Value of Immunohistochemical Markers in Preinvasive Bronchial Lesions in Risk Assessment of Lung Cancer[1]

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ABSTRACT

Purpose: **Bronchial carcinogenesis is a multistep process characterized by accumulation of genetic and molecular abnormalities, which precedes and accompanies the preinvasive lesions known as dysplasia and carcinoma *in situ* (CIS). We hypothesized that the level of accumulated molecular abnormalities in dysplasia assessed by immunohistochemical markers might reflect the severity of the carcinogenic process, thus allowing for risk assessment in smokers.**

Experimental Design: **We performed a prospective analysis of bronchial biopsies in 48 former smokers who had at least one area of metaplasia. Twenty-two of the patients had a previous history of lung cancer. Eighty bronchial lesions were recorded at baseline, including 31 metaplasia, 12 mild dysplasia, 9 moderate dysplasia, 9 severe dysplasia, and 19 CISs. Forty-one percent of the patients had multiple preinvasive lesions. Immunohistochemical analysis of P53, cyclin D1, cyclin E, Bax, and Bcl2 was performed. Aberrant expression of one of these proteins as compared with normal bronchi was recorded as one molecular alteration.**

Results: **After 18 months, 17 patients were diagnosed with lung cancer. No isolated parameter, including dysplastic grade or any isolated molecular alteration, was significantly associated with cancer occurrence at 18 months follow-up, using a logistic regression statistical analysis. In contrast, considering CIS and cancer as end point, more than two immunohistochemical abnormalities were associated with cancer or CIS occurrence ($P = 0.02$).**

Conclusions: **We concluded that the cumulative index of immunohistochemical abnormalities in a random dysplasia is associated with CIS or lung cancer in the cancerization field of symptomatic smokers, independently of the histopathological grade of dysplasia. This set of histopathological biomarkers might be useful in risk assessment and provide intermediate end points for chemopreventive trials.**

INTRODUCTION

Lung carcinogenesis is a multistep process characterized by accumulation of successive molecular genetic and epigenetic abnormalities, resulting in epithelial cell malignant transformation (1). The main carcinogens responsible for 85% of lung cancers are contained in tobacco smoke (2). Numerous genetic and molecular abnormalities have been shown in very early stages like hyperplasia and metaplasia and even in normal appearing epithelial bronchial and alveolar cells in smokers (3, 4). Molecular lesions are thus preceding the morphological transformation known as preinvasive lesions that are observed in smokers. These preinvasive lesions are multicentric, reflecting the fact that the carcinogenic process may randomly affect any anatomical site in the lung. Concomitant lesions are not necessarily of the same age and might have different dynamic progression. This typically represents the field cancerization process previously defined in upper airways (5, 6). The current hypothesis is that one of these lesions will progress into lung cancer and that patients surgically treated for a first primary lung cancer are at high risk for development of a second primary lung cancer from any random lesion in the cancerization field. Based on loss of alleles of tumor suppressor genes, the cumulative index of genetic abnormalities in terms of fraction loss of specific chromosomal arms such as 3p and 9p (7–9) increases with the severity and grade of the histological lesions. Although the risk of cancer progression increases with histological grade of preinvasive lesions, these lesions such as SD[3] and CIS may also regress (10–12). Also, whether a low-grade lesion like metaplasia or mD progresses more slowly to lung cancer than high-grade dysplasia (MD and SD) is unknown. Consequently, the histological grade of any preinvasive bronchial lesion might not be predictive of lung cancer.

In addition, the chronology of acquisition of molecular events and their correlation with morphological grades is unknown.

Several molecular abnormalities have been described in lung cancer and their preinvasive lesions. We have previously validated the immunohistochemical application of P53, Bax-*Bcl*2, P16, and cyclin D1 for the study of preinvasive lesions in

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[3] The abbreviations used are: SD, severe dysplasia; CIS, carcinoma *in situ*; mD, mild dysplasia; MD, moderate dysplasia; BBR, Bax:Bcl2 ratio.

the vicinity of lung carcinoma in surgical samples (13, 14). The aim of the present study is to validate the use of these immunohistochemical markers on bronchial biopsies for lung cancer risk assessment. *P53* is a tumor suppressor gene acting as a transcription factor regulating the activity of several genes involved in cell cycle control, apoptosis, and DNA repair. *P53* inactivation by mutation contributes to tumor growth by loss of both cell cycle control and escape from apoptosis (15, 16). Most *P53* mutations are missense mutations (~85%), leading to P53 protein stabilization. This stabilization allows immunohistochemical detection (17). Positive immunoreactivity of P53 correlates fairly with *P53* missense mutations and is endorsed with biological significance when stringent criteria are required for the threshold value of P53 positivity. It has been assumed that at least 20% of immunopositive nuclei within a cell population allows a correlation of ~90% with the presence of missense mutation (18). Although a few studies have demonstrated the presence of *P53* mutation in preinvasive lesions in the lung, we and others (13, 19, 20) have previously shown that P53 protein stabilization and immunohistochemical expression in preinvasive bronchial lesions is predictive of synchronous or metachronous lung cancer.

