**Advances in Brief**

**Altersations of Rb Pathway (Rb-p16\(^{INK4a}\)-Cyclin D1) in Preinvasive Bronchial Lesions\(^1\)**

Elisabeth Brambilla,\(^2\) Sylvie Gazzeri, Denis Moro, Sylvie Lantuejoul, Sylvie Veyrenc, and Christian Brambilla


**Abstract**

Lung cancer results from a stepwise accumulation of genetic and molecular abnormalities with unknown temporal relationships to precursor bronchial lesions. In a search for biomarkers of malignant progression, we analyzed the expression of the tumor suppressor gene Rb and of the proteins regulating its phosphorylation and function in G\(_1\) arrest, p16\(^{INK4a}\) and cyclin D1, in preinvasive bronchial lesions accompanying cancer in 75 patients, in comparison with similar lesions in 22 patients with no cancer history. Rb was constantly expressed in preinvasive lesions, including carcinoma in situ (CIS). In contrast, p16 expression was lost in moderate dysplasia (12%) and in CIS (30%) in patients with lung cancer. p16 loss occurred exclusively in patients who displayed loss of p16 expression in their related invasive carcinoma. Loss of p16 expression was not seen in nine patients with dysplasia but no cancer progression. Cyclin D1 overexpression was seen in hyperplasia and metaplasia (6%), mild dysplasia (17%), moderate dysplasia (46%), and CIS (38%) in patients with cancer but was lost in 5% of the patients during the process of invasion; it was also observed in patients with no cancer progression (14%). Our results indicate that Rb protein function can be invalidated before invasion through alteration of the Rb phosphorylation pathway, by p16 inhibition, and/or by cyclin D1 overexpression and suggest a role for p16 and cyclin D1 deregulation in progression of preinvasive bronchial lesions to invasive carcinoma.

**Introduction**

Lung cancer is the leading cause of cancer-related death in industrial countries, and cigarette smoking is its main risk factor. Most patients cannot be cured because they present with advanced stages of the disease, and prognosis remains poor despite therapeutic improvements (1–3). Much evidence has been provided that invasive lung cancer is the end result of the stepwise accumulation of genetic alterations. The accumulation of 10–20 successive mutations should allow progression to invasive carcinoma (4). Morphological changes accompanying this transformation process have been described in detail in smokers (5). They progress from hyperplasia to metaplasia, which are rather common reactive lesions, to dysplasia and CIS, which are considered to be at risk for cancer development (5, 6). However, despite increasing risk of malignant transformation with histopathological grade, all these lesions are able to regress, including CIS (7, 8). In contrast, minimal lesions, such as hyperplasia and dysplasia, have been shown to display genetic and molecular changes (9, 10), and two recent studies demonstrated loss of one allele of chromosomes 3p, 9p, and 17p in normal bronchial mucosa of current and former smokers (11, 12). Thus, the morphological classification has a predictive value but cannot predict exactly for each individual case. It is believed that multiple intraepithelial lesions develop at various times in patients exposed to carcinogens, which supports the idea that the entire bronchial mucosa is damaged by carcinogens. This phenomenon is referred to as the “field cancerization” process. At present, neither the temporal sequence of the genetic abnormalities nor their relationship to specific morphological states has been precisely established. Because effective chemoprevention may be the most promising clinical approach, elucidating intermediate biomarkers to stratify patients for individual risk of progression and measure the success of these therapies is of importance.

The malignant transformation of bronchial epithelial cells is driven by activation of oncogenes and growth factors and even more evidently by tumor suppressor gene inactivation. In this regard, genes of the p53-Rb pathway of G\(_1\) arrest are the most commonly affected genes in lung cancer. Rb gene inactivation, reflected by absence of Rb protein expression, has been reported in a minority of NSCLC (13, 14) but in the majority of small cell lung carcinoma. Although Rb expression is maintained in at least 80% of NSCLC, Rb functions on G\(_1\) arrest can be invalidated by mechanisms that alter the Rb phosphorylation pathway. Only the underphosphorylated form of Rb protein is able to mediate G\(_1\) arrest. Rb phosphorylation at G\(_1\)-S transition is driven by Cdk5 Cdk4 and Cdk6, in protein complexes with cyclin D1. These complexes are controlled by potent inhibitors.

