**Advances in Brief**

**p53 Mutant Immunophenotype and Deregulation of p53 Transcriptional Pathway (Bcl2, Bax, and Waf1) in Precursor Bronchial Lesions of Lung Cancer**

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**Abstract**

Lung cancer is the end result of a multistep process in which genetic and molecular changes accompany, in an unknown temporal sequence, histological precursor (preinvasive) bronchial lesions. Biomarkers allowing prediction of the rate of progression of precursor lesions at different locations in the anatomical field may be clinically useful. Toward this aim, we analyzed, using immunohistochemistry, the expression of the p53 gene and of its transcriptional target genes bax, bcl2, and waf1 in preinvasive bronchial lesions from 69 patients with lung cancer and in similar lesions from 20 patients with no cancer progression. p53 accumulation occurred with increasing frequency, from 19% in mild dysplasia to 36% in moderate dysplasia and 59% in carcinoma in situ, and was exclusively observed in patients with p53-positive carcinoma. The higher frequency of the p53-positive immunophenotype in lesions adjacent to the p53-positive carcinoma, as compared to lesions distant from it, suggests that p53 mutant preneoplastic lesions had a higher rate of progression to invasion than did p53-negative lesions. Similar lesions in patients with no progression to lung cancer were all p53 negative. Bcl2 overexpression and Bax down-regulation, as shown by immunostaining, occurred in preinvasive lesions and were mainly maintained during invasion. The expressions of bax, bcl2, and waf1 did not correlate with p53 status. We conclude that p53 stabilization in preinvasive lesions has high predictive value for progression to invasion and that Bax/Bcl2 imbalance contributes to the clonal expansion during premalignant states.

**Introduction**

Lung cancer is now the leading cause of cancer death in men living in industrialized countries and is becoming so in women in the United States. Cigarette smoking is the main risk factor, responsible for 90 and 78% of lung carcinomas in men and women, respectively (1). Despite therapeutic efforts, the prognosis of lung cancer has changed little during the last 20 years, and survival rate is <5% at 10 years. The only curable cancers are those diagnosed in the early stages and treated surgically. Thus, there is a shift of interest toward diagnosis and study of early and premalignant states. Because early detection and effective chemoprevention may be the most promising clinical approach, elucidating intermediate biomarkers to stratify the patients in clinical trials and monitor the success of chemoprevention may be a promising strategy.

Evidence is accumulating that invasive lung cancer is the end result of a multistep process in which molecular changes accompany or even precede histological changes. The malignant transformation of lung epithelial cells involves mutation of oncogenes and resultant deregulation of their encoded proteins. Perhaps more importantly, inactivation of tumor suppressor genes, such as p53 (2-8), Rb1 gene (9-15), and cyclin-dependent kinase inhibitors (16-21), through inhibition of their protein products removes important regulatory constraints on cell cycle at the G1 checkpoint. Moreover, clonal expansion to reach a critical size necessary for tumor growth and progression could also be influenced by the level of cell death. Thus, cell death susceptibility factors and their antagonists of the Bcl2 family of proteins are potential regulators of clonal expansion and tumor progression in lung cancer (22-24).

In addition to genetic and molecular events, experimental and in vivo studies indicate that carcinogens induce bronchial epithelial cell transformation through stepwise morphological changes, including hyperplasia, metaplasia, dysplasia of progressive severity (mild, moderate, and severe), and CIS (25-28). Dysplasia and CIS have been proven to be true premalignant lesions associated with an increased risk of lung cancer, whereas basal hyperplasia and squamous metaplasia are very common reactive lesions in adults. However, because genetic abnormalities have been found in these common lesions in smokers, they should be included in a study of potential preinvasive lesions (29, 30). Moreover, because preinvasive and even severe (severe
dysplasia and CIS) lesions could eventually regress spontaneously (31–34), there is no definitive predictive value for invasion provided by morphological examination alone. The temporal sequence of the genetic and molecular events along this morphological transformation pathway has not been established. According to Vogelstein and Kinzler (35), 10–20 successive mutations would be necessary to allow clonal expansion and progression to invasive cancer, and p53 mutation is the most frequent mutation in lung cancer.

