abnormal (nuclear staining; see “Materials and Methods”).
Table 2: group A ($n = 22$) was normal for all three markers; group B ($n = 31$) showed normal expression of p53, but loss of pRB or p16 expression; conversely, in group C ($n = 21$), pRB and p16 were normally expressed, whereas p53 was overexpressed; and group D ($n = 29$) was characterized by loss of either pRB or p16 and p53 overexpression. Thus, the various possible expression patterns were approximately equal in number.

Clinical Correlations. We asked whether expression of pRB, p16, and p53 was correlated with a variety of clinical

| Table 3 Individual immunohistochemical marker expression correlated with clinical data in 103 NSCLCs |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                     | pRB             | p16             | p53             |
| Mean age (years)$^a$ | Normal 60.7 63.2 | Abnormal 0.411 | Normal 59.4 63.1 | Abnormal 0.076 | Normal 62.3 59.9 | Abnormal 0.256 |
| Pack-years$^a$       | 43.9 32.9 0.226  |                  |                  |                  |                  |
| Sex$^a$              | Male 64 9                  | 39 34 0.559 | 17 13 0.764 | 17 13 0.497 |
|                      | Female 25 5                 |                  |                  |                  |
| Smoking history$^b$  | Non-smoker 5 3 0.039/0.075 | 5 3 0.631 | 5 3 0.411 |
|                      | Smoker 84 11                 |                  |                  |                  |
| TNM stage$^b$        | I 51 7                      | 31 27 0.831 | 29 29 0.737 |
|                      | II, III 38 7                | 25 20 0.013 | 24 21 0.082 |
| Histologic subtype$^b$ | Squamous cell carcinoma 35 5 | 18 22 0.133 | 18 22 0.279 |
|                      | Adenocarcinoma 39 5          | 27 17 0.374 | 25 19 0.279 |

$^a$ t tests used for comparisons.

$^b$ x$^2$ tests.

$^c$ Fisher’s exact test.

Fig. 2 Kaplan-Meier cumulative survival curves as a function of pRB, p16, p53, and combined IHC marker status. A, survival as a function of pRB IHC expression [abnormal, (−); normal, (+); $P = 0.443$, not significant]. B, survival as a function of p16 IHC expression [abnormal, (−); normal, (+); $P = 0.135$, not significant]. C, survival as a function of p53 IHC expression [abnormal, (−); normal, (+); $P = 0.702$, not significant]. D, survival as a function of IHC status of the four groups [group A ($n = 22$), normal for pRB, p16, and p53]; group B ($n = 31$), abnormal for pRB or p16 and normal for p53]; group C ($n = 21$), normal for pRB and p16 and abnormal for p53]; group D ($n = 29$), abnormal for pRB or p16 and abnormal for p53]. $P$s for all possible comparisons between groups are not statistically significant, including $P = 0.28$ for comparison of groups A and C.
parameters (Table 3). There was no statistically significant association of abnormal expression of any of these markers, either individually or in combination, with gender, smoking habits, stage of disease, or histological tumor type. However, tumors from patients over the age of 65 were significantly more likely to show loss of p16 expression ($P = 0.00593$) or loss of p16/pRB expression ($P = 0.00052$) than those from patients younger than 65. Survival data were available on all 103 cases. Neither pRB (Fig. 2A), p16 (Fig. 2B), nor p53 (Fig. 2C) status stratified the tumors into significantly favorable and unfavorable subgroups. Grouping the tumors (groups A-D) into categories with different abnormality patterns in the RB/p16 and p53 pathways also was of no prognostic significance (Fig. 2D). The biggest survival differences occurred between groups A (pRB, p16, and p53; all normal and, surprisingly, with the worse survival) versus group C (pRB and p16 normal, but p53 abnormal). However, this difference was not statistically significant.

![Figure 3](image.png)

**Fig. 3** Kaplan-Meier cumulative survival curves as a function of LOH at various 3p regions. A, survival as a function of LOH at any possible 3p locus (No LOH = 42; LOH = 61). B, survival as a function of 3p14 LOH (six markers; No LOH = 48; LOH = 55); C, survival as a function of 3p21 LOH (D3S1029; No LOH = 44; LOH = 26). D, survival as a function of 3p25–26 LOH (D3S1038; No LOH = 53; LOH = 31). The ticks on the curves represent censored observations. $P$s for the comparisons are not statistically significant.

