Prognostic Implications of Molecular and Immunohistochemical Profiles of the Rb and p53 Cell Cycle Regulatory Pathways in Primary Non–Small Cell Lung Carcinoma

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ABSTRACT

Purpose: Many studies have highlighted the aberrant expression and prognostic significance of individual proteins in either the Rb (particularly cyclin D1, p16Ink4a, and pRb) or the p53 (p53 and p21Waf1) pathways in non–small cell lung cancer. We hypothesize that cumulative abnormalities within each and between these pathways would have significant prognostic potential regarding survival.

Experimental Design: Our study population consisted of 106 consecutive surgically resected cases of predominantly early-stage lung cancer. We recognize as a common occurrence during carcinogenesis, with NSCLC being no exception. Many studies have highlighted the aberrant expression, due to genetic or epigenetic alterations, of at least one key control element in primary lung tumors (3–6), occasionally with prognostic significance (7). However, few studies have compared multiple molecular markers of the complex interaction among the cellular control elements and their prognostic implications.

INTRODUCTION

Non–small cell lung cancer (NSCLC), even in resectable and early-stage lesions, maintains a very poor 5-year survival (1, 2), and much work is now being focused on identifying factors with predictive potential to improve therapeutic strategies for these patients. Cell cycle control deregulation has been recognized as a common occurrence during carcinogenesis, with NSCLC being no exception. Many studies have highlighted the aberrant expression, due to genetic or epigenetic alterations, of at least one key control element in primary lung tumors (3–6), occasionally with prognostic significance (7). However, few studies have compared multiple molecular markers of the complex interaction among the cellular control elements and their prognostic implications.

Effective control by the cell cycle checkpoints ensures repair of damaged DNA before replication and prevents the maintenance of deleterious genetic abnormalities (8). It is not surprising, therefore, that abnormalities of the key proteins involved in the cell cycle and its control are among the most commonly altered in human cancers (9–11). Two major pathways involved in the cellular progression from G0–S phase include the Rb (Rb, cyclin D1, and p16Ink4a) and p53 (p53/p21Waf1) cell cycle pathway and the G1/S checkpoint arrest pathway. In the Rb pathway, the essential components include cyclin D1 complexed with cdk4/cdk6 (the enzymatically active complex capable of phosphorylating pRb), a specific cdk4 inhibitor such as p16Ink4a, and pRb itself (12) that, on phosphorylation in mid-to-late G1, allows for cellular progression (13). The main effector protein of the p53 G1 checkpoint arrest pathway is the universal
cell cycle cyclin-dependent kinase inhibitor, p21Waf1 (14, 15), which inhibits kinase activity and subsequently pRb phosphorylation in situations of genetic damage (15, 16). pRb is a common denominator link between the two pathways, and cellular progression and ultimately tumor propagation can be achieved by deregulated pRb phosphorylation, genetic mutation, or functional inactivation with sequestration of the pRb gene product (13). In NSCLC, genetic mutation or loss of the Rb gene and gene product is uncommon but is more commonly seen in small cell lung cancer cases (3), whereas alterations of the phosphorylation status seem to play a more substantive role. Data strongly implicate at least the D-type cyclins and cyclin E in this phosphorylation activity (13, 17–20), and in keeping with clonal expansion, selective maintenance of abnormalities of these proteins would be advantageous to tumor progression (21).

Cyclin D1 (G1-phase cyclin) abnormalities are a common occurrence in human carcinomas, including NSCLC (3, 4, 6, 11, 22). This protein functions as a growth factor sensor (23) as well as being involved in cell growth and proliferation (24, 25). Its stimulatory role has also been speculated from the presence of protein overexpression in many tumors, including NSCLC by a variety of mechanisms [e.g., gene amplification, chromosomal rearrangement, or as yet undefined (11, 22–28)], and the sometimes significant association of it with proliferation indices such as Ki-67 (29) and proliferating cell nuclear antigen (6). Its oncogenic potential has been shown in vitro (30, 31), and its possible oncogenic role in vivo is deduced from identifying alterations in human tumors (22). Indeed, it has been proposed that one of the mechanisms by which NSCLC evaded cell cycle controls was by the abnormal expression of cyclin D1 (32). However, analysis of cyclin D1 in isolation does not allow us to categorically document its exact effect in carcinogenesis, as confounding data exist on its effect on cellular growth. Cyclin D1 overexpression can cause cell cycle arrest (33) and induce apoptosis (34). Pagano et al. (35) showed that the down-regulation of cyclin D1 was necessary to start DNA replication, and Han et al. (36) reported that stable high expression of exogenous cyclin D1 markedly inhibited, rather than enhanced, the growth of breast cancer cell lines. Therefore, it seems the timing and amount is just as important as the presence of overexpression. Cyclin D1 cooperation with other proteins also cannot be understated, because it can be an effector of either growth suppression or growth progression. For instance, it has been shown that cyclin D1 and p21Waf1 together are involved in the normal p53 growth suppression pathway (33, 37). Therefore, given the varied roles of cyclin D1 and its complex interaction with other proteins, it is not surprising that isolated cyclin D1 correlations gave such varied results and further emphasized the need for complete pathway analysis to fully understand the malignant potency of any given tumor.