Although wt p53 is produced at low level, has a short half-life (20 min), and is undetectable by IH, missense mutations, which account for 80% of the mutations in lung cancer, produce abnormally stable p53 protein, which is detectable by IH (2–8). Previous studies have provided sufficient evidence that p53 abnormal stabilization, indicative of missense mutation, precedes invasion (36–42). These studies were carried out on dysplastic lesions in patients surgically treated for a synchronous non-small cell lung carcinoma. However, the anatomical location of a preinvasive lesion in relation to invasive carcinoma was not considered, and most studies obviously reported on bronchial lesions that were adjacent to invasive carcinoma. It is thought that the entire mucosa of upper and lower airways is the target of carcinogens (tobacco and others, such as radon) and that active mutagenesis involves more than one site at a time, referred to as the “field cancerization” process. It is, thus, of interest to study different sites of the field cancerization to establish their interrelationships with regard to p53 abnormalities.

Moreover, previous studies on p53 in histologically classified preinvasive bronchial lesions have not included a control group of patients with no previous, synchronous, or metachronous lung cancer history. To evaluate the potential predictive value for cancer progression of p53 stabilization, we analyzed its expression in intraepithelial preneoplastic bronchial lesions of a large series of patients with synchronous cancer, compared to those observed in patients without any lung cancer history.

Because p53 is a transcription factor that is induced by several kinds of genotoxic stresses that allow G1 arrest and/or apoptosis of cells bearing DNA damage, functional p53 protein actively prevents cells with abnormal DNA from clonal proliferation, thus acting as a “gate keeper” (43). The main target gene of p53 transcription for G1 arrest is waf1/cip1, encoding the protein p21-Waf1 (44, 45), which functions as a cyclin-dependent kinase inhibitor to prevent Rb protein phosphorylation, thus keeping Rb protein in its hypophosphorylated form, where it is able to mediate G1 arrest. p53 transcriptional target genes for apoptosis, thus far identified for the presence of a p53 response element in their promoter, include Bax and Bcl2 (46). wt p53 can induce the transcription of Bax and repress Bcl2 transcription, at least in some cell types, such as fibroblasts, allowing apoptosis by changing the BBR. Thus, the effects of wt p53 on apoptosis may be mediated in part through its effect on the expression of Bax and Bcl2, and p53 mutation may allow Bcl2 overexpression and Bax down-regulation (46).

An additional goal of this study was to investigate the protein expression of target genes of p53 transcription (waf1, bax, and bcl2), aimed at demonstrating an early deregulation of these factors in preinvasive bronchial lesions.

**Materials and Methods**

**Patients and Samples.** Bronchial intraepithelial preinvasive lesions were detected in 42 surgical lung resection specimens for cancer with at least one area of metaplasia, and studies on formalin-fixed tissue sections and on frozen sections were systematically performed on cancer and bronchial resection margins. Preinvasive lesions could be observed by chance on this frozen material in only 20 cases. Only 4 of these 42 patients never smoked. Tobacco consumption ranged from 7 to 125 pack-years for the 38 others.

In addition, 53 bronchial biopsies presenting at least one area of metaplasia, obtained at light fiberoptic bronchoscopy from 47 patients, were also included in the study. In 27 of these patients, a lung cancer was diagnosed in a period of time extending from 4 years before to 1 year after biopsy. Two of these 27 patients never smoked. In the 20 other patients, previous, synchronous, and metachronous (within a 2-year minimum follow-up period) cancers were not diagnosed; these patients with no cancer history were considered the control group. In this control group, 10 patients were nonsmokers, and bronchial biopsy was performed because of respiratory symptoms and abnormal chest X-ray in the clinical setting due to benign diseases, including infectious or inflammatory process, sarcoidosis, amyloidosis, chronic obstructive bronchopulmonary diseases, and benign tumors. Bronchial biopsies were fixed in Bouin’s fixative and embedded in paraffin, for conventional histopathological diagnosis.