### Table 4  Individual immunohistochemical marker expression correlated with genetic changes in 103 NSCLCs

<table>
<thead>
<tr>
<th>Genetic change</th>
<th>pRB</th>
<th>p16</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Abnormal</td>
<td>$P^a$</td>
</tr>
<tr>
<td>3p LOH (−) $^b$</td>
<td></td>
<td>39</td>
<td>3</td>
</tr>
<tr>
<td>3p LOH (+) $^b$</td>
<td>50</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>17q LOH (−)</td>
<td>45</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>17q LOH (+)</td>
<td>36</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>11p LOH (−)</td>
<td>58</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>11p LOH (+)</td>
<td>21</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5q LOH (−)</td>
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<tr>
<td>5q LOH (+)</td>
<td>19</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>18q LOH (−)</td>
<td>61</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>18q LOH (+)</td>
<td>12</td>
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<td></td>
</tr>
<tr>
<td>K-ras wild type</td>
<td>79</td>
<td>13</td>
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</tr>
<tr>
<td>K-ras mutant</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $x^2$ tests used for categorical data.

$^b$ For 3p LOH all eight markers considered, whereas there were also no significant differences for individual consideration of 3p14.2, 3p21, and 3p25–26 region LOH.
Table 5  Inverse correlation of abnormal expression of p53, pRB, or p16 and K-ras codon 12 mutations

<table>
<thead>
<tr>
<th>K-ras codon 12 mutation</th>
<th>No</th>
<th>Yes</th>
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<tbody>
<tr>
<td>p53, pRB, or p16 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Abnormal</td>
<td>76</td>
<td>5</td>
</tr>
<tr>
<td>$P = 0.00449 \ (\chi^2)$</td>
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<td></td>
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<tr>
<td>$P = 0.01115 \ (\text{Fisher's test})$</td>
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</table>

pathways (group D) did not behave significantly more aggressively than those expressing all three proteins at normal levels (group A).

**Molecular Correlations.** Expression of the three TSGs was correlated with previously obtained molecular genetic data, as well as additional 3p LOH data in this study (20, 22–25). Previously, we had found in this same series that LOH at 17q, 5q21, and 11p were associated with worse survival and often higher tumor stage, whereas LOH at 18q was not associated with impaired prognosis (22, 23, 25). In this study of regions 3p21 and 3p25–26, combined with our previous study of 3p14 (FHIT region; Ref. 20), we found no correlation with clinical stage or survival of various 3p regions LOH individually or combined (Fig. 3). Expression of the individual proteins was not statistically correlated with LOH at commonly altered chromosomal regions including 3p (all regions), 5q, 11p, 17q, and 18q (Table 4), although loss of p16 tended to be more common in tumors without allelic loss at 3p ($P = 0.052$) and 17q ($P = 0.047$). There was no association between individual TSG expression and presence of a mutation in codon 12 of the K-ras gene previously tested in this cohort (Ref. 30; Table 4). However, tumors showing a normal expression pattern of all three genes (group A) were significantly more likely to have a K-ras mutation compared with those tumors abnormally expressing at least one gene (groups B-D; Table 5). Finally, when TSG expression was stratified into groups A-D as described above, no significant correlation between these groups with other molecular genetic data was found (data not shown).

**DISCUSSION**

Abnormalities of the p53 and RB-p16 tumor suppressor pathways are frequent molecular events in lung cancers (3, 4). There have been several studies examining the clinical associations and prognostic value of such alterations in lung cancer, particularly NSCLC. However, nearly all of these studies have examined each of these markers in isolation or in dual combinations, such as RB with p53 (7–14, 16, 17, 31–35). Thus, the potential interaction of these events has not been well addressed, although other studies suggest that additional information may be gained with the study of combinations of biologically important molecular markers, such as Ras and p53 expression, as well as c-erbB-2 expression and K-ras mutations (15, 36). Indeed, the few studies of the combination of altered RB and p53 status indicate that they may act synergistically to affect prognosis in some NSCLCs (8, 9). An independent study correlated pRB expression with Ras and p53 expression, and suggested that combined results of pRB and Ras or pRB and p53 may be useful for predicting survival and outcome in adenocarcinomas (8). The same group also reported that loss of p16 or pRB expression was associated with an increase in proliferative activity in the p53-positive tumors but not in the p53-negative tumors (11). This would be consistent with a paradigm, whereby alteration of the p16/pRB pathway is potentially synergistic with altered p53 protein in affecting the proliferative activity of NSCLC. For this study we used a clinically well characterized cohort of patients with resected NSCLCs for which we had formalin-fixed and paraffin-embedded material, as well as full pathological staging and clinical follow-up. In addition, we had performed other molecular analyses on these specimens. Thus, we are able to give a comprehensive overview of molecular abnormalities at the genomic and protein expression level in a reasonably large cohort of well characterized NSCLCs. Specifically, we tested whether abnormal expression of p53 in combination with abnormalities of pRB or p16 expression, and/or 3p LOH were related to advanced clinicopathological features and adverse outcome. However, we did not find any significant clinical associations.