---

\(^1\)To whom requests for reprints should be addressed, at Laboratoire de Pathologie Cellulaire, BP 217, 38043 Grenoble Cedex 9, France. Phone: (33) 4 76 76 54 86; Fax: (33) 4 76 76 59 49; E-mail: Elisabeth.Brambilla@ujf-grenoble.fr

\(^2\)Supported by grants from Conseil Régional Rhône-Alpes, Groupement des Entreprises Françaises dans la Lutte contre le Cancer, Association pour la Recherche contre le Cancer, and Projet Hospitalier de Recherche Clinique.

\(^3\)The abbreviations used are: CIS, carcinoma in situ; NSCLC, non-small cell lung carcinoma; Cdk, cyclin-dependent kinase.
the Cdk inhibitors p16<sup>INK4A</sup>, p15<sup>INK4b</sup>, and p18<sup>INK4c</sup>, the inactivation of which may deregulate Rb phosphorylation.

Cumulative results implicate p16<sup>INK4A</sup> as a tumor suppressor gene, because p16<sup>INK4b</sup> is frequently inactivated in lung cancer through loss of one allele (80–100%) and inactivation of the remaining allele by three alternative mechanisms: homozygous deletion, hypermethylation of the 5′ end of the gene, and mutation, in decreasing order of frequency (15–19). In a previous study, we showed that loss of protein expression was highly concordant (95%) with one of these mechanisms of inactivation (20). An inverse correlation between alterations in the expression of Rb and p16 are observed in many tumor types, including lung cancer (21–26), which reflects a functional redundancy of Rb and p16 on a common p16/Rb growth suppressor pathway.

Cyclin D1 gene product is part of the family of Cdk-cyclin complexes that allow Rb phosphorylation at G1-S transition. Somatic deregulation of cyclin D1 either by amplification or by transcriptional up-regulation has been demonstrated in many tumor types (27–31) and may play a role in the progression of lung cancer (32, 33). This is, after p16 inactivation, the second-most reported mechanism responsible for Rb epigenetic inactivation in lung cancer through inappropriate phosphorylation.

Although increased cyclin D1 protein expression has been recently shown in proliferative and preinvasive breast lesions (34), neither Rb nor p16 or cyclin D1 overexpression has been reported in mucosa and specifically bronchial preneoplasia. The aim of this study was to investigate preinvasive lesions for Rb, p16<sup>INK4A</sup>, and cyclin D1 overexpression to assess their role in lung cancer initiation and early progression. Because early lesions such as hyperplasia and metaplasia have been shown to carry genetic abnormalities and, specifically, loss of allele (loss of heterozygosity) at 3p and 9p chromosomes (9, 10), we included them in the spectrum of preinvasive lesions studied. To investigate the specificity of these phenotypic abnormalities for cancer progression in preinvasive lesions, we compared the frequency of p16<sup>INK4A</sup> and cyclin D1 alterations in patients with previous, synchronous, or metachronous lung cancer with those observed in identical intraepithelial lesion of patients without previous cancer history or cancer development over a 3-year follow-up period.

Materials and Methods

Patients and Samples. Bronchial specimens with preinvasive lesions were obtained from lung resection performed for lung cancer in 46 patients. Preinvasive lesions were classified according to classical criteria (35, 36). Because both severe dysplasia and CIS are sometimes difficult to differentiate and cannot be distinguished only by thickness, we grouped them in a common group of severe preinvasive lesions. Four of the 46 patients with surgical resection were nonsmokers. Tobacco consumption ranged from 7 to 125 pack-years for the 38 others. Tissues were fixed in formalin and embedded in paraffin. Frozen sections were systematically performed on cancer and bronchial resection margins, but preinvasive lesions could be observed by chance on this frozen material in only 20 cases.

In addition, 57 bronchial biopsies presenting at least one area of metaplasia obtained at fiberoptic bronchoscopy in 51 patients were also included in the study. In 29 of these patients, a lung cancer was diagnosed over a period of time extending from 4 years before to 1 year after biopsy. Two of these 29 patients never smoked. In the 22 other patients, neither previous or synchronous cancer nor metachronous cancer had been diagnosed within a 3-year follow-up. This was considered the control group with no cancer history. In this control group, 12 patients were nonsmokers, and bronchial biopsies were performed because of respiratory symptoms and abnormal chest X-ray in the clinical setting of benign disease, including infectious or inflammatory process, sarcoidosis, amyloidosis, chronic obstructive bronchopulmonary diseases, and benign tumors. Bronchial biopsies were fixed in Bouin fixative and embedded in paraffin, for conventional histopathological diagnosis.