Preinvasive lesions were histologically classified according to previous criteria (47): hyperplasia (basal cell hyperplasia) consists of an expansion and stratification of basal cells in the lower part of the epithelium; metaplasia (squamous metaplasia) includes basal cell hyperplasia associated with squamous transformation of suprabasal layers; dysplastic changes superimpose pleomorphism, nuclear irregularities, and increased mitotic activity to hyperplasia and/or metaplasia, expanding to the lower one-third (mild dysplasia), lower two-thirds (moderate dysplasia), or upper one-third (severe dysplasia) of the epithelium; and CIS is marked by lack of progression of maturation from base to luminal surface. Because both severe dysplasia and CIS were sometimes difficult to differentiate and could not be distinguished only on thickness, we confounded them in a common group of severe preinvasive lesions.

There was obviously more than one lesion per patient because one to five sections from distinct anatomical areas of preneoplastic lesions were examined for each patient. The distance of each area of intraepithelial lesion from any invasive carcinoma was appreciated by the thickness separating serial blocks in paraffin section and the 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 >1 cm from the margin of invasive carcinoma and separated from it by strands of normal epithelium were considered distant from invasive carcinoma.

Invasive lesions were classified according to the most recent 1981 WHO classification of lung cancer (47). Basaloid carcinoma refers to a more recently described class of lung cancer (48). Large cell neuroendocrine carcinoma is a high-
Table 1  Numbers of bronchial preinvasive areas according to histological type and distance from invasive carcinoma in patients surgically treated for synchronous lung cancer (42 patients)

<table>
<thead>
<tr>
<th>Histological Grade</th>
<th>No.</th>
<th>Adjacent (&lt;1 cm)</th>
<th>Distant (≥1 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous carcinoma</td>
<td>24</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Basaloid carcinoma</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>6</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>LCNEC*</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>CIS**</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

* Number of carcinomas according to histological type with associated areas of preinvasive lesions.

** LCNEC, large cell neuroendocrine carcinoma.

CIS was the most advanced lesion in these patients.

IH. Antibodies used in the study were as follows: D07 (1:800; DAKO, Glostrup, Denmark) and PAB 1801 (1:5000; Calbiochem, Cambridge, United Kingdom) for p53 immunostaining, N19 (1:300; Santa Cruz Biotechnology, Santa Cruz, CA) for Bax, clone 124 (1:100; DAKO) for Bcl2, and EA 10 (1:50, Calbiochem, Cambridge, MA) for p21-Waf1. IH was performed on frozen sections fixed with 4% paraformaldehyde and on formalin-fixed sections. On paraffin sections, endogenous peroxidase was quenched using 0.3% hydrogen peroxide in distilled water for 5 min at room temperature, prior to incubation with primary Ab. After overnight incubation at 4°C with the primary Ab, slides were washed in PBS and then exposed to the secondary Ab, biotinylated donkey Ab F(ab’)2, antirabbit Ab (1:1000; The Jackson Laboratory, West Grove, PA), or antimouse Ab (1:400; The Jackson Laboratory) for 1 h at room temperature. They were then washed in PBS and incubated with the streptavidin, biotin, and peroxidase complex (1:400; DAKO) for 1 h at room temperature. The chromogenic substrate of peroxidase was a solution of 0.05% 3,3’-diaminobenzidine tetrahydrochloride, 0.03% H2O2, and 10 mmol/liter imidazole in 0.05 mol/liter Tris buffer (pH 7.6). The slides were counterstained with Harris’ hematoxylin. Normal rabbit or mouse IgG at the same concentration as the primary antibodies served as a negative control. The threshold values for p53 immunostaining were chosen at 20 and 10% for invasive carcinoma and preinvasive lesions, respectively, as described below. Bax and Bcl2 staining were scored, taking into account percentage of positive cells (from 1 to 100%) and intensity of staining (from 1 to 3), as compared with the Bcl2 intensity level 3 of the background lymphocytes. Total score was established in multiplying the percentage by intensity, giving scores of 0 to 300. Final scores of 1, 2, and 3 were given for total scores of 10–50, 50–100, and 100–300 respectively. Scores were assessed by two independent observers (E. B. and S. L.) without knowledge of the follow-up status of the patients.