IHC is a sensitive and reliable method for detecting RB and p16 gene inactivation because of the diverse molecular lesions that down-regulate their function (27). The frequency of abnormalities we found for pRB (14%) and p16 (46%) are in keeping with previously published data (Table 1). RB and p16 IHC expression showed a strong inverse correlation ($P = 0.002$), consistent with their common growth regulatory pathway and other studies (10–12). The absence of pRB expression has been reported to be associated with poor prognosis, particularly in stage I and II disease, in some, but not all, studies of NSCLC (8–10, 21). Our findings add to this controversy as our data indicate that pRB immunohistochemical expression was not a significant predictor of outcome in this group of patients. In NSCLC, p16 may be inactivated by homozygous deletion, promoter methylation, or mutation; most of these events lead to loss of protein expression (34, 37). We found no correlation of p16 loss of expression with advanced disease stage or survival in this cohort in agreement with the findings of Kinoshita et al. (11). In contrast, other studies found a correlation with increased stage of disease and adverse survival with absent p16 immunostaining (10, 13). Marchetti et al. (38) found p16 mutations in metastatic, but not in nonmetastatic, NSCLCs. In our series, the trend for tumors without detectable p16 expression to behave aggressively did not reach statistical significance ($P = 0.13$). The differences between studies may be related to a different genetic background or competing environmental factors in the present cohort of tumors from Australian patients. Other explanations include differences in the patient (e.g., stages of disease), sample (primary tumors or cell lines; higher frequencies of genetic events have been observed in cultured cell lines and metastatic sites compared with primary lesions; Ref. 31), and treatment (single or combined modality). Finally, methodological differences and biological heterogeneity may also play a role. The combination of pRB and p16 IHC changes also did not correlate with survival. It is noteworthy that a significant proportion (42%) of our resected NSCLCs were normal for both pRB and p16 expression. Therefore, other members of this growth control pathway (i.e., cyclin D1 and/or CDK4) may be implicated as the...
alternative targets for disrupting the G1 cell cycle checkpoint in NSCLC. In support of this, cyclin D1 was found by some to be overexpressed in lung tumors with normal RB protein expression (39, 40). Although CDK4 gene amplification has been reported in 10–15% of malignant gliomas and certain other malignancies, its role in lung cancer has not yet been described.

In our study, 49% of the NSCLCs overexpressed (presumably mutant) p53, and we found p53 overexpression to be strongly correlated with p53 mutations (P = 0.00001). It is well recognized that p53 missense mutations generally result in positive staining because of increased protein stability, whereas complete gene deletion, frameshift, or nonsense mutations are not usually identified immunohistochemically and immunostaining in this study would not have detected these types of abnormalities (41). However, we found neither p53 IHC nor SSCP changes (from our prior study) predicted survival in this reasonably large cohort. Positive p53 staining also did not correlate with other clinicopathological features. There was no difference in the frequency of p53-positive staining in lung cancers with squamous (22 of 40) compared with adenocarcinoma histology (19 of 44) despite the fact we had previously found a preponderance of p53 gene mutations in the squamous cancers in this cohort of NSCLCs (25). A summary of 14 studies of the prognostic importance of p53 mutations or overexpression in NSCLC [mutational analysis (four studies); immunostaining (eight studies); and both techniques (2 studies)] yielded controversial results (5). With respect to the 10 immunohistochemical studies, aberrant p53 expression correlated with a shortened survival in 5 studies, improved survival in 3 studies, and had no survival effect in 2 studies. In one study that analyzed both gene mutation and protein expression, only the latter predicted shortened survival. Complicating the interpretation of published data are possible methodological differences as discussed above, including the fact that different antibodies have been used. Moreover, various p53 mutants or wild-type p53 overexpression, perhaps, have different effects on lung cancer behavior, and it is possible that wild-type p53 overexpression may be immunohistochemically detectable in certain tumors; the biological significance of the latter is unclear.