$P16^{INK4}$ and cyclin D1 are two proteins regulating the phosphorylation of Rb protein, which is the main protein involved in the control of G_1 arrest (14). $P16^{INK4}$ prevents Rb phosphorylation by cyclin D1-kinase complexes. Three different mechanisms account for $P16^{INK4}$ inactivation: promoter methylation; homozygous deletion; and missense mutation with a decreasing order of frequency. We previously showed that all three mechanisms can be identified in non-small cell lung cancer concordantly with loss of $P16^{INK4}$ protein expression in 50% of the cases (21, 22) and that $P16^{INK4}$ loss occurs in preinvasive bronchial lesions, starting at the level of MD (14). Overexpression of cyclin D1 induces hyperphosphorylation of Rb protein and thus loss of Rb function in G_1 arrest in releasing E2F1, an essential transcription factor for the G_1-S transition (23, 24). We have previously shown that cyclin D1 overexpression occurs in 45% of lung cancer and in 30% of dysplasias (14, 22). Cyclin E is also involved in achievement of Rb protein phosphorylation at the end of G_1 phase in association with cyclin-dependent kinase 2. A few studies have shown cyclin E overexpression in non-small cell lung carcinoma as well as in preinvasive lesions (25). The clinical significance of these abnormal protein expressions or their association in preinvasive lesion is currently unknown.

Besides cell cycle deregulation, loss of susceptibility for apoptosis leads to cell accumulation and tumor growth. Bcl2 and Bax are respectively antiapoptotic and apoptotic factors of the Bcl2 family of proteins, and their equilibrium in the cell cytoplasm is known to influence the susceptibility for apoptosis (26, 27). Whereas Bcl2 expression is confined to the basal cell layer in normal bronchial epithelium, Bax is expressed in all bronchial epithelial cells. An extension of Bcl2 expression and/or loss of Bax expression leads to the inversion of BBR ratio to become <1. Bcl2 overexpression has been demonstrated in ~30% of non-small cell lung carcinoma (28, 29), as well as an inversion of BBR ratio in precursor bronchial lesions of lung cancer (13, 30).

Symptomatic smokers and patients surgically treated for primary lung cancer are at risk for developing lung cancer. We hypothesized that accumulation of molecular abnormalities as assessed by aberrant protein expression in a large series of correctly classified preneoplastic bronchial lesions in these high-risk patients might reflect the severity of the carcinogenic process. We postulated that the level of accumulation of molecular abnormalities at each step of the morphological transformation might give more insight in their chronology and help to assess a molecular fingerprint of the field cancerization process characteristic of progression into lung cancer. Thus, the aim of the present study was validation of immunohistochemical markers for lung cancer risk assessment.

PATIENTS AND METHODS

Patients. We performed a prospective study in 48 patients (45 males, 3 females) mean age of 63 years (35–82 years) who presented at least one lesion of bronchial metaplasia. These 48 patients at high risk for lung cancer included 32 former smokers and 15 current smokers. Among them, 27 were heavy smokers (25–95 pack-years). Twenty-one patients had a previous history of surgically treated lung cancer 2–10 years before the biopsy. They did not receive prior chemotherapy or radiotherapy. The bronchial biopsies were performed under white light or fluorescent bronchoscopy at more than one site according to macroscopic suspicion. The indication for bronchoscopy was pulmonary symptoms such as cough, dyspnea, and phlegm or systematic during follow-up of the primary lung cancer. Patients were suspected of cancer but without clinical overt cancer. The lesions included in the present study were detected and biopsied at distance from the primary site in the cancer treated patients. When several sites were biopsied, all lesions of the grade of metaplasia or worse were studied. The clinical follow-up duration ranged from at least 18 to 36 months. Seventeen patients presented a lung cancer, all squamous cell carcinoma, considered as synchronous when diagnosed within 6 months after the baseline biopsy (in 16 patients) or metachronous (in 1 patient 12 months after detection of the preinvasive lesion) to the presence of the preinvasive lesions. Patients with a second primary at a different site underwent careful pathological review to exclude lung metastasis. Thirty-one patients did not present invasive cancer during the follow-up time.

Histopathological Classification of Preinvasive Lesions. Slides were reviewed separately by two pathologists well experienced in lung cancer pathology using criteria for preinvasive lesions according to the WHO 1999 classification (31), without knowledge of the clinical context. In case of discrepancies a consensus was achieved, involving the staff of the pathology laboratory. Dysplastic lesions were separated in two groups for statistical analysis. Metaplasia and mD were considered as low-grade lesions, and MD, SD, and CIS were considered as high-grade lesions. Although CIS may regress, it is usually treated as a new tumor site with local treatment. Therefore, in a alternative calculation, CIS was considered as a neoplastic lesion and grouped with invasive lung cancer as an end point for the study.