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

Results

Histological Distribution of Bronchial Cancer and Preneoplasia. Squamous and basaloid carcinoma were the most frequent types of carcinoma associated with preinvasive lesions (Table 1). Both types displayed an even distribution of adjacent and distant preinvasive lesions, whereas only preinvasive lesions distant from invasive carcinoma were seen in other histological types. These multiple lesions typified the field cancerization process, as defined above. The grades of preneoplastic lesions that were encountered were evenly distributed according to distance from invasive carcinoma, but CIS was more frequently found adjacent than distant from invasive carcinoma (Table 2).

Bronchial biopsies were selected for the study on the basis of at least one area of metaplasia. As shown in Table 2, a lower incidence of dysplasia was seen in biopsies than on surgical samples, obviously due to the small size of these lesions, which could be missed on a small biopsy. In 20 patients with no cancer history, dysplasias were rare, and no CISs were recorded. CIS was the most advanced lesion in bronchial biopsies from three patients who had been successfully treated by surgery for lung cancer 1, 2, and 4 years before the discovery of CIS on biopsy.

p53 Immunoreactivity in Preinvasive Bronchial Lesions from Patients with Lung Cancer. p53 IP was considered positive (positive IP) in carcinoma and indicative of mutation when at least 20% of nuclei were stained with at least two different antibodies. Because intraepithelial lesions were small and positive nuclei were confined to clusters in the basal and suprabasal areas in early lesions, the threshold value of 10% of p53-positive cells occurring in clusters within an histologically defined preinvasive area was adopted for p53-positive IP. In this regard, normal mucosa, hyperplasia, and metaplasia were p53 negative in all groups of patients. p53-positive immunostaining was observed in 19% of mild dysplasia, 36% of moderate dysplasia, and 59% of severe dysplasia and CIS and in 69% of corresponding invasive carcinoma in the group of patients with surgical resection (Fig. 1, A–C). This frequency was slightly lower in the group of 22 cancer patients with bronchial biopsies.
Fig. 1  
A, p53 immunostaining with PAB 1801 in a mild dysplasia, showing stained nuclei in clusters in basal and suprabasal layers. ×200.  
C, p53 immunostaining with DO7 in a CIS. ×200.  
D, Bcl2 immunostaining of normal
(Table 3), probably due to the small size of biopsies, which could lead to clusters of few cells being missed. There was a 100% concordance between p53 reactivity on frozen and paraffin sections from the same patients. There was a significant increase in the frequency of p53-positive IP with increasing histological grade of the lesions in the group of surgical patients, as well as in both group of cancer patients together ($P = 0.0036$ and $P = 0.0037$ respectively). Two of the five patients with CIS as the most advanced lesion were p53 positive. One of these was surgically treated and the other by endobronchial radiotherapy, and none had recurrence after 2 years. The three others were p53 negative and cured by surgery for one and locally cured by cryotherapy for the two others, one of which recurred at another bronchial location.

P53-positive cells occurring as isolated cells were not considered to be reflecting mutant IP. They were detected in hyperplastic and metaplastic cells on bronchial biopsies in 8 of 47 patients, 6 with synchronous cancer and 2 without, and in 16 of 42 patients with surgical resection for cancer. Overall, they were seen in two normally appearing mucosa, 14 hyperplastic and metaplastic lesions, 8 mild dysplastic, 6 moderate dysplastic areas, and 1 CIS.

As shown in Table 3, there was a significant higher rate of p53 immunoreactivity in preinvasive lesions adjacent to than in lesions distant from the invasive carcinoma ($P = 0.0006$), and this difference was greater for mild and moderate dysplasia ($P = 0.0029$) than it was for severe dysplasia and CIS ($P = 0.026$).