The directional mechanism of the cdk4 molecule, complexed to cyclin D1 and to its substrate pRb, is a major effector pathway for this cyclin D1 protein (20). Indeed, all of the cyclin-dependent inhibitors play major roles in maintaining tight cellular control. The main G1-phase cyclin-dependent inhibitors of interest to this study that impinge on the Rb pathway are the cyclin-dependent kinase-selective inhibitor p16INK4A and a universal inhibitor such as p21Waf1. p16INK4A, a selective cdk4/ cdk6 inhibitor, has an intimate association with cyclin D1/cdk4 and pRb (38). p16INK4A can control and also seems to be under pRb regulatory control itself. In cells in which pRb was constitutively inactive (phosphorylated), p16INK4A expression increased with consequent cdk4 inhibition and shutdown of G1 kinase activity (38). This underlies the identification of a reciprocal arrangement between these proteins often seen in NSCLC (39–41). Therefore, loss of p16INK4A allows for continued, uncontrolled tumor proliferation and is an abnormality that is found quite often in NSCLC (3, 4, 40–42). p53 is a prominent growth suppressor and is commonly mutated in diverse tumor types (9), p21Waf1 is the main intermediary of the wild-type, p53-induced checkpoint control (14), and the wild-type p53 tumor suppressor gene (14) directly regulates its gene expression. p21Waf1 inhibits growth when levels are increased because it not only functions as a potent universal inhibitor of cyclin-dependent kinases (15, 43), including the cyclin D1 cdk4/cdk6 but also directly inhibits proliferating cell nuclear antigen-dependent DNA replication (44). Thus, by increasing the amount of p21Waf1, p53 indirectly inhibits pRb phosphorylation and achieves growth arrest. However, growth arrest may also involve cyclin D1, as discussed previously, a known growth promoter, further increasing the complexity of the protein interactions in any one pathway.

The cell cycle control of the G0-S phase involves a complex interplay of various cell proteins. To date, many studies have looked at individual or small numbers of components within this cell cycle phase, including those of the two major cellular control pathways. Some have found synergistic relationships among protein abnormalities and survival of their patients. However, the interrelationship between the proteins within each pathway and between both pathways is necessary to fully understand the mechanisms at play in any given tumor. We addressed both the Rb and the p53 pathways in our analysis of 106 cases of NSCLC and addressed the significance of abnormalities in one or both pathways in tumor development together with survival data, where the underlying hypothesis is that abnormalities or accumulations of abnormalities may have prognostic potential.

MATERIALS AND METHODS

Sample Population Details. The study population consisted of 106 consecutive surgically resected cases of primary NSCLC collected from the Mayo Clinic (Rochester, MN) between 1991 and 1992. These cases were collected in a prospective fashion by selection criteria that included all cases where patients went to thoracic surgery for removal of lung tumor and nontumor tissue that could be saved in frozen samples and where blood could be drawn for research studies. All patients were thought to be resectable before the operation, and the large majority was completely resected at the time of operation. Some were found to have a metastatic lesion in another lobe (stage IV) at the time of operation and had this metastatic lesion resected. No induction therapy was done on any patient, and surgery was done by one of three Mayo Clinic thoracic surgeons. No patient had received previous chemotherapy or radiation therapy before diagnosis and entry into this study. The patients signed a consent form to participate in the study according to an approved Mayo Clinic institutional review board research protocol and they completed an extensive...
immunostained slides for cyclin D1, pRb, p16INK4A, and p21Waf1 were evaluated by at least two pathologists: cyclin D1 (L.B. and W.P.B.), pRb and p16INK4A (D.B.F. and E.B.), and p21Waf1 (R.T.J., A.B., and W.P.B.). Immunohistochemical analysis for p53 was described previously (48). Any differences in initial independent evaluations were resolved at a multiheaded scope and a consensus reached. The following criteria were used to assess distribution and intensity of positive tumor cell staining, whereas nuclear coloration was recognized as the primary standard for demonstrating a positive reaction (Supplemental Figs. 1, 2, and 3).

**Distribution.** Absent tumor cell staining was scored as 0, <10% of positive tumor cells staining were scored as 1, 10% to 50% of cells staining were scored as 2, 50% to 90% of cells staining were scored as 3, and >90% cells staining were scored as 4.

**Intensity.** Absent staining in tumor cells was scored as 0, equivocal was scored as 1, clearly positive was scored as 2, and strongly positive staining was scored as 3.