Immunohistochemistry. Immunohistochemical analysis was performed on formalin-fixed bronchial biopsies. Primary antibodies directed against P53, cyclin D1, cyclin E, $P16^{INK4}$, Bax, Bcl2 are described on Table 1. After overnight incubation at 4°C with the primary antibody, slides were rinsed in PBS and exposed to the secondary antibody, or a biotinylated donkey

Table 1 Technical conditions of immunostaining

Antigen	M/P[a]	Antibody clone	Source	Retrieving	Dilution
P53	M	DO7	Dako, Glostrup, Denmark	MW 10 min. (pH 6)	1/20
$P16^{INK4}$	P	P16-C20	Santa Cruz Biotechnology, Santa Cruz, CA	No	1/200
Cyclin D1	M	DCS-6	Calbiochem, Cambridge, United Kingdom	MW 10 min. (pH 6)	1/4000 kit tyramine CSA; Dako
Cyclin E	M	13 A3	Novocastra L., Newcastle, United Kingdom	MW M. S. Unmasker (pH 8)	1/50
Bcl2	M	124	Dako	MW 10 min. (pH 6)	1/25
Bax	M	N19	Santa Cruz Biotechnology	MW 10 min. (pH 6)	1/200

[a] M, mouse monoclonal antibody; P, rabbit polyclonal antibody; MW, microwave heating.

antimouse antibody (1/500; Jackson Laboratory) for monoclonal antibodies, or a biotinylated antirabbit antibody (1/1250; Jackson Laboratory) for polyclonal antibodies for 1 h at room temperature. Slides were then rinsed in PBS and exposed to the avidin biotin peroxidase complex (1/400; Dako) for one hour at room temperature. The chromogenic substrate of peroxidase was developed using a 0.05% solution of 3.3′ diaminobenzidine tetrahydrochloride, 0.03% H_2O_2 and 10 mM imidazole in 0.05 mM, and Tris HCl buffer (pH 7.6) and counterstained with H&E. In the negative controls, the primary antibody was replaced by nonspecific, nonimmune immunoglobulin of the same isotype at equivalent final concentration. Positive external controls were tissues known to express the given antigen (P53, P16, cyclin D1, cyclin E, *Bcl*II, or Bax). Internal controls on slides were lymphocytes for *Bcl*II and Bax and endothelial cells and fibroblasts for $P16^{INK4}$. In these conditions of immunostaining, P53, cyclin, D1 and cyclin E were negative in normal cells present in the submucosa of bronchial biopsies.

Statistical Analysis. The association of immunohistochemical and histological parameters have been measured by Fisher's exact test.

To determine whether MD shared similarity of molecular profile either with low-grade lesion (metaplasia or dysplasia) or with high-grade lesion (SD and CIS), a Mann-Whitney test was performed to compare the number of immunohistochemical abnormalities (from 0 to 4) between MD/metaplasia and mD and between MD/SD and CIS.

To evaluate the predictive value of aberrant protein expression and histological grade or any kind of association for the presence of lung cancer, a multivariate logistic regression analysis was done. The tested parameters were at least one of the high-grade lesions (MD, SD, and CIS) and the presence of more than two molecular abnormalities in any lesion. Only P53, cyclin D1, cyclin E, and the ratio of BBR were considered as valid parameters; they were evaluated on 100% of lesions. In contrast, $P16^{INK4}$ could not be interpreted in 17 of the cases, and this marker was discarded from the statistical analysis.

The statistical analysis was performed on all patients followed for a minimum of 18 months. Because CIS patients were all treated, an alternative analysis was performed, considering CIS as an end point as well as lung cancer, and were indeed excluded from the tested parameters. The age, sex, and number of pack-years did not differ statistically between groups and were not included in the present model.

Statistical analysis was performed with SPSS 6.0. Differences were recognized as significant when their P was <0.05.

RESULTS

Distribution of Preinvasive Bronchial Lesions. Eighty bronchial lesions were recorded by histological examination of biopsies from 48 patients. Eight patients had more than one biopsy at 2–13 months internal time. The number of lesions/patient varied from 1 to 5 with a mean of 1.6. Distribution of recorded lesions was: 31 metaplasias; 12 mDs; 9 MDs; 9 SDs; and 19 CISs. All of the lesions were immunohistochemically studied and the results reported as one case.

The distribution of recorded lesions/patient was as follows: 26 patients had metaplasia; 11 had mD; 7 had MD; 9 had SD; and 16 had CIS.

Among the patients with at least one high-grade lesion, 10 of 24 (41%) exhibited multiple lesions and 9 of 16 patients (56%) presenting a CIS had another associated high-grade lesion.

P53 Immunohistochemical Reactivity. P53 immunohistochemical reactivity was considered as positive when at least 20% of nuclei were clearly stained and formed clusters of positive basal and suprabasal cells. Dispersed positive cells along the basal cell layer were not considered as significant of P53-positive reactivity. All lesions could be evaluated for P53 reactivity relative to internal and external controls. In P53-positive lesions, 20–80% nuclei were stained leading to 29 of 80 (36%) P53-positive lesions. These included 1 of 31 metaplasia (3%), 6 of 12 mDs (50%), 3 of 9 MDs (33%), 7 of 9 SDs (78%), and 12 of 19 CISs (63%; Fig. 1*A*). P53 reactivity was significantly more frequent in high-grade dysplasia than in low-grade dysplasia ($P < 0.001$; Table 2).