There was obviously more than one lesion per patient because one to five distinct anatomical area of preinvasive lesions present on the same or different blocks from bronchial margins were examined for each patient and selected for the study. The distance of each area of intraepithelial lesion from invasive carcinoma was determined by the thickness separating serial blocks in paraffin section and precise localization of the frozen section. Lesions present on the same section as invasive carcinoma were always considered adjacent to invasive carcinoma. Preinvasive lesions located more than 1 cm distant from the margin of invasive carcinoma and separated from it by normal strands of bronchial epithelium were considered distant from invasive carcinoma.

Invasive carcinoma were classified according to the WHO classification (35). Basaloid carcinoma refers to a recently described histological class accounting for 5% of NSCLC (37). Large cell neuroendocrine carcinoma is a high grade neuroendocrine lung tumor recently individualized by Travis et al. (38).

Immunohistochemistry. The sources of primary antibodies and dilutions used in the study as well as retrieval methods are indicated in Table 1. Immunohistochemistry was performed on frozen and formalin-fixed sections or Bouin-fixed bronchial biopsies. On paraffin sections, endogenous peroxidase activity was quenched with 3% hydrogen peroxide at room temperature for 10 min. After overnight incubation at 4°C with the primary antibody, slides were washed in PBS and then exposed to the secondary antibody, biotinylated donkey F(ab′)<sub>2</sub> antirabbit (1:1000; The Jackson Laboratory, West Grove, PA) or antimouse (1:400; The Jackson Laboratory), for 1 h at room temperature. They were then washed in PBS and incubated with the streptavidin-biotin-peroxidase complex (1:400; DAKO, Copenhagen, Denmark) for 1 h at room temperature. They were then washed in PBS and incubated with the streptavidin-biotin-peroxidase complex (1:400; DAKO, Copenhagen, Denmark) for 1 h at room temperature. The chromogenic substrate of peroxidase was a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride, 0.03% H<sub>2</sub>O<sub>2</sub>, and 10 mmol/liter imidazole in 0.05 mol/liter Tris buffer (pH 7.6). Normal rabbit or mouse IgG at the same concentration as the primary antibodies served as negative controls. For cyclin D1, immunostaining was enhanced using a tyramine kit (DAKO) according to manufacturer’s instructions, with minor modifications: 0.5 dilution of the amplification reagent and the streptavidin-peroxidase solution.

Statistical Analysis. Differences between independent groups were determined by means of the Kruskal-Wallis test. Differences between proportions were evaluated using Fisher’s exact test.
Results

Histological Distribution of Bronchial Neoplasia and Preneoplasia. Squamous cell carcinoma (27 cases) and basaloid carcinoma (6 cases) were the most frequent type of carcinoma associated with preinvasive lesions. They displayed an equal distribution of adjacent and distant preinvasive lesions, whereas only intraepithelial lesions distant from invasive carcinoma were seen in other histological types: 7 adenocarcinoma, 2 large cell neuroendocrine carcinoma, and 1 large cell carcinoma. In one case, CIS was the most advanced lesion. These disseminated lesions identified the field cancerization process as defined previously. The distribution of preinvasive lesions of different grade and their distance from invasive carcinoma are shown in Table 2. Severe dysplasia and CIS were more frequently found beside invasive carcinoma than distant from it.

Bronchial biopsies were selected for the study on the basis of at least one area of metaplasia. A lower incidence of dysplasia was found in these biopsies as compared with that observed on surgical samples, obviously due to the small size of intraepithelial lesions, which could be missed on a small biopsy (Table 2). No CIS was included in this group of 22 patients who did not develop cancer in the 3-year follow-up because CIS is an intraepithelial cancer. However, CIS was the most advanced lesion in three patients who had been treated by surgery for a first lung cancer that was resected 1, 2, and 4 years before the time of discovery of CIS on biopsy, and these lesions were included in the group of lesions from patients with lung cancer history.

No CIS was included in this group of 22 patients who did not develop cancer because CIS is an intraepithelial lesion, which could be missed on a small biopsy (Table 2). Surgical samples, obviously due to the small size of intraepithelial lesions, sometimes associated with mild cytoplasmic staining.