For cyclin D1, p53, and p21Waf1, the results for intensity and distribution were summed and a “score” assigned as follows: sum of 0, no staining (score 0); sum of 1 to 3, slight staining (score 1); sum of 4 to 5, moderate staining (score 2); and sum of 6 to 7, marked staining (score 3). Overexpression for each of these three antibodies was assigned when a score of 2 to 3 was achieved. For pRb and p16INK4A, the results for distribution alone were used and positive staining was assigned to the percentage of nuclei staining positively with the antibody (4, 42). For each assay, a distribution value of 0 to 1 (negative or <10% of cells staining positively) was taken as negative, with all other cellular distributions regarded as positive.

**Statistical Analysis.** Statistical analysis was carried out using the SPSS Statistical Program version 9.0 for Windows. Associations between each of these proteins with clinical, pathologic, epidemiologic, and survival data were explored and assessed using the χ² and Fisher Exact tests depending on the cellular frequencies observed in 2 × 2 tables.

Associations were considered statistically significant if two-tailed P < 0.05. Kaplan-Maier curves were used to estimate survival probability as a function of time, and patient survival differences were analyzed by the log-rank test. Death from tumor was regarded as a censored event. As this study is an ongoing research project, the results were analyzed in relation to previous data for other genetic markers and protein

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**Immunohistochemical Analysis.** All immunohistochemical assays were carried out on 10% formalin-fixed, paraffin-embedded tissue sections cut to 5-μm thickness. Immunohistochemical staining for cyclin D1 used antigen retrieval in Antigen Retrieval CITRA solution (Biogenex, San Ramon, CA). The immunostaining using the mouse monoclonal antibody clone, DCS-6 [cyclin D1 (Ab-3); Oncogene Science, Inc., Boston, MA], was done on the Ventana automated immunostainer (Ventana Corp., Tucson, AZ) using specified procedures and reagents. The primary antibody, at a dilution of 1:40, was incubated for 32 minutes, and the streptavidin-biotin complex immunoperoxidase detection system was used. The chromogenic substance for the immunoperoxidase was 3,3’-diaminobenzidine tetrachloride, and the counterstain used was 1% methyl green (Becton Dickinson, San Jose, CA). Formalin-fixed sections of a murine tumor explant grown from human cornea epithelial cells with transfected cyclin D1 were used as the positive control tissue. Negative controls involved immunohistochemical staining of all sample tissue sections and tumor explant tissues, with buffer replacing the primary antibody.

The p16INK4A immunostaining used the polyclonal antibody p16INK4A C-20 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200. Endogenous peroxidase activity was blocked with H2O2 in H2O (0.3%) for 5 minutes. Suppression of nonspecific immunostaining was achieved using an incubation step with low-fat milk 2% in H2O for 30 minutes. After overnight incubation at 4°C with the primary antibody, slides were washed in PBS and then exposed to the biotinylated secondary, donkey anti-rabbit antibody at a dilution of 1:1,000 (The Jackson Laboratory, Bar Harbor, ME) for 1 hour at room temperature. The chromogenic substrate for the peroxidase solution was 0.05% 3,3’-diaminobenzidine tetrahydrochloride/0.03% H2O2/10 mmol/L imidazole on 0.05 mol/L Tris buffer (pH 7.6). The slides were counterstained with Harris hematoxylin. Normal rabbit immunoglobulin G, at the same concentration as the primary antibody, served as a negative control.

pRb immunohistochemical analysis was also carried out by hand and used the rabbit polyclonal Rb C-15 (Santa Cruz Biotechnology) antibody at a dilution of 1:2,000. This antibody recognizes both the phosphorylated and the nonphosphorylated pRb110 protein. The methodology used was as described for the p16INK4A assay, with the exception of the addition of microwave antigen retrieval in citrate buffer for 2 × 5 minutes (pH 6) before the incubation with the primary antibody. The staining methods and results of the immunohistochemical analyses on these tumor cases for p21Waf1 and p53 have been described previously (47, 48). The p21Waf1 and p53 antibodies used were the EA10 monoclonal antibody (1:50, Oncogene Science) and the DO-7 monoclonal antibody (1:50, DAKO Corp., Carpinteria, CA), respectively.
analyses studied, including genetic polymorphisms (48–50), transforming growth factor-β (47), and allelic deletion analysis of FHIT gene (51) and p53 (48, 52).