Among the 20 patients presenting with a P53-positive lesion, 8 presented a synchronous invasive lung cancer and 11 did not within 18 months. One patient died without lung cancer 1 year after the biopsy. The predictive positive value of P53 reactivity for the occurrence of an invasive lung cancer was 40 and 66% (10 of 15) when CIS and invasive cancer were considered together as end point.

Loss of $P16^{INK4}$ Protein Expression. $P16^{INK4}$ expression was considered as positive when cell nuclei were stained regardless of cytoplasmic staining. Nuclear staining in endothelial cells and fibroblasts was the required internal control. In the absence of $P16^{INK4}$ expression in endothelial cells or fibro-

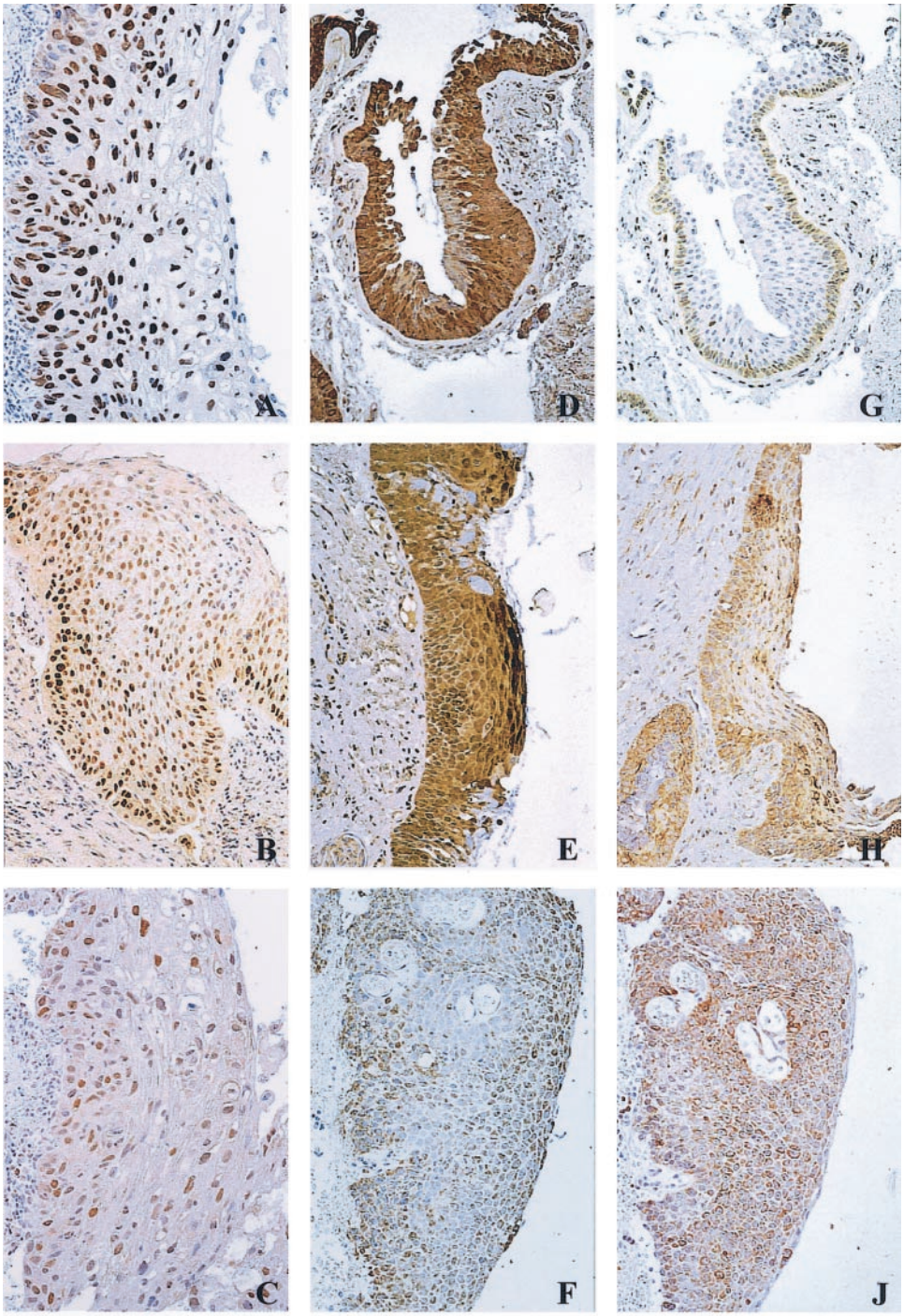

Fig. 1 Immunostaining of P53, cyclin D1, cyclin E, Bax, and *Bcl*II in bronchial mucosae. *A*, P53-positive nuclear immunostaining in a CIS. *B*, cyclin D1-positive nuclear immunostaining in a SD. *C*, cyclin E-positive immunostaining in a CIS. *D* and *G*, Bax (score 3) immunostaining in a normal (*left*) and hyperplastic (*right*) bronchial mucosae (*D*). *Bcl*II (score 1) immunostaining restricted to the basal cells (*G*) on a serial section of the same bronchial mucosae as *D*. *E* and *H*, Bax immunostaining (score 3) on a squamous cell metaplasia with preserved normal Bax expression (*E*) and decrease of Bax in several areas of another squamous metaplasia (score 1; *H*). *F* and *J*, Bax (*F*) and *Bcl*II (*J*) immunostaining on serial sections of the same severe angiogenic dysplasia, showing loss of Bax in several areas (*F*) in contrast with increased *Bcl*II staining (*J*) as compared with normal bronchi (*D* and *G*). In this case, the BBR ratio was <1.