Frequency of p16 Expression in Preinvasive Bronchial Lesions. p16 immunostaining was assessed by comparison of type II pneumocytes, normal bronchial cells, and some endothelial cells of the stroma, type II pneumocytes, and normal bronchial epithelial cells, seen in the vicinity of tumor cells.

Three tumors lacked Rb protein expression (6.5%; one large cell neuroendocrine carcinoma, one large cell carcinoma, and one basaloid carcinoma), whereas all other invasive carcinomas included in this study expressed Rb (Rb positive). All preinvasive lesions were intensely immunostained with 30–80% of cell nuclei stained. Rb immunostaining was as intense in dysplasia of any grade and CIS as in normal and hyperplastic bronchi (Fig. 1, A and B).

Rb Immunoreactivity in Preinvasive Bronchial Lesions. Rb immunoreactivity was considered as negative in tumors (loss of Rb expression) when all tumor cells in the section showed no nuclear staining with Rb antibodies. There was a general agreement in results of immunostaining obtained with C15 on paraffin section and IF8 on frozen sections. Internal positive controls required to interpret the staining on tumor cells nuclei were endothelial cells of the stroma, type II pneumonocytes, and normal bronchial epithelial cells, seen in the vicinity of tumor cells.

Table 1: Dilution, retrieval method, and source of primary antibodies used in this study

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Antibody</th>
<th>Retrieval method</th>
<th>Dilution according to fixation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb</td>
<td>C15 (P)</td>
<td>MW, 10 min</td>
<td>Form, 1:2000</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
</tr>
<tr>
<td>Rb</td>
<td>1F8 (M)</td>
<td>Fz, 1:100</td>
<td></td>
<td>Novocastra (Newcastle, United Kingdom)</td>
</tr>
<tr>
<td>p16</td>
<td>C20 (P)</td>
<td>Fz, 1:750</td>
<td></td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>DCS6 (M)</td>
<td>MW, 10 min</td>
<td>Form, 1:4000</td>
<td>Calbiochem (Cambridge, MA)</td>
</tr>
</tbody>
</table>

*a* P, polyclonal; M, monoclonal.

*b* MW, microwaving in citrate buffer (pH 6).

*c* Form, formalin-fixed sections; Fz, frozen sections.

Table 2: Distribution of preinvasive lesions according to histological grade and distance from invasive carcinoma in surgical samples and to the presence or absence of lung cancer history in bronchial biopsies.

<table>
<thead>
<tr>
<th>Surgical samples (n = 42)*</th>
<th>Bronchial biopsies (n = 47)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjacent, &lt; 1 cm</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>27</td>
</tr>
<tr>
<td>Metaplasia</td>
<td>15</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>11</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>19</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>34</td>
</tr>
</tbody>
</table>

*a* n, number of patients.

*b* Three of these patients had CIS as the most advanced lesion.

Rb immunoreactivity was considered as negative in tumors (loss of Rb expression) when all tumor cells in the section showed no nuclear staining with Rb antibodies. There was a general agreement in results of immunostaining obtained with C15 on paraffin section and IF8 on frozen sections. Internal positive controls required to interpret the staining on tumor cells nuclei were endothelial cells of the stroma, type II pneumonocytes, and normal bronchial epithelial cells, seen in the vicinity of tumor cells.

Three tumors lacked Rb protein expression (6.5%; one large cell neuroendocrine carcinoma, one large cell carcinoma, and one basaloid carcinoma), whereas all other invasive carcinomas included in this study expressed Rb (Rb positive). All preinvasive lesions were intensely immunostained with 30–80% of cell nuclei stained. Rb immunostaining was as intense in dysplasia of any grade and CIS as in normal and hyperplastic bronchi (Fig. 1, A and B).