When the concordance of the p53 status (positive or negative IP) between preneoplasia and related invasive carcinoma was examined (Table 4), p53-positive IP was exclusively observed in dysplasia and in CIS associated with a p53-positive carcinoma in both groups of patients with surgical samples and with bronchial biopsies, in which preinvasive and invasive lesions were observed on the same section (eight patients). Accordingly, preneoplasias accompanying p53-negative cancer were all p53 negative. Concurrent p53-positive IP in preinvasive lesions and carcinoma was much higher in severe dysplasia and CIS, 80% of which shared the p53-positive IP of the invasive lesion, than it was in early dysplasias, only 18% of which shared p53-positive IP with invasive carcinoma. So, the direct relationship between preneoplasia and neoplasia, with regard to p53 mutation, was better in CIS than it was in dysplasia. Isolated p53-positive cells were seen in 24 patients, 12 of whom had a negative p53 invasive carcinoma, indicating no correlation between the isolated p53-positive cells in the mucosa and the p53 status of the corresponding invasive carcinoma.

P53 IP in Preinvasive Lesions of Patients with or without Lung Cancer History. There was no case of p53-positive IP in preinvasive lesions in the group of 20 patients who underwent a bronchial biopsy in the setting of a benign disease, had no previous lung cancer history, and did not have invasive lung cancer and did not develop it within the 2-year follow-up after biopsy (Table 3). Consequently, the difference in the frequency of p53 immunoreactivity between dysplastic lesions (of any grade) in patients with or without cancer history, was highly significant ($P < 0.0032$). Moreover, the positive predictive value for concomitant invasion of p53-positive immunostaining was 100% in this series, whereas p53-negative IP had no negative predictive value. Isolated p53-positive cells in preinvasive bronchial lesions had low predictive value, as shown by their occurrence in two patients with benign disease.

Bcl2 and Bax Levels of Expression in Preinvasive Bronchial Lesions and Corresponding Invasive Carcinoma. Because Bcl2 expression was more consistently interpretable in frozen and formalin-fixed paraffin sections than it was in sections fixed with Bouin’s fixative, only surgical samples were used in this comparative study. Bcl2 gave inconsistent staining.
Immunohistochemistry of Bronchial Preneoplasia

Table 4: Concordance of p53 IP between invasive carcinoma and corresponding dysplasias of the same patients

<table>
<thead>
<tr>
<th>p53 status of invasive carcinoma</th>
<th>Dysplasia (mild and moderate)</th>
<th>Severe dysplasia and CIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53-positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53-positive</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>p53-negative</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>p53-positive</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>p53-negative</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Probability</td>
<td>P = 0.0029^a</td>
<td>P = 0.0006^a</td>
</tr>
</tbody>
</table>

* Probabilities for differences in proportions according to Fisher exact test.

(50%) of bronchial basal cells in Bouin’s-fixed biopsies, whereas a consistent staining was obtained for both Bax and Bcl2 on formalin-fixed biopsies, and the BBR could be more consistently assessed. The concordances between frozen and formalin-fixed paraffin sections for Bax and Bcl2 immunostaining were 88 and 85%, respectively. The distributions of Bax, Bcl2 immunostaining, and BBR in areas of preinvasive lesions according to their localization in relation to invasive lesion are shown in Table 5.

Bcl2 immunostaining was cytoplasmic and granular and was restricted in normal bronchial epithelium to the basal epithelial layer and to some epithelial cells that were perpendicularly oriented to the basal lamina, with their cytoplasm extending to the apical lumina (Fig. 1D). These “perpendicular” cells were very dispersed and represented <1% of epithelial bronchial cells. On serial sections immunostained with neuroendocrine makers (chromogranine, Leu 7, synaptophysine, and neural cell adhesion molecule), no concordant staining of Bcl2 and neuroendocrine markers was observed on these cells, which, thus, could not be identified as neuroendocrine cells. We supposed that these perpendicularly oriented Bcl2-positive cells in normal bronchi and bronchioli could represent another variant of reserve cells, in addition to those basally located. A score of 1 was given to this normal pattern. All areas of hyperplasia had this same staining pattern. Nineteen % of metaplasia, 43% of mild dysplasia, and 40% of moderate dysplasia, as well as 53% of severe dysplasia in CIS, had increased Bcl2 staining (scores of 2 and 3; Table 5 and Fig. 1, E and F). Bcl2 immunostaining was rather heterogeneous in suprabasal layers, but it rarely stained >50% of cells, except in some CIS (Fig. 1E). Bcl2 overexpression was more frequently observed in metaplasia and mild dysplasia adjacent to invasive carcinoma than distant from it (P = 0.0018), but there was no difference in Bcl2 expression according to distance in moderate and severe dysplasia and CIS (P = 0.5). Bcl2 was overexpressed (score of 2 or 3) in 17 of 40 invasive carcinomas (42%), and immunostaining was low or negative (score of 0 or 1) in 23 of 40. In the 17 patients with Bcl2 overexpression in invasive carcinoma, 13 had Bcl2 overexpression in one or several preinvasive lesions. However, of the 23 (77%) patients with low or no Bcl2 expression in invasive carcinoma, 5 (22%) showed Bcl2 overexpression in one or several preinvasive lesions, indicating that Bcl2 overexpression in preneoplasia was not always maintained in neoplasia.