blasts, lesions were considered as nonevaluable for $P16^{INK4}$ expression. This occurred in 17% of the cases (13 of 80). When loss of $P16^{INK4}$ protein expression was considered as a molecular abnormality, large areas of epithelial cells lacked staining, and <10% cell nuclei were stained Thus 21% of the lesions presented loss of the protein expression (14 of 67), including 3 of 25 metaplasias (12%), no mDs, 2 of 9 MDs (22%), 1 of 7 SD (14%), and 8 of 17 CISs (47%). $P16^{INK4}$ loss was more frequently observed in high grade than in low-grade dysplasia ($P < 0.01$; Table 2).

In 11 patients that presented at least one lesion with loss of $P16^{INK4}$ expression, 3 were diagnosed with lung cancer. The positive predictive value of $P16^{INK4}$ loss for the occurrence of lung cancer was of 27% and of 50% (3 of 6) when CIS was grouped with lung cancer as an end point at 18 months of follow-up.

Table 2 Distribution of molecular abnormalities according to histological grade of preinvasive bronchial lesions

Lesions	P53	$P16^{INK4}$	Cyclin D1	Cyclin E	BBR $< 1^a$
Metaplasia	3% (1/31)	12% (3/25)	6% (2/31)	3% (1/31)	3% (1/31)
MD	50% (6/12)	0	17% (2/12)	25% (3/12)	25% (3/12)
MD	33% (3/9)	22% (2/9)	67% (6/9)	33% (3/9)	67% (6/9)
SD	78% (7/9)	14% (1/7)	56% (5/9)	55% (5/9)	78% (7/9)
CIS	63% (12/19)	47% (8/17)	53% (10/19)	89% (17/19)	53% (10/19)
P^a	$P < 0.001$	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$

[a] *P*, probability of difference in distribution between low-grade (metaplasia and MD) and high-grade lesions (MD, SD, and CIS; Fisher's exact test).

Cyclin D1 Overexpression. Cyclin D1 expression could be evaluated compared with positive external controls and negative internal controls in all 80 bronchial lesions. Cyclin D1 was considered as overexpressed when at least 5% of cell nuclei were stained because the immunohistochemical method was standardized to be cyclin D1 negative in normal control tissues. Twenty-five of 80 bronchial lesions (31%) were cyclin D1 positive, including 2 of 31 metaplasias (6%), 2 of 12 mDs (17%), 6 of 9 MDs (67%), 5 of 9 SDs (56%), and 10 of 19 CISs (53%; Fig. 1*B*). The frequency of cyclin D1 overexpression was significantly higher in high grade than in low grade dysplasia ($P < 0.001$; Table 2). Among 16 patients who presented at least one lesion with cyclin D1 overexpression, 5 had confirmed lung cancer. The positive predictive value of cyclin D1 overexpression for occurrence of invasive lung cancer was of 31%, and of 50% when CIS was considered as an end point with lung cancer (6/12).

Cyclin E Overexpression. Cyclin E expression was evaluated in the 80 lesions. It was considered as overexpressed when >5% of cell nuclei were labeled, immunohistochemical method being standardized for a negative background on normal cells. Thirty-six percent of lesions were positive (29 of 80), including 1 of 31 metaplasia (3%), 3 of 12 mDs (25%), 3 of 9 MDs (33%), 5 of 9 SDs (55%), and 17 of 19 CISs (89%; Fig. 1*C*). The frequency of cyclin E overexpression was significantly higher in the high grade than in the low-grade dysplasia ($P < 0.001$; Table 2).

Among the 18 patients that presented at least one cyclin E-positive lesion, 9 had lung cancer. The positive predictive value of cyclin E overexpression for occurrence of invasive lung cancer was 50% at 18 months follow-up, and of 81% (9 of 11) when CIS and/or lung cancer was the common end point.

Forty-two lesions presented both cyclin D1 and E overexpression. Among those, 29 of 42 (69%) were positive for one or the other of these cyclins, whereas 13 of 42 (31%) overexpressed both cyclins D1 and E concomitantly.

Bax and Bcl2 Expressions. All lesions were evaluated for Bax and bcl2 immunostaining, relative to internal and external controls. *Bcl*II immunostaining was cytoplasmic and granular and restricted to the basal layer in normal bronchial epithelium. A score of 1 was applied to this normal expression pattern: 62% of the lesions showed a normal (score 1) *Bcl*II pattern (Fig. 1*G*). A *Bcl*II immunostaining extending to several layers of the epithelium was given a score 1 for ⅓, 2 for ⅔, and 3 for the whole thickness immunostained. Accordingly, 1 of 31 metaplasia (3%), 4 of 12 mDs (33%), 5 of 9 MDs (55%), and 9 of 9 SDs (100%), as well as 11 of 19 CISs (58%) presented a score of *Bcl*II > 1 (Fig. 1*J*).