Various combinations of the three markers also failed to stratify for survival (Fig. 2D). Although there seems to be a possible difference in survival between groups A and C, this did not reach statistical significance (P = 0.28). This may possibly be due to relatively small numbers in the different groups, limiting statistical power. Future studies of biomarkers would benefit from larger datasets, preferably recruited multi-institutionally. In any case, these data would be consistent with the notion that abnormalities of these TSGs are perhaps early and critical events for oncogenesis rather than for tumor progression. Such a model would also be consistent with the notion that 3p allele loss is the earliest change in preneoplastic bronchial lesions associated with lung cancers, followed by 9p allele loss (p16 locus), 17p allele loss (p53 locus), 5q allele loss, and ras mutations (42–46). K-ras mutations were significantly more common in tumors showing a normal expression pattern of RB, p16 and p53 (Table 5). One could, thus, speculate that K-ras oncogene activation by mutation could represent an alternative critical step to p53 or RB/p16 TSG inactivation. This would also be consistent with the observations that K-ras mutations are very rare in small cell lung cancers, which are nearly always abnormal for pRB and mutant for p53 (4).

Our present study and our previous detailed study of 3p14.2 (FHIT) LOH (20) in 103 resected NSCLCs suggests no influence on survival of 3p LOH at any region and no correlation of 3p LOH at any region with IHC abnormalities in pRB, p16, or p53. Burke et al. (47), in a study of 106 resected stage I and II Mayo Clinic NSCLCs, found that LOH at the FHIT locus at 3p14.2 (markers D3S1300 and D3S1234) was associated with poor survival (P = 0.01), p53 missense mutations (but not with nonmissense mutations), and nonadenocarcinoma histology. The poor survival was independent of tumor stage, size, grade, histological subtype, and p53 mutation status. However, the number of cases actually informative for 3p14.2 LOH was only 38 (no LOH in 25 and LOH in 13). Mitsudomi et al. (48) studied 98 informative and resected NSCLCs from Japan and found an increased frequency of 3p LOH in squamous cell carcinoma compared with adenocarcinomas, but no significant association between 3p LOH and sex, disease stage, or grade of differentiation. They found no significant survival impairment for their cohort as a whole but impaired survival in adenocarcinoma patients with 3p LOH. Nelson et al. (49), in 40 primary lung cancers, found no associations of age, sex, p53, or K-ras mutation and 3p14.2 LOH. Pifarre et al. (50) studied 64 resected NSCLCs, and although finding significant prediction of poor survival from a tumor having the replication errors phenotype (RER) at 2q and 3p markers, they found no association of 3p LOH with impaired survival. Field et al. (51) tested 45 NSCLCs for 3p LOH and found an association with high frequency LOH at other loci (9p and 17p) but not with other sites of LOH. Thiberville et al. (52), in studies of 86 NSCLCs, found no survival or stage information from 3p21–22 LOH. Horio et al. (53), in 71 curatively resected NSCLCs, found 3p LOH to have a nonsignificant trend toward poorer prognosis. Thus, overall there is either no striking correlation or a very mild effect of 3p LOH on survival and/or the occurrence of other genetic abnormalities in lung cancer. This would be consistent with 3p LOH being an early step in the pathogenesis of many lung cancers, as suggested by studies of preneoplastic lesions associated with lung cancers (44, 45).

In summary, we have found nearly all possible combinations of normal and abnormal immunohistochemical expression for pRB, p16, and p53 and LOH or retention of heterozygosity for 3p in approximately equal numbers in a relatively large panel of resected NSCLCs. These various patterns were not of prognostic significance and, with a few exceptions, the molecular abnormalities seemed to occur independently of one another. The data are consistent with the notion that these pathways are important for tumor development rather than clinical progression. They also point to the importance of discovering new genetic abnormalities in NSCLCs that seem normal for pRB, p16, p53, or are without 3p LOH.

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REFERENCES


Correlation of Abnormal RB, p16\textsuperscript{ink4a}, and p53 Expression with 3p Loss of Heterozygosity, Other Genetic Abnormalities, and Clinical Features in 103 Primary Non-Small Cell Lung Cancers

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