RESULTS

Results of Individual Assays and Univariate Analysis: Cyclin D1 Immunohistochemical Analysis. Statistical analyses for 105 of 106 cases were available: one case lacked an identifiable tumor on the pertinent immunohistochemical slide. In total, 50 of 105 (48%) cases displayed D1 overexpression (score 2–3). This percentage approximately correlated to >10% of tumor cell nuclei staining positively (4). In the univariate analysis exploring relationships to clinicopathologic variables, no significant associations to cyclin D1 levels were identified in relation to gender, family history of any cancer, smoking history, tumor grade, tumor size, or necrosis. Although overexpression was identified in all tumor stages, a statistically significant association between higher-stage tumors (stages III and IV) and cyclin D1 overexpression was found (P = 0.02). Stage I and II tumors combined were more likely to have negative levels of cyclin D1 overexpression. When you compare stage I versus stage II versus stage III and IV combined, statistical significance is maintained (P = 0.06). However, statistical significance was not identified when stage I was compared with all other stages combined (P = 0.2). Although not statistically significant, an interesting trend regarding tumor histology was seen with cyclin D1 overexpression occurring more commonly in SQCC (see Table 1). Significant relationships to the presence of genetic polymorphisms (CYP1A1, CYP2E1, and GSTM1), immunohistochemical analyses for CerbB2, and proliferation index Ki-67 or to apoptotic pathway proteins were not identified. Cyclin D1 levels did not show any significant association to affiliated immunohistochemical assays for proteins such as transforming growth factor-βRII and p27 or to allelic deletion of the FHIT gene or Ras mutations. Cyclin D1 levels were not associated with p53 immunopositive assays or mutation. Of the 92 analyzed cases, we noted that there was a bias toward cyclin D1 overexpression occurring in 10 of 15 cases with a G:C to T:A p53 mutation (P = 0.2). On analyzing the relationship between p53 mutations with aberrant expression of cell cycle proteins, it was found that the majority of cases with immunopositive cyclin D1 were also positive for pRb [84 of 102 (82%)]. Only 7 of 18 cases negative for pRb displayed cyclin D1 overexpression. No significant associations between cyclin D1 expression and pRb (P = 0.4) or p16INK4A (P = 0.5) were found.

pRb Immunohistochemical Analysis. Statistical analysis for 103 of 106 cases was evaluated. Seventeen percent of the cases were immunonegative (loss of protein expression) for pRb. The majority of cases, 85 of 103 (83%), were pRb positive. Statistically significant associations of pRb expression to the following clinicopathologic variables were not identified in the initial univariate analysis: gender, age, smoking history, family history of cancer or any history of cancer in first-degree relatives, and tumor stage. Although not statistically significant, it does seem that pRb negative was more commonly associated with poorly differentiated tumors, because 14 of 18 cases clustered within this latter category, whereas the four remaining cases were classified as moderately differentiated lesions (P = 0.4; trend test, P = 0.25). A significant association with histology was identified. As illustrated in Tables 1 and 3, tumors were more likely to be pRb positive than negative, with negative being more significantly associated with SCC (P = 0.05) and pRb positive significantly associated with SQCC (P = 0.02).

p16INK4A Immunohistochemical Analysis. Immunohistochemical analysis of 96 cases revealed a p16INK4A immunonegative rate of 53% (51 of 96 cases). Positive staining of >10% of the cells was observed in 47% (45 of 96) of the cases. The latter staining was heterogeneous with 10% to 90% of the cells stained positively. On univariate analysis, there was no significant association with CerbB2, p27kip1, or transforming growth factor-βRII immunopositive, FHIT loss of heterozygosity, or Ras mutations. There was no relationship to gender, tumor necrosis, or smoking history. In the analyzed genetic polymorphisms, a significant association with CYP1A1 was noted (P = 0.04). p16INK4A-positive cases were more likely to be CYP1A1 mutant or at-risk heterozygotes, whereas p16INK4A-negative cases were normal for CYP1A1. There was no significant association to the other genetic polymorphisms examined (i.e., GST or CYP1E1). In relation to a positive history for lung or other cancers, these patients were more often p16INK4A negative (P = 0.03 and 0.005, respectively). A significant association between p16INK4A and a family history of lung cancer was not identified. As illustrated in Table 1, and

Table 1

<table>
<thead>
<tr>
<th>Histologic Subtype</th>
<th>Cyclin D1+</th>
<th>pRb−</th>
<th>p16INK4A−</th>
<th>p21^kip1+</th>
<th>p53+</th>
</tr>
</thead>
<tbody>
<tr>
<td>SQCC</td>
<td>24/54 (44)</td>
<td>10/52 (19)</td>
<td>27/49 (55)</td>
<td>23/55 (42)</td>
<td>23/55 (43)</td>
</tr>
<tr>
<td>LCC</td>
<td>17/29 (59)</td>
<td>1/29 (3)</td>
<td>10/26 (39)</td>
<td>12/29 (48)</td>
<td>17/29 (59)</td>
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</table>

| Cyclin D1+ | 9/22 (41) | 7/22 (32) | 10/22 (46) | 13/22 (59) |

NOTE: +, immunopositive for p16INK4A and pRb and overexpression of cyclin D1, p21^kip1, and p53; −, immunonegative for p16INK4A and pRb and negative for overexpression of cyclin D1, p21^kip1, and p53. *P = 0.02. †P = 0.05.