Bax immunostaining was also cytoplasmic, granular, and intense in all bronchial epithelial cells (score = 3; Fig. 1*D*). Forty-three percent (34 of 80) of lesions disclosed a loss of Bax expression as compared with normal bronchial epithelium (Fig. 1, *D*, *E*, and *H*). They comprised 2 of 31 metaplasias (6.5%), 4 of 12 mDs (33%), 6 of 9 MDs (66%), 7 of 9 SDs (78%), and 15 of 19 CISs (79%).

The ratio BBR was calculated for each lesion as compared with the normal epithelium where the score of Bax was constantly higher than that of Bcl2 (BBR > 1). A BBR < 1 was regarded as an inversion of the normal BBR ratio. Inversion of BBR was observed in 34% of cases, comprising 1 of 31 metaplasia (3%), 3 of 12 mDs (25%), 6 of 9 MDs (67%), 7 of 9 SDs (78%), and 10 of 19 CISs (53%; Fig. 1*F–J*). Inversion of BBR correlated with the histological grade of the lesions. It was significantly more frequent in high-grade dysplasia than in low-grade lesions ($P < 0.001$). No correlation was shown between the immunoreactivity of p53 and increase of Bcl2 expression, decrease of Bax, or inversion of BBR, thus confirming our previous results.

Among the 19 patients who presented with at least one lesion with inversed BBR ratio (<1), 7 had lung cancer. The predictive positive value of BBR < 1 for invasive lung cancer was of 37% at 18 months follow-up and of 64% (9 of 14) when CIS was considered as an end point together with lung cancer.

The Place of MD according to Molecular Grade. Fig. 2 illustrates the number of molecular abnormalities (1–4) according to the grade of histological preinvasive lesions concerning those abnormalities that could be evaluated in 100% of cases: P53 overexpression; inversion of BBR ratio; cyclin D1; and cyclin E overexpression.

To evaluate whether MD should be considered as a low-grade dysplasia or a high-grade dysplasia according to its molecular profile (cumulative index of molecular abnormalities; from 1 to 4), a Mann-Whitney median test was used, considering the cumulative index of molecular abnormalities in the different lesion groups. This cumulative index was significantly higher in MD than in mD and metaplasia ($P = 0.002$) but not significantly different between MD, SD, and CIS ($P = 0.229$). These results imply that MD should be considered as a high-grade dysplasia as regard to its accumulative index of molecular abnormalities.

Prospective Clinical Follow-Up of Patients. During the follow-up, 16 patients had lung cancer synchronous to baseline

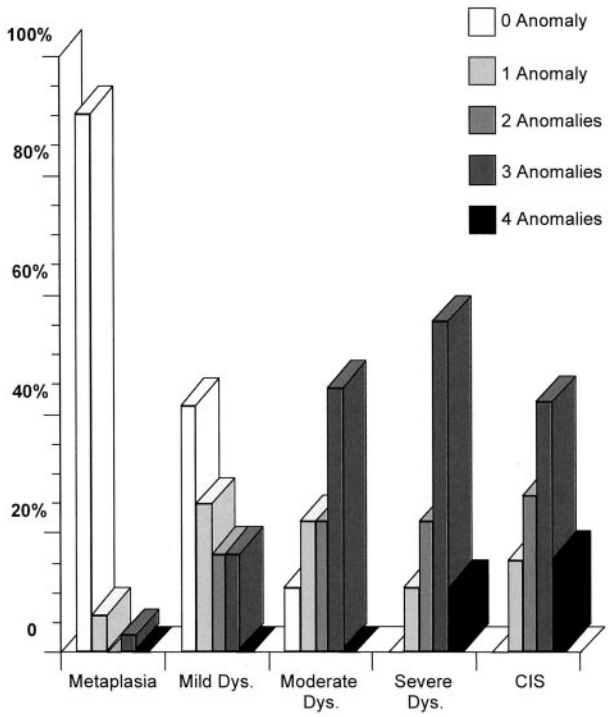

Fig. 2 Distribution (percentage) of the number of immunohistochemical anomalies (P53, cyclin D1, cyclin E, BBR < 1) by histological grade of preinvasive bronchial lesion.

preneoplastic lesions showed here, and 1 patient developed a metachronous lung cancer (12 months). Among the 31 patients that did not have lung cancer during this period, 8 presented a CIS that was treated (7 by bronchoscopical treatment and 1 by a surgically limited bronchial resection). Among the 17 patients with cancer, 8 presented a CIS at baseline biopsy, of which 3 developed at distance from cancer and 5 at the same bronchial location as CIS but on different biopsies. In 24% of cases (4 of 17), the biopsied lesion was contralateral to the cancer, in 13 of 17 (76%) homolateral, and in 6 of those 13 (46%) located in another lobe.