Frequency of p16 Expression in Preinvasive Bronchial Lesions. p16 immunostaining was assessed by comparison of type II pneumocytes, normal bronchial cells, and some endothelial cells with the positive internal controls. p16 immunostaining was interpretable in invasive carcinoma of 42 surgically treated patients and 1 CIS as the most advanced lesion. A tumor was considered as p16 negative when large areas or <10% of nuclei were stained. p16-positive tumors had heterogeneous nuclear staining in 10–70% of tumor cells. Twenty-three of 42 carcinomas (55%) were p16 negative. When combined with Rb phenotype, 23 of 42 (55%) invasive carcinomas displayed Rb+/p16− phenotype, 16 (38%) displayed Rb+/p16+ phenotype, and 3 (7%) displayed Rb−/p16+ phenotype. p16 immunostaining was in good agreement with that on frozen and formalin-fixed frozen sections.

p16 immunostaining on frozen and formalin sections was constant, mild, or moderate in basal and suprabasal nuclei of normal, hyperplastic, and metaplastic bronchi and in mild dysplasia, sometimes associated with mild cytoplasmic staining. Only 2 moderate dysplasia (8%) from 2 patients were p16 negative, but 10 severe dysplasia and CIS from 6 patients (20%) were p16 negative (Table 3; Fig. 1). On surgical samples overall, 12 of 66 moderate dysplasia and CIS (18%) had lost p16 protein expression. The frequency of p16 loss of expression increased with their grade (P = 0.002). When the concordance of p16 expression between preinvasive and invasive carcinoma was examined, p16 loss of expression was exclusively observed.
Rb Pathway in Bronchial Preinvasive Lesions

[Images A to H showing different sections of tissue with varying degrees of staining]
in preinvasive lesions associated with p16-negative invasive carcinoma. In 23 patients with p16-negative invasive carcinoma, 8 (35%) had one or several preinvasive lesions that displayed concurrent loss of p16 expression. The loss of p16 expression was as frequent in the vicinity of invasive carcinoma as it was at a distance from invasive carcinoma.

Due to inconsistent immunostaining of normal cells on Bouin-fixed bronchial biopsies, p16 immunostaining of preinvasive lesion was interpretable in bronchial biopsies from 28 patients of 51 patients: 14 with cancer history and 14 without cancer history. p16 loss of expression was found in preinvasive lesions in 5 of 14 (36%) bronchial biopsies from 5 patients with lung cancer history, 2 moderate dysplasia, and 4 severe dysplasia and CIS (1 patient had 1 moderate and 1 severe dysplasia that were concurrently p16 negative).

Overall, in patients with lung cancer history p16 loss of protein expression was found in 4 of 33 moderate dysplasia (12%) and in 14 of 47 severe dysplasia and CIS (30%). p16 loss of protein expression was not seen in 14 bronchial biopsies performed in the context of benign lung disease on 9 mild and moderate dysplasia from 9 patients where p16 was interpretable.

**Cyclin D1 Overexpression in Bronchial Preinvasive Lesion and Related Invasive Carcinoma.** Cyclin D1 immunostaining could be validated in paired invasive and preinvasive lesions of 39 patients on bronchial surgical resections and in preinvasive lesions on bronchial biopsies from 29 patients with lung cancer history and 22 patients without such history. Immunostaining could not be obtained on frozen sections.

Cyclin D1 was considered overexpressed when >5% of cell nuclei were stained, given that normal bronchial, bronchiolar, and parenchymal cells were generally negative. Cyclin D1 was overexpressed in 19 of 40 (47%) invasive carcinoma in the group of surgical bronchial resections. Cyclin D1 was overexpressed in preinvasive lesions (on surgical samples) in 3 of 45 (7%) hyperplasia (in 3 patients), 2 of 30 (7%) metaplasia (in 2 patients), 2 of 23 (9%) mild dysplasia (in 2 patients), 12 of 26 (46%) moderate dysplasia (in 10 patients), and 15 of 41 (37%) severe dysplasia and CIS (in 12 patients; Table 4; Fig. 1). The frequency of cyclin D1 overexpression increased significantly with the grade of the preinvasive lesion from hyperplasia and metaplasia to moderate and severe dysplasia (P = 0.0001) and was not different in the vicinity of invasive carcinoma or at a distance from invasive carcinoma, except for CIS. Frequency of cyclin D1 overexpression was similar in preinvasive lesions on bronchial biopsies from patients with cancer history and was seen in 8 patients: 4 of 12 mild dysplasia, 7 of 15 moderate dysplasia, and 4 of 9 severe dysplasia or CIS. Overall, in patients with lung cancer cyclin D1 overexpression was noted in 6% of hyperplasia and metaplasia, 17% of mild dysplasia, 46% of moderate dysplasia, and 38% of severe dysplasia and CIS.