Bax immunostaining was also intense and granular in the cytoplasm in the normal and hyperplastic bronchial epithelium and was given a score of 3 (Fig. 1G). Bax immunostaining was reduced compared to that of normal epithelium in 6.6% of hyperplasia and metaplasia, 19% of mild dysplasia, 14% of moderate dysplasia, and 21% of severe dysplasia and CIS (Fig. 1H). Loss of intensity of Bax immunostaining compared to normal epithelium was more frequent in preinvasive lesions observed in the close vicinity of invasive carcinoma than away from it (P = 0.0013; Table 5). Bax expression was as high as that in normal epithelium (score of 2 or 3) in 27 of 40 (67%) invasive carcinoma and was lower (score of 0 or 1) in 13 of 40. This was the distribution of high or low bax expression observed in a larger series of 165 cases of non-small cell lung carcinoma (data not shown). In the 13 patients with low Bax expression in invasive carcinoma, 8 (62%) had one or several preinvasive lesions (6 dysplasias and 8 CISs) showing low Bax expression. In the 27 patients with high Bax expression in invasive carcinoma, 25 had high bax expression, and only 2 had low Bax expression in preinvasive lesions.

The BBR in individual preinvasive lesions was compared with that of normal epithelium. Because the score of Bax was always higher than that of Bcl2 immunostaining, BBR was less than unity in normal bronchial epithelium. A BBR equal to or higher than unity (BBR ≥ 1), reflecting an inversion of BBR, was never found in hyperplasia but was observed in metaplasia (22.6%), mild and moderate dysplasia (42%), and CIS and severe dysplasia (65%; Table 5). BBR was more frequently inversed in the metaplasia and mild and moderate dysplasia that were adjacent to the invasive area than it was in lesions that were distant from the invasive area (P = 0.0018). No difference was noted in BBR of severe dysplasia and CIS according to distance from invasive carcinoma (P = 0.4). An abnormal BBR (BBR ≥ 1) was seen in 16 of 40 (29%) invasive carcinomas and was concordantly seen in preinvasive lesions in 13 of these 16 (81%) patients. BBR was reversed (BBR ≥ 1) in five patients with normal BBR (12.5%) in the carcinoma and was low (BBR < 1), in concordance with corresponding cancer, in 19 of 24 (79%). Thus, Bax down-regulation and disturbed BBR, as compared to normal epithelium, were more often maintained from preinvasive lesions to invasive carcinoma than Bcl2 up-regulation.

No correlations were found between Bcl2 overexpression, Bax loss of expression, and BBR, with p53 IP in preinvasive as well as invasive carcinoma.

Waf1 Expression in Preinvasive Lesion and Corresponding Invasive Carcinoma. Waf1 Ab gave nuclear immunostaining in paraffin sections fixed with formalin or Bouin’s solution. From 0 to 5% of cells were stained in normal and hyperplastic bronchi. There was a wide range of variation from 1 to 50% labeled cells in dysplasia and CIS and from 1 to 70% labeled cells in corresponding invasive carcinoma, without any relation with the grade of severity of the lesion. When <30% of cells were labeled in preinvasive lesions, the positive cells were confined to the upper part of dysplastic epithelium, whereas when >30% of cells were labeled, they were dispersed in all layers. There was a low concordance and no statistical relationship between the level of Waf1 expression in preinvasive and related invasive lesions, and no correlation of Waf1 immunostaining with p53 IP or with any other studied factor, such as Bax or Bcl2 level. A high frequency of Waf1-stained
cells (≤20% of stained cells) could be observed in either p53-positive or -negative preinvasive lesions.