Among the 10 patients presenting metaplasia associated with one or several molecular abnormalities, 5 developed invasive lung cancer or CIS. Eleven patients presented a mD, 7 of which showed at least one of the recorded molecular abnormalities (Table 3*A*). At 18 months follow-up, 3 of these 7 patients presented an invasive carcinoma and 3 others a CIS. Of these 6 patients, 5 presented a mD with 1–3 molecular abnormalities including one with a P53-positive expression as a solely molecular abnormality. Two patients presenting a mD with molecular abnormalities did not develop lung cancer at 2 years follow-up. Among the 4 patients that presented a mD without molecular abnormality, one presented a synchronous carcinoma, and 3 others had no cancer detected during the follow-up.

MD was diagnosed in 7 patients with molecular abnormalities (Table 3*B*); two developed a lung cancer and 2 others a CIS. All of those 4 patients had a MD associated with 1–3 molecular abnormalities. The 3 other patients presenting with a

Table 3 Occurrence of CIS or invasive cancer in patients with: MD (A), MD (B), and SD (C) at baseline biopsy

A. MD

Patient	D[a]	No. of molecular abnormalities	Carcinoma[b]	CIS[b]	Total
1	1	0	0	0	0
2	1	3	0	0	0
3	1	0	0	0	0
4	1	1	1	0	1
5[c]	2	1	0	1	1
6	1	0	0	0	0
7	1	2	0	0	0
8[c]	1	0	1	0	1
9[c]	1	2	0	1	1
10[c]	1	3	0	1	1
11[c]	1	2	1	0	1

B. MD

Patient	D	No. of molecular abnormalities	Carcinoma	CIS	Total
1	1	3	0	0	0
2	1	1	0	0	0
3[c]	2	0 and 3	0	1	1
4	1	3	1	0	1
5	2	2 and 2	0	0	0
6	1	1	1	0	1
7[c]	1	3	0	1	1

C. SD

Patient	D	No. of molecular abnormalities	Carcinoma	CIS	Total
1	1	3	1	0	1
2	1	3	0	0	0
3	1	4	0	1	1
4	1	1	0	0	0
5	1	3	0	1	1
6	1	3	0	0	0
7	1	2	1	1	2
8	1	3	0	1	1
9	1	3	0	1	1

[a] Number of dysplastic area.
[b] Carcinoma and CIS are diagnosed additionally after baseline biopsy.
[c] Patients with another high-grade lesion.

MD with molecular abnormalities did not develop cancer or CIS during the 18 months of follow-up.

SD was present in 9 patients with 1–4 molecular abnormalities (Table 3*C*); two of them developed a lung cancer and 4 others a CIS only. Three of these patients that presented SD with molecular abnormalities did not develop lung cancer or CIS at 18 months follow-up.

Overall, among the 18 patients that presented at least one lesion with more than two molecular abnormalities, regardless of histological grade, 7 patients presented a synchronous lung cancer and 6 presented a CIS during the 18 months follow-up.

Multivariate Analysis. The logistic regression analysis included as variables each of the high-grade dysplasias (MD, SD, CIS), the presence of any lesion with more than two molecular abnormalities and, considering an invasive carcinoma as an end point, did not show a significant association for any of these variables with cancer occurrence. However, because local

treatment is recommended for CIS and always performed in our series, CIS, in an alternative statistical approach, was not included as a variable but considered as an end point with invasive carcinoma. A significant association was found between a preinvasive lesion not considering CIS with more than two molecular abnormalities and occurrence of invasive carcinoma and/or CIS; $P = 0{,}02$; risk ratio = 9.2; 95% confidence interval: 1.4–60.5.

DISCUSSION

According to the current concept, the preneoplastic or preinvasive bronchial lesions eventually progress to squamous cell carcinoma. However, the natural history of these lesions and their timing toward progression are far from well known. The time delay for a low-grade lesion or a CIS to progress into lung cancer may be extremely variable. As an example, in experimental animals exposed to carcinogens, high-grade lesions in the lung have been shown to progress or regress (10–12). The risk of developing lung cancer might depend upon both the number of molecular changes accumulated to a certain point in time and the rate of accumulation of new changes. Field cancerization is a multistep and a multicentric process. In recent series (32) among patients presenting CIS, 44% presented several lesions, the mean number of preinvasive lesions in patients presenting with a high-grade lesion was >2, and 15% of patients with high-grade lesions had at least 3 (33). Our results are in complete agreement with these previous observations because 41% of the patients exhibited multiple lesions and more than half CIS patients had another high-grade lesion. This reflects the multicentric transformation of epithelial cells along the bronchial tree, otherwise defined as field cancerization.

In this study, we have chosen as biomarkers susceptible to discriminate a severe field cancerization process the aberrant expression of five proteins playing key roles in the carcinogenic process: P53; cyclin D1 and cyclin E involved in cell cycle regulation; and P53, Bax, and Bcl2 major players of susceptibility to apoptosis. These markers were indeed chosen with respect to their clear association with the oncogenic process and definite increasing abnormalities from the normal epithelium through low-grade and to high-grade dysplasia. Our results as regard to the respective frequency of these abnormalities in preinvasive lesions are in concordance with our previous studies, and the levels of these five proteins could be assessed in all of the lesions. In contrast, 17% of lesions could not be evaluated for $P16^{INK4}$ on formalin-fixed paraffin section. The reason for this failure is unavailability of internal controls. Knowing the low basal level of P16 in normal epithelial cells and in submucosal normal cells, it is not surprising that P16 was negative in the normal epithelium and thus could not serve as internal positive control. In contrast with a previous study by Lonardo *et al.* (25), we found overexpression of cyclin E in some low-grade lesions, indicating that this could be an early molecular lesion during the course of carcinogenesis. Interestingly, cyclin E or D1 overexpression was infrequently concomitant, suggesting that only one of these cyclins involved in G_1-S transition could be sufficient to provide advantage for growth.