When cyclin D1 expression was compared in bronchial preinvasive and invasive lesions in the same patient, there was a concurrent positive or negative cyclin D1 overexpression in preinvasive and invasive lesions in 33 of 39 patients (84%).

**Table 3** Loss of p16 expression in preinvasive bronchial lesion and corresponding invasive carcinoma

<table>
<thead>
<tr>
<th>p16-negative surgical samples</th>
<th>p16-negative bronchial biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With cancer history (n = 14)</td>
</tr>
<tr>
<td>Adjacent</td>
<td>Distant</td>
</tr>
<tr>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>0/7</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>1/14</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>8/28</td>
</tr>
<tr>
<td>and CIS</td>
<td>23/42</td>
</tr>
<tr>
<td>P = 0.0020&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> p16-negative indicates that most cell nuclei are not immunostained with p16 antibody.

**Table 4** Cyclin D1 overexpression in intraepithelial lesions and corresponding invasive carcinoma

<table>
<thead>
<tr>
<th>Cyclin D1-positive surgical samples</th>
<th>Cyclin D1-positive bronchial biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With cancer history (n = 29)</td>
</tr>
<tr>
<td>Adjacent</td>
<td>Distant</td>
</tr>
<tr>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>3/24</td>
</tr>
<tr>
<td>Metaplasia</td>
<td>1/13</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>1/9</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>7/16</td>
</tr>
<tr>
<td>Severe dysplasia and CIS</td>
<td>12/29</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>19/40 (47%)</td>
</tr>
</tbody>
</table>

<sup>b</sup> Cyclin D1 positive means that at least 10% of cells are immunostained.

<sup>b</sup> Probability for increased frequency of p16 loss of expression with the increasing severity of the preinvasive lesion (Fisher’s exact test).
Moreover, in nine patients with concomitant lesions of the same type, the lesions were concurrently cyclin D1 positive in three and concurrently cyclin D1 negative on six. Only four patients had cyclin D1 overexpression in their invasive carcinoma but not in their preinvasive lesions, and two patients had cyclin D1 overexpression in preinvasive lesions, which was not maintained in their invasive carcinoma. Cyclin D1 overexpression was observed in 3 of the 22 patients with no synchronous or metachronous cancer, 2 of 22 mild dysplasia, and 2 of 22 moderate dysplasia (Table 4).

Comparison of Cyclin D1 Overexpression and p16 Loss of Expression in Preinvasive and Invasive Lesions. There was no relationship linking cyclin D1 overexpression and p16 loss of expression in preinvasive lesions or invasive carcinomas or in all lesions considered together. In the preinvasive and invasive lesions with interpretable immunostaining for both p16 and cyclin D1, none of these abnormalities were observed in 31 of 74 lesions (42%); of 105 dysplasia and CIS observed on surgical sample) or in 10 of 40 (25%) related invasive carcinoma, including the 3 Rb-negative cases. Thus, only 14% of invasive carcinoma had no distinct alterations on Rb-cyclin D1-p16 pathway. Both alterations were observed in 6 of 74 (8%) dysplasia and CIS and 9 of 40 (22%) invasive carcinoma. Overall, 37 of 114 (32%) preinvasive and invasive lesions had one of these phenotypical abnormalities. There was neither a direct nor inverse relationship between these two molecular abnormalities that were statistically independent in this small series.

Discussion

The focus of this study has been placed on molecular pathology of the Rb phosphorylation pathway, aimed at demonstrating a deregulation of Rb phosphorylation control before invasion in bronchial neoplasia. Both cyclin D1-dependent phosphorylation pathway and the control of this phosphorylation by Cdk inhibitors such as the p16INK4a gene product could be deregulated as a result of genetic alterations, leading to cyclin D1 overexpression and/or p16INK4a inhibition. Evidence of both abnormalities have been alternatively provided in lung cancer, rather exclusively in the tumors in which Rb protein expression was retained (19, 21, 23, 31), consistent with our results in invasive carcinoma and preinvasive lesions. Despite the constant evidence of strong Rb immunostaining in preinvasive lesions in our series, we cannot conclusively assert that the loss of Rb protein expression never occurs, because a few Rb-negative tumors were examined beside preinvasive lesions. It is, however, generally accepted that loss of Rb protein expression is of low occurrence in non-small cell nonneuroendocrine carcinoma and improvement in immunohistochemical techniques aimed at determining its frequency (39), show an overall occurrence of <20% (23). Occurrence of Rb loss in a large series of NSCLC otherwise demonstrated a 10% loss of Rb protein expression in squamous and baselaid carcinoma (data not shown). We thus focused our study on the epigenetic mechanism of Rb inactivation through deregulation of Rb phosphorylation pathway.