Discussion

This is the largest series of preinvasive lesions thus far examined for p53 accumulation or stabilization and the first study providing a comparison between histologically classified preinvasive lesions in patients with or without lung cancer progression on follow-up and between preinvasive lesions adjacent to or distant from invasive carcinoma within the cancerization field.

p53 stabilization has been widely described in lung cancer of all histological types (4, 24, 50–53). Despite small numbers of invasive cancer included in this study, the focus of which has been placed on preinvasive lesions, the incidence of p53 mutant IP was similar to that reported previously in large series of lung carcinomas. Eighty % of the p53 mutations are missense mutations inducing long half-lives and stabilization of the protein product. Thus, p53 immunoreactivity is highly indicative of missense mutation in the gene sequence, at least, when stringent criteria for "p53 positivity" are observed (4, 6, 24). Naturally, IH cannot discriminate normal unstable wt p53 from the absence of protein product resulting from p53 mutations such as stop or nonsense codons or mutations at splicing sites, which generate mRNA truncation and absence of protein expression. This accounts for ~20% of lung cancer in all histological types (6, 54). Stabilized p53 protein in preinvasive lesions could also be the result of a transitorily stabilized reactive p53 protein because stabilization is an intrinsic property of wt p53. In contrast with an irreversible stabilization induced by missense mutation, the wt p53 can be transiently stabilized in a reversible way in reactive processes that induce its wt function (55, 56). This probably accounts for the few scattered p53-positive cells dispersed among the basal cells in hyperplasia and metaplasia. These were considered in only two reports (41, 57), but evidence that these isolated p53 positive cells displayed mutant p53 was not provided. In this series, we have not considered them indicative of mutation in the p53 gene sequence and did not include them within the cases with p53-positive IP. Indeed, these dispersed p53-positive cells were encountered mainly in hyperplasia and metaplasia that were more prone to be of a reactive nature, consistent with the hypothesis that they reflect a reactive phenomenon. Whenever p53 mutations were sought in

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>Adjacent (%)</th>
<th>Distant (%)</th>
<th>Adjacent (%)</th>
<th>Distant (%)</th>
<th>Adjacent (%)</th>
<th>Distant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2 ≥ 2</td>
<td>17/40 (42)</td>
<td>13/40 (33)</td>
<td>16/40 (40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bax &lt; 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBR ≥ 1</td>
<td></td>
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</table>

*Bcl2 final score ≥2 signifies a numerical score of >100 [numerical score = percentage of labeled cells multiplied by intensity of staining (1–3)].

*Bax final score <2 signifies a numerical score of <50.

*BBR ≥ 1 means that the Bcl2 score was higher than the Bax score.
pears as a marker of irreversibility of a preneoplastic bronchial lesion.

The relative concordance of p53 IP between preneoplasia and neoplasia, previously observed by Nuorva et al. (40), was confirmed here at a higher level of significance (P = 0.0003). All carcinomas synchronous with p53-positive preinvasive lesions, adjacent or distant, were p53 positive, whereas preneoplasia in patients with p53-negative carcinoma (distant or adjacent) were all p53 negative. This could suggest that preneoplasia and neoplasia belong to the same p53-positive or -negative clones and goes along with an apparent clonal relationship between anatomically different lesions observed synchronously in the cancerization field (29, 30, 59–61). However, this clonal relationship could only be assessed by sequence analysis. It could be inferred that scattered p53-positive cells are likely to reflect a reactive process against an active mutagenesis occurring in the mucosa exposed to carcinogens because they were previously encountered in smoking patients with lung cancer (41). It is, however, unlikely that they belong to the clonal expansion because they occurred indiscriminately in patients with p53-positive or -negative invasive carcinoma and in two patients without lung cancer history.