We found a significant association between the grade of histological lesions as discriminated between low grade and high grade and the individual frequency and cumulative rate of four molecular markers. These findings are in keeping with the concept of progressive and stepwise accumulation of the genetic and epigenetic abnormalities driving cell transformation. Reproducibility analyses conducted by the pathologists with the regard to subclassification of preinvasive lesions have questioned the possibility of eight classes of lesions between hyperplasia and CIS, compared with two classes of low-grade and high-grade lesions (34). In this setting, whether MD should be considered as low grade or high grade had to be decided. It was considered as a high-grade lesion in the study cited above. Interestingly, we found here that the cumulative rate of molecular abnormalities observed in MD allow them to be regarded as high-grade dysplasia, along with SD and CIS.

The presence of a high-grade lesion or of a lesion presenting more than two molecular abnormalities regardless of histological grade was not found to be associated with invasive lung cancer in our study at 18 months follow-up. However, because CIS is generally treated in our and other institutions by local treatments (phototherapy or cryotherapy; Ref. 35), CIS could be considered as an end point as well as invasive lung cancer. In addition, pathologists have experienced the high frequency of discovery of a microinvasive carcinoma on serial sections 100–300 μm away from CIS, reflecting the frequent underestimation of invasion in the vicinity of CIS. Thus, considering both CIS and invasive lung cancer as one end point seems reasonable. The presence of a lesion presenting more than two molecular abnormalities (excluding CIS) was statistically associated with occurrence of invasive lung cancer or CIS.

With the exception of P53 (17, 19, 20), the predictive positive value for the evolvement of or association with lung cancer of other histochemical biomarkers is not clear for the moment. In this study, we found a predictive positive value at 18 months follow-up for the development of lung cancer of 40% for P53, 37% for an inversion of the BBR ratio, 31% for cyclin D1, and 50% for cyclin E overexpression. These predictive values deny the significance of any of these isolated markers as valuable end point biomarkers. Their inclusion in a molecular profile renders them interesting because immunohistochemistry is easy to perform, albeit scrutiny is necessary for their interpretation and consistent results. The predictive value calculated here is indeed likely to be underestimated by the short minimal follow-up of 18 months. Interestingly, the majority of invasive synchronous and metachronous cancers developed at a different site from that of the studied dysplasia and in 24% contralaterally. This suggests that the protein biomarkers applied here are more valuable for evaluating the field cancerization process than the progression of a lesion at a given site because the biopsies would influence follow-up and outcome of a given lesion. In this respect, this study differs widely from another longitudinal study of identified lesions (36). Our findings are more consistent with the potential power of oncogenic mutations (37–40), aberrant promoter methylation (38, 40, 41), or loss of heterozygosity alterations (42, 43) assessed in peritumoral or tumoral epithelial cells shed into body fluids (BAL, sputum) to predict cancer progression at a random location in the respiratory tract. Thus, a molecular signature or grading reflected in the cumulative rate of molecular lesions has clinical value.

It should be noted that several lesions considered as low-

grade lesions presented several molecular abnormalities. Interestingly among 17 patients with metaplasia or mD, 11 patients with at least one molecular abnormality developed lung cancer or CIS consistent with a much higher rate of progression in these patients than in the overall population presenting metaplasia or mD. These findings suggest that the profile of molecular lesions allows identification of low-grade dysplasia associated with a higher lung cancer risk than those associated simply with the morphological grade of the given lesion. In patients with low-grade lesions, the severity of the cancerization field process and the risk of lung cancer is reflected by the molecular grade.

In conclusion, we have shown the feasibility of a prospective approach using molecular pathology on bronchial biopsies, the stepwise accumulation of these abnormalities along the process of cancerization, and the place of MD among high-grade lesions. Molecular biomarkers *in situ* might provide a way to select a subpopulation of patients with particularly active mutagenesis, progressive field cancerization process, and at very high risk to develop CIS or lung cancer. The present data would encourage thorough screening for cancer detection in patients with preinvasive bronchial lesions associated with several molecular abnormalities chosen in a constellation of potential biomarkers. These patients should be intensively evaluated by other means, *i.e.*, low-dose spiral computerized tomodensitometry in association with fluorescent bronchoscopy, to enable earlier detection and therapeutic intervention of early lung cancer. Low-grade lesions with a severe molecular grade would benefit from a closer follow-up than that usually applied in this setting. These observations support the notion that risk assessment should be based not only on smoking consumption and on histopathology of bronchial lesions but also on the basis of *in situ* molecular biomarkers.

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