p16 loss of protein expression was demonstrated in 51% of a large series of NSCLCs (23), and an inverse relation between Rb and p16 protein expression was observed. Our results of p16 immunostaining on frozen paraffin sections suggest that p16 loss of protein expression may precede invasion. It seems to be a rather late event arising in a few moderate dysplasia with increasing frequency in severe dysplasia and CIS (30%). The frequency of 55% of p16 loss of protein expression observed in the present series of invasive carcinoma goes along with previous results (19, 21, 23). Moreover, p16 loss of expression, acquired in preinvasive lesions, was always maintained in invasive carcinoma. Its magnitude increased with grade of dysplasia. In contrast, p16 loss of expression was not observed in preinvasive lesions when related invasive carcinoma had retained p16 expression. This suggests that p16 loss of protein expression in preinvasive lesions was accounted for by somatic p16 inactivation and was specific for malignant clonal expansion. This hypothesis is supported in this study by the maintain of normal p16 expression in similar but reactive intraepithelial lesions observed on bronchial biopsies from patients with benign lung disease, although the small size of the control sample analyzed for p16 expression (14 cases) precludes any definitive conclusion. Moreover, only three of these control patients were smokers. p16 loss may be a reflect of field cancerization in smokers. Thus, we cannot ascertain that p16 loss is predictive for cancer progression. This abnormality was not restricted to areas adjacent to invasion but was seen with a comparable frequency in moderate and severe dysplasia and CIS lesions distant from invasive areas, suggesting that anatomically distinct high-grade preinvasive lesions shared the same propensity for being driven by p16 genetic alteration. Interestingly, 9p21 loss of allele (loss of heterozygosity), in which p16 and p15 genes jointly map, has been shown in preinvasive lesions (from hyperplasia to CIS) with a high incidence of 30% in hyperplasia or metaplasia to reach 80–100% in related invasive carcinomas in the same patients (10, 11), which is far higher than the frequency of p16 loss of protein expression that we observed in this study. Whether this loss of allele specifically targeted p16 at 9p21 locus could not be ascertained because a molecular approach using polymorphic probes of the 9p21 region rather than specific probes for p16 gene was made. It cannot be excluded yet that p16 silencing was due to loss of one allele occurring early and specific methylation or loss of the second allele occurring further in late lesions.

In contrast with p16 loss of protein expression, cyclin D1 overexpression occurred early in hyperplasia and metaplasia, with an accelerated rate in moderate and severe dysplasia. There was a high concordance of presence or absence of cyclin D1 overexpression between preinvasive lesions and invasive carcinoma, except in two patients in whom cyclin D1 overexpression in preinvasive lesion was not maintained in invasive carcinoma. This suggests that some transient overexpression in preinvasive lesions could not be related to clonal outgrowth because somatic genetic abnormalities conferring a growth advantage should be maintained during clonal expansion. Accordingly, three patients with no lung cancer history in the 2-year follow up had areas of dysplasia overexpressing cyclin D1. Consequently, although cyclin D1 overexpression was mostly observed in preinvasive lesions of patients with a field cancerization process, it could sometimes be evidence of a reversible process.

Because cyclin D1 is thought to drive cell cycle division...
(40) mediated by extracellular mitogens, any constitutive over-
abundance may accelerate cell growth. However, recent results
suggest a possible involvement of cyclin D1 expression in
the apoptotic pathway. Waf1 was shown to mediate p53 wild-type
G1 arrest and could induce cyclin D1 expression during p53-
induced G1 arrest (41). Moreover, cyclin D1 expression was
found to correlate with terminal deoxynucleotidyl transferas-
mediated nick end-labeled apoptotic cells in head and neck and
oral mucosa but not with proliferating cells (42). These results
suggest that cyclin D1 overexpression could not only be the
result of a constitutive somatic cyclin D1 deregulation through a
genetic phenomenon such as amplification, as shown previ-
ously (43), but could also occur as a response to p53 wild-type-
Waf1 mediated G1 arrest in these lesions. Similarly, it could also
explain that overexpression of cyclin D1 in preinvasive lesions
was sometimes, although rarely, absent in invasive carcinoma
or present in reversible or nonprogressing intraepithelial lesions.