Table 2

<table>
<thead>
<tr>
<th>p53 Mutation</th>
<th>Cyclin D1+</th>
<th>pRb−</th>
<th>p16INK4A−</th>
<th>p21^kip1+</th>
<th>p53+</th>
</tr>
</thead>
<tbody>
<tr>
<td>No p53 mutation, n (%)</td>
<td>22/47 (47)</td>
<td>12/30 (40)</td>
<td>10/15 (67)</td>
<td>7/29 (24)</td>
<td>1/13 (77)</td>
</tr>
<tr>
<td>Non-G:C to T:A, n (%)</td>
<td>23/44 (52)</td>
<td>13/26 (50)</td>
<td>7/29 (24)</td>
<td>6/13 (46)</td>
<td></td>
</tr>
<tr>
<td>G:C to T:A, n (%)</td>
<td>18/47 (38)</td>
<td>11/30 (37)</td>
<td>1/13 (77)</td>
<td>9/15 (60)</td>
<td></td>
</tr>
</tbody>
</table>

Note: +, immunopositive for p16INK4A and pRb and overexpression of cyclin D1, p21^kip1, and p53; −, immunonegative for p16INK4A and pRb and negative for overexpression of cyclin D1, p21^kip1, and p53. *P = 0.2. †P = 0.4.
although not statistically significant, there was an increased frequency of p16\(^{INK4A}\) negative in adenocarcinoma. Regarding tumor grade and differentiation, p16\(^{INK4A}\) negative was seen more often in higher-grade tumors (grades 3 and 4; \(P = 0.02\)). There was, however, no significant association with tumor stage. There was no association to pRb, nor was there a reciprocal arrangement between these two proteins. In addition, there was no significant association with cyclin D1 (\(P = 0.5\)), p21\(^{Waf1}\) (\(P = 0.1\)), p53 mutations (\(P = 0.7\)) or p53 immunopositive (\(P = 0.5\)), nor was there any significant association with the presence of G:C to T:A mutations (\(P = 0.9\)).

**p21\(^{Waf1}\) Immunohistochemical Analysis.** The results of this assay have been published previously (47). However, for the purposes of this study, stratification of immunohistochemical results was changed to mirror p53 and cyclin D1. In total, 106 cases were analyzed, and of those cases, 59 (56%) were negative for p21\(^{Waf1}\) overexpression, whereas 47 (44%) cases were considered positive for overexpression. In the univariate analysis, there was no significant association with p53 protein, cyclin D1, p16\(^{INK4A}\), or pRb. In addition, there was no significant association with smoking history, apoptotic tertiles, bax score, bcl2, transforming growth factor, transforming growth factor-pRbII, genetic polymorphisms (CYP1A1, GSTM1, or CYP2E1), FHIT loss of heterozygosity, Ki-67, or Ras. There was a significant association seen with p27 (\(P = 0.05\)). There is no significant association with stage, grade of the tumor, or histology. Interestingly, p21\(^{Waf1}\) levels negative for overexpression were significantly associated with larger tumor volume (\(P = 0.01\)). p21\(^{Waf1}\) overexpression was also significantly associated with males rather than with females. In addition, there was no significant association with p53 missense mutations (\(P = 0.3\); Table 2).

**p53 Mutation and Protein Immunohistochemical Analysis.** The results of this assay have been published previously (48). There was a significant correlation between the highest level of p53 immunohistochemical staining (score 3) and p53 missense mutations in exons 5 to 8 (\(P < 0.001\)). A total of 45 mutations were identified in the cases analyzed. p53 immunohistochemical data of negative (score 0–1) versus overexpression (score 2–3) revealed that there were 52 (50%) and 54 (50%) cases, respectively. In the univariate analysis, significant relationships of this protein with cyclin D1 (\(P = 0.2\)), p21\(^{Waf1}\) (\(P = 0.4\)), p16\(^{INK4A}\) (\(P = 0.5\)), and pRb (\(P = 0.3\)) were not identified as outlined previously. p53 protein analysis was not significantly related to tumor volume (\(P = 0.5\)) or histology (\(P = 0.2\)). The p53 protein score, however, does seem to be significantly associated with stage (\(P = 0.038\), Fisher’s exact test). In stage I lesions, there was an equal preponderance of p53-positive and p53-negative expression cases [26 of 52 (50%) in each category]. In stage II tumors, the majority was p53 positive [14 of 18 (78%) cases]. In stage III and IV combined, 12 of 33 (36%) cases show p53 protein overexpression, with 21 of 33 (67%) cases maintaining levels considered negative for overexpression by immunohistochemistry (\(P = 0.02\)). In both stage I and II lesions, p53 tended to be overexpressed [40 of 70 (57%) versus 30 of 70 (43%)], whereas stage III and IV lesions favored p53 levels negative for overexpression as indicated above (\(P = 0.05\)). An association between p53 immunohistochemical analysis and tumor grade was noted. p53 overexpression was significantly associated with high-grade tumors (\(P = 0.04\)).