Tumor growth is the net result of intrinsic proliferation and escape from active cell death. This could be true for clonal expansion during stepwise progression of preinvasive lesions. Only one previous report was made on Bcl2 expression in preinvasive lesions (62), whereas Bax expression has never been examined. As previously shown (62), the prominent basal staining with Bcl2 was lost in preinvasive lesions from metaplasia to CIS. In contrast, about half of dysplasias showed increased Bcl2 expression in suprabasal cells. Because susceptibility to cell death is governed by intracytoplasmic equilibrium between factors of the Bcl2 family, such as Bcl2 and Bax, a disequilibrium of these factors in preinvasive lesions could contribute to increased cell survival and establishment of cell clones. Bcl2 overexpression seemed to occur before or concomitantly with disorganization that accompanies dysplasia but was not obligatorily maintained in related invasive carcinoma. In contrast, Bax expression that was maintained at a high level in normal and hyperplastic lesions was down-regulated in a proportion of metaplasia and dysplasia, and this down-regulation was highly maintained in related invasive carcinoma. Because of its ability to promote cell death, Bax would be expected to function as a tumor suppressor gene, and reduction of Bax level could provide tumor cells a selective survival advantage and, along with Bcl2 overexpression, contribute to their expansion. A change of BBR in favor of Bc12 was obvious in these lesions and was more frequent in early lesions in the vicinity of invasion. Along with the hypothesis that Bcl2 and Bax ratio could govern cell turnover, these results suggest that preinvasive lesions contiguous to invasion had higher propensity for clonal expansion due to escape from apoptosis. Inversely BBR could be regarded, in addition to p53 mutation, as another sign of high progression rate. A better concordance rate between preinvasion and invasion with regard to Bax down-regulation and BBR than to Bcl2 overexpression suggests that Bcl2/Bax balance is more important in cell death/or turnover than Bcl2 alone. We cannot infer from these results that this Bcl2-Bax imbalance was specific for malignant transformation because Bcl2 could not be consistently measured in the control groups on Bouin’s-fixed bronchial biopsies. However, aggravation of this imbalance in the most severe lesions and in the vicinity of invasion favors its role in malignant transformation.

We could not find any relationship between Bcl2, Bax level, and BBR ratio with p53 status in preinvasive or invasive lesions. In contrast, two previous studies reported an inverse relationship between Bcl2 overexpression and p53 mutation in non-small lung carcinoma (22, 23). Bcl2 and Bax appear as independent regulators of clonal expansion in preneoplastic bronchial lesions.

Waf1 expression was highly variable in preinvasive lesions and invasive carcinoma with no relation with p53 status, demonstrating that Waf1 expression cannot be regarded as an indicator for wt p53 function. This independency of P21 expression to p53 status was demonstrated in several tumor types (63–65). No relationship could be established between Waf1, p53 immunological status, and Bcl2 or Bax level and their ratio in preinvasive or invasive lesions. Waf1 was mainly expressed in the most differentiated cells in the preinvasive and invasive carcinoma, suggesting that it could be related to the G1 arrest that is necessary for terminal differentiation and maturation.

In conclusion, although p53 and apoptotic factor deregulation may allow clonal cell expansion in preinvasive lesions by disrupting stringent control on proliferation, differentiation, and apoptosis, none are obligatory events in the progression to invasion. p53-positive IP, however, appears specific for malignant transformation and has an extremely high predictive value for progression to invasive carcinoma. Bax/Bc12 imbalance probably contribute to clonal expansion at early stage. Suitable biomarkers for preneoplasia should ideally identify the field cancerization process, allow determination of the intensity of genetic damage and the rate of cancerization process, and enable the impact of chemoprevention on the genetic damage and on lung cancer incidence to be measured. p53-positive IP, along with telomerase reactivation (66), stromelysin 3, and urokinase-type plasminogen activator production (67), are the most promising prognostic factors for synchronous invasion when they are detected in a preinvasive lesion.

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References


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p53 mutant immunophenotype and deregulation of p53 transcription pathway (Bcl2, Bax, and Waf1) in precursor bronchial lesions of lung cancer.

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