Our results suggest that the Rb pathway of G1 arrest was
 disrupted in preinvasive lesions through Cdk inhibitor inactiva-
tion and/or cyclin D1 overexpression, although this latter phe-
nomenon could have two opposite potential significances in
respect to proliferation or apoptosis.

It is not surprising that cyclin D1 and p16 could be dereg-
ulated independently because they enter in a binary protein
complex with Cdk4 or Cdk6, in which p16 displaces cyclin D1.
The deregulation can naturally affect Rb functions indepen-
dently or in combination, as is the case in a proportion of invasive
carcinoma.

Overall, these results show that p16<sup>INK4</sup> functions can be
affected before invasion as reflected by loss of p16 protein
expression in 30% of high-grade preinvasive lesions. cyclin D1
can also be deregulated before invasion in half of preinvasive
lesions, and this cannot be definitively assigned to a genetic
abnormality. Although Rb protein expression is preserved in the
vast majority of NSCLC and all their precursor lesions, impair-
ment of Rb function can precede invasion. Although immuno-
histochemical analysis suffers from limitations related to the
fact that protein expression level does not demonstrate genetic
alterations in the lung of current and former smokers. J. Natl. Cancer

J. J., Ro, J. Y., Broxson, A., Yu, R., Morice, R. C., Kemp, B. L., Khuri,
F. R., Walsh, G. L., Hinekman, W. N., and Hong, W. K. Clonal genetic
alterations in the lung of current and former smokers. J. Natl. Cancer

12. Wistuba, I. I., Lam, S., Behrens, C., Virmani, A. K., Fong, K. M.,
LeRiche, J., Samet, J. M., Srivastava, S., Minna, J. D., and Gazdar, A. F.
Molecular damage in the bronchial epithelium of current and former

13. Reissmann, P. T., Koga, H., Takahashi, R., Figlin, R. A., Holmes,
E. C., Piantadosi, S., Cardon-Cardo, C., and Slam, D. J. Inactivation
of the retinoblastoma susceptibility gene in non small cell lung cancer.

expression in resected non-small cell lung tumors. Cancer Res., 56:
3415–3420, 1996.

15. Cairns, P., Mao, L., Merlo, A., Lee, D. J., Schwab, D., Eby, Y.,
Tokino, K., Van der Riet, P., Blaugrund, E., and Sidransky, D. Rates of
p16 (MTSI) mutations in primary tumors with 9p loss. Science (Wash-

16. Cairns, P., Polascik, P., Eby, T. J., Tokino, K., Califano, J., Merlo,
A., Mao, L., Herath, Y., Jenkins, R., and Westra, W. Frequency of
homologous deletion at p16/CDK2 in human primary tumours. Nat.

17. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E.,
Burger, P. C., Baylin, S. B., and Sidransky, D. 5’ CpG island methyl-
ating is associated with transcriptional silencing of the tumor suppressor

18. Marchetti, A., Buttitta, F., Pellegrini, S., Bertacca, G., Chella, A.,
Caminelli, V., Tognoni, V., Filardo, A., Angeletti, C. A., and Bevilacu-
qua, G. Alterations of p16 (MTS1) in node positive non small cell lung

M. R., Demetrick, D. J., Serrano, M., Hannon, G. J., Shiseki, M., and
Zarinala, M. Mutations in the p16<sup>INK4/A+/MTS1</sup> and p15<sup>INK4B/MTS2</sup>
and p18 genes in primary and metastatic lung cancer. Cancer Res., 55:

20. Gazzett, S., Gouy, V., Vour’t ch, C., Brambilla, C., and Brambilla,
C. Mechanism of p16<sup>INK4/A</sup> inactivation in non small cell lung

Kaye, F. Absence of p16<sup>INK4/A</sup> protein is restricted to the subset of lung

Immunohistochemical detection of the cyclin-dependent kinase inhibitor
2/multiple tumor suppressor gene 1 (CDK2/MTS1) product p16<sup>INK4/A</sup>
Alterations of Rb Pathway (Rb-p16\textsuperscript{INK4}-Cyclin D1) in Preinvasive Bronchial Lesions

Elisabeth Brambilla, Sylvie Gazzeri, Denis Moro, et al.