**pRb Pathway Protein Abnormalities (cyclin D1, p16\(^{INK4A}\), and pRb).** In each of the univariate analyses, there was no statistically significant association between any two proteins of the Rb pathway (i.e., cyclin D1, pRb, and p16\(^{INK4A}\)); however, we observed an interrelationship of all three proteins. The analysis of 94 cases is highlighted (Supplemental Table 1).

Tumor abnormalities of all proteins were assessed as follows: only 3% (3 of 94) of the cases had an abnormality of all three proteins within the pathway, with cyclin D1 overexpression, and the losses of pRb and p16\(^{INK4A}\) proteins. Thirty-two percent (30 of 94) of the cases had an abnormality of two proteins within the pathway, and in this subset of 30 cases, 70% (21 of 30) of the cases contained a concurrent abnormality of both cyclin D1 and p16\(^{INK4A}\). In 48% (45 of 94) of the cases, there was an abnormality of one protein only, the most common being p16\(^{INK4A}\) (47%, 21 of 45) followed by cyclin D1 (44%, 20 of 45). Only 17% (16 of 94) of the cases did not contain an abnormality of any of the three main proteins of the pRb pathway.

**p53 Pathway Abnormalities (p53 and p21\(^{Waf1}\)).** There is no association of either p53 (\(P = 0.5\)) or p21\(^{Waf1}\) (\(P = 0.55\)) alone with survival. In the 105 cases immunohistochemically analyzed, 52 of 105 (50%) cases showed p53 overexpression, with p21\(^{Waf1}\) overexpression in 25 of 105 (24%).

**Relationship of Rb Pathway Abnormalities with the p53 Pathway.**

This was also of interest to look at the relationship of abnormalities of the Rb pathway with the presence or absence of p53 mutations in 83 tumors in which simultaneous results were available (Supplemental Table 2). Abnormalities of only the Rb pathway were seen in 37 of 83 (45%) cases, whereas 8 of 83 (10%) cases harbored only a p53 mutation. Only 7 of 83 (8%) cases of NSCLC analyzed failed to show an abnormality of either the Rb or p53 pathway, whereas in 31 of 83 (37%) cases there was a concurrent abnormality of both pathways highlighting their cooperation in NSCLC development.

**Rb and p53 Pathway Analysis in Relation to Survival.**

With the knowledge from the above results that Rb and p53 pathway abnormalities are critical in this series, we hypothesized that their identification alone or in specific combinations may be able to predict patient survival. Interesting and somewhat complex results emerged, our contention that multiple proteins of the cell cycle need to be examined concurrently to more accurately assess and understand the complexity of their interactions and their influence on survival.

Abnormalities of each of the Rb pathway proteins, cyclin D1, pRb, and p16\(^{INK4A}\), in isolation were not associated with a

### Table 3  pRb immunohistochemical analysis according to the histologic subtype of NSCLC

<table>
<thead>
<tr>
<th>Histologic subtype</th>
<th>pRb−, n (%)</th>
<th>pRb+, n (%)</th>
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<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>10 (19)</td>
<td>42 (81)</td>
</tr>
<tr>
<td>SQCC</td>
<td>1 (3)</td>
<td>28 (97)</td>
</tr>
<tr>
<td>LCC</td>
<td>7 (32)</td>
<td>15 (68)</td>
</tr>
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</table>

NOTE: −, immunonegative for pRb; +, immunopositive for pRb.
poorer survival ($P = 0.1, 0.3$, and 0.4, respectively). Abnormalities of the p53 pathway proteins analyzed here (i.e., p53 and p21$^{Waf1}$) in isolation were also not associated with a poorer survival ($P = 0.5$ and 0.6, respectively). However, what revealed possible prognostic markers and therapeutic targets were specific combinations of protein abnormalities that gave statistically significant results with survival time.

Analysis of the Rb pathway proteins revealed the following results. Survival analysis of various combinations of pRb and p16$^{INK4A}$ abnormalities did not identify any significant combination with prognostic potential ($P = 0.3$). In addition, although we did identify cases with a concurrent abnormality of cyclin D1 and p16$^{INK4A}$, survival analysis with various combinations of cyclin D1 and p16$^{INK4A}$ abnormalities did not identify any significant associations/prognostic potential ($P = 0.7$). However, the specific combination of pRb negative and cyclin D1 protein overexpression was significantly associated with a poor survival in these patients ($P = 0.004$; Fig. 1). All seven patients in this group were dead by 4.7 years, with a mean survival of 2.3 years and a median survival of 1.6 years. All other combinations of pRb and cyclin D1 had a mean survival of 4.1 years, with a median survival of 6.1 years. Knowing that abnormalities of both Rb and p53 pathways occurred in several (37%) patients, we addressed the significance on survival of a concurrent p53 mutation in these cases. The survival time of this cohort group of seven patients was significantly worse. In three of the seven patients who also concurrently contained a p53 mutation, the mean survival time decreased to 0.9 years, with a median of 0.8 years, and all three patients were dead by 1.4 years after diagnosis. With all other combinations, the mean survival time was 4.0 years ($P = 0.007$; Fig. 2). This relationship was also maintained when analyzed with p53 immunohistochemical analysis ($P = 0.006$).

Neither p53 mutation nor abnormal p53 immunohistochemical expression is significantly associated with poorer survival ($P = 0.8$ and 0.5, respectively), nor is p21$^{Waf1}$ analysis ($P = 0.6$). However, when we consider abnormalities involving the two pathways coincidentally, some interesting results emerged. In any variation of the Rb pathway abnormalities (taking all three proteins analyzed into consideration), the presence or absence of a p53 mutation did not make a significant difference on survival. However, examination of the combination of a p53 overexpression/mutation with that of cyclin D1 overexpression and pRb negative alone, as discussed previously, was associated with a significant effect on survival (Fig. 2). However, looking at individual protein relationships, interesting results emerged. There was no significant association with survival in those cases containing a p53 mutation in association with p16$^{INK4A}$ or pRb negative ($P = 0.6$ and 0.9, respectively). However, the presence of p53 overexpression or a p53 mutation in association with cyclin...
D1 overexpression displayed a significant relationship with survival ($P = 0.004$ and $0.003$; Figs. 3 and 4, respectively). For instance, in the subgroup of patients with overexpression for p53 and cyclin D1, the mean survival was 2.8 years, whereas that for all other combinations of the proteins was 4.3 years. With a p53 mutation, the cyclin D1 overexpression group did significantly worse, with a mean survival of 2.6 versus 4.9 years for those cases with negative cyclin D1 overexpression). Although p21$^{\text{War}}$ is the effector molecule of wild-type p53 function and occasionally in conjunction with cyclin D1, we analyzed the cyclin D1 results to see if p21$^{\text{War}}$ displayed any effect. There was no significant relationship between p21$^{\text{War}}$ protein levels and cyclin D1 and survival ($P = 0.1$).

DISCUSSION

p53 and Rb genes and their pathways involving the G1-S phase transition are commonly affected genes in lung cancer (3, 4, 9), and our study supports this observation as only 7 of 83 (8%) cases in our series of NSCLC failed to show an abnormality of either pathway. Conceptually, however, these data also raise the possibility that other mechanisms important in a few NSCLC cases exist. Forty-five percent and 10% of cases showed an abnormality of the pRb and p53 pathways, respectively, whereas 31 of 83 (37%) cases harbored a concurrent abnormality of both pathways. Only 16 of 94 (17%) cases had no abnormality of the Rb pathway, a finding somewhat similar to that of Geradts et al. (53) and Betticher et al. (54). Geradts et al. categorized their NSCLC cases into four categories based on the analysis of three proteins (pRb, p16, and p53). Twenty-one percent of tumors were normal for all three proteins, 30% were abnormal for pRb or p16 and normal for p53, 20% were normal for pRb or p16 and abnormal for p53, and 28% of tumors showed an abnormality in both pathways (53). Betticher et al. meanwhile showed that only ~10% of cases had no detected abnormality of the Rb pathway in his series (54). In our analysis of the relevant proteins, cyclin D1 overexpression was identified in 47.6%, with loss of p16 and pRb in 53% and 17%, respectively, somewhat similar to other studies (4, 5, 29, 40, 41, 53–58). p21 was overexpressed in 44% of cases with p53 overexpression in 50% of cases and a total of 45 mutations identified within the tumor series, again somewhat similar to that already documented using standard methodologies (53, 56, 58–62).

The exact role played by each of the proteins analyzed in tumor development in our series is not easy to define. Cyclin D1 seems to be not only involved in tumor growth initiation, as it was overexpressed in 40% of stage I and II tumors, but also significantly involved in the continued growth of tumors, as cyclin D1 overexpression was more common in higher-stage lesions ($P = 0.02$). The exact method by which cyclin D1 exerts its effect cannot be ascertained from our results, as there was no association identified between it and the proliferation index Ki-67 immunolabeling or to the apoptotic indices examined. However, we do know that cyclin D1 can exert its tumorigenic affect through other mechanisms, such as failure of DNA repair (35), etc., and these factors may be at play here. Cyclin D1 immunopositive showed a bias toward tumors of the squamous cell histologic subtype, and interestingly, there was a bias toward cyclin D1 overexpression in cases with a G:C to T:A transversion ($P = 0.2$), although a significant association with any of the smoking variables analyzed was not identified.

Loss of pRb function in NSCLC is uncommon, and our rate of loss of 17% is in line with the 6.5% to 60% range of many other studies reviewed (4, 5, 53, 55, 58–60), with the highest rate of loss being identified in a study of advanced lung cancer cases (stage IIIA-IV inclusive; ref. 61). An increase of pRb-negative cases within the poorly differentiated category was noted. In addition, pRb negativity was statistically more associated with the LCC histologic subtype, as noted previously (55), a subtype of carcinoma that is associated with a poorer survival.

In keeping with the functional redundancy between Rb and p16, we noted a higher rate of p16 negativity. Interestingly, p16 negativity was associated with a history of cancer other than that of lung, and in those cases in which there was mutant or heterozygote CYP1A1, p16 was maintained. p16 negativity was more commonly seen in cases without this genetic polymorphism, indicating that maintenance of p16 was advantageous for the preserving some control within the cell cycle framework. p16 negativity from other studies was particularly in the adenocarcinoma histologic subtype (41). In addition, p16 was more often negative than positive in adenocarcinoma and LCC than in SQCC, although this result did not reach statistical significance and p16 showed no particular association to the presence of a G:C to T:A smoking-induced transversions (65). Although we did not observe a statistically significant reciprocal arrangement between pRb and p16 abnormalities in any histologic subtype, as seen in other studies (5, 39–41), we did observe that simultaneous loss of both of these proteins only occurred in 8 of 95 (7%) cases. On analyzing further the reciprocal arrangement observed in other studies, many but not all of the studies showed a p16-negative rate higher in SQCCs than in adenocarcinomas, a finding that was contrary to our findings (5, 40, 41, 64, 69). Indeed, Kelley et al. (39) and Geradts et al. (66) both showed that not all tumors exhibit this reciprocal arrangement as did our study; indeed, Leversha et al. (64) also showed that the strong inverse correlation between pRb and p16 expression seen in SQCCs was essentially absent in adenocarcinoma (64). Interestingly, our study cohort consisted predominantly of adenocarcinoma (55 versus 29).

In the pRb pathway itself, the most common pathway abnormality in our series is that of p16 loss, followed by cyclin D1 overexpression and lastly by pRb loss. It is also interesting to document that only three (3%) cases contained an abnormality of all three proteins, suggesting that it is functionally and biologically redundant to affect all proteins within this same area of the cell cycle. There was no association between survival and any of the individual protein abnormalities in this study.

A concurrent abnormality of two proteins, however, within the same pathway was observed. In 25% of cases, we had a concurrent abnormality of both p16 and cyclin D1 protein, an abnormality that did not have a significant affect on survival. Concurrent abnormality, however, between cyclin D1 and pRb

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did significantly affect patient survival. Negative for pRb in association with cyclin D1 overexpression, although only seen in seven cases, significantly predicted a poor survival for patients harboring this abnormality (P = 0.004; mean survival 2.3 years, median 1.6 years compared with 4.1 and 6.1 years, respectively, for all other combinations), mirroring results identified in another study whereby two or three abnormalities of proteins analyzed displayed a significantly poorer prognosis (cyclin D1, p21Waf1, and pRb analyzed; ref. 60). In our study, cyclin D1 overexpression is associated with higher-stage tumors and pRb loss is more often seen in poorly differentiated tumors. Some authors, but not all, view Rb binding as dispensable for the oncogenic function of cyclin D1 (30); indeed, Hinds et al. (19), in an earlier article, were of the view that cyclin D1 overexpression itself could result in a profound depression of pRb leading to the conclusion that this protein combination is biologically sustainable within any tumor. When a p53 mutation is also associated with this combination, the negative effect on survival is even more dramatic, with all three patients dying within 1 year. This combination is particularly interesting given that a recent study by Gregore et al. (61) showed that abnormalities of p53 and pRb were not associated with any survival difference in any combination; however, our additional investigation of this relationship in the presence of cyclin D1 overexpression with demonstration of a survival difference further highlights the complex nature of these protein interactions.

The effect of a p53 mutation is also significant when associated with an abnormality of cyclin D1 of itself, a finding observed in 31 of 83 cases. Cyclin D1 overexpression associated with a p53 mutation or p53 protein overexpression was associated with a poorer survival in this group of patients in contrast with all other combinations of these proteins (P = 0.0033 and 0.004, respectively). This is not surprising because p53 overexpression was associated more with higher-grade lesions. We have shown that both abnormalities of the Rb and the p53 pathway play substantial roles in the development of NSCLC; however, we have also shown that cumulative abnormalities affecting more than one protein within the same pathway or from a different pathway may have significant effects on patient survival. Because the numbers analyzed in these subsets are small, caution is needed in their interpretation. These results, especially when noted that the significant survival differences were not maintained when all proteins of both pathways were analyzed in conjunction with one another, highlight the complexity of the interrelationships of these proteins at this critical phase of the cell cycle, a finding similar to that observed also by Geradts et al. in their analysis of Rb, p16, p53, and 3pLOH (53). These results raise the element of caution in overinterpreting survival results when specific protein abnormalities are taken in isolation.

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Prognostic Implications of Molecular and Immunohistochemical Profiles of the Rb and p53 Cell Cycle Regulatory Pathways in Primary Non–Small Cell Lung Carcinoma

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