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Immunohistochemical markers of proliferation and vascularisation in preneoplastic bronchial lesions and invasive non-small cell lung cancer

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Autofluorescence bronchoscopy (AFB) has been shown to be sensitive to detect preneoplastic lesions in central lung airways system. In early stages of carcinogenesis, up-regulation of cyclooxygenase (COX)-2, Ki67 and/or increased angiogenesis may play a role by promoting the proliferation of tumoral cells and their resistance to apoptosis, as well as angiogenesis, tumor cell invasion and setting up of the metastatic process. The present study compared the expression of proliferative (COX-2, Ki67 and PCNA) and angiogenic markers (CD34 and NG2) between preneoplastic bronchial squamous dysplasia lesions and invasive squamous cell carcinoma. Biopsies obtained during AFB [preneoplastic lesions: low-grade (lesions up to moderate dysplasia), n=13; high-grade lesions (severe dysplasia), n=12] and surgical specimens (resections of bronchogenic carcinoma, n=11) were stained with COX-2, Ki67, PCNA, CD34 and NG2 monoclonal antibodies. Microvessel density (MVD) was analysed based on anti-CD34 immunostaining. Lesions were positive for COX-2 in 12 out of 25 preneoplastic lesions, and in 10 out of 11 invasive carcinomas (p=0.025). In preneoplastic lesions, the mean percentage of Ki67 positive cells was lower compared to invasive carcinomas (37.4±5.8 versus 58.6±8.4%, p=0.043). In addition, significant differences in MVD were observed between preneoplastic and NSCLC specimen [35.3 (25.9, 61.9) versus 22.1 (20.1, 32.6), p=0.016]. No differences were observed in the mean percentage of PCNA or NG2 positive cells between preneoplastic lesions and invasive carcinomas. Findings of the present study indicate that increases in COX-2 and Ki67 expression may be associated with the development of bronchogenic carcinomas and possibly with acquisition of an invasive phenotype. In contrast, increased CD34 expression in preneoplastic lesions suggests that increased MVD may represent an early marker of lung carcinogenesis.

Key words: cyclooxygenase-2, PCNA protein, Ki-67, microvessel density, non-small cell lung cancer, autofluorescence bronchoscopy

Lung cancer is the most common malignancy in the developed countries worldwide, and accounts for more deaths than prostate, breast and colorectal cancers together [1]. Despite tremendous effort to improve outcome of patients with lung cancer, the prognosis remains grim – with at best only 10 to 14% patients patients with non-small cell lung cancer (NSCLC) alive after 5 years, and two-thirds of death accurring within 12 months after diagnosis [2]. Mortality in NSCLC worsens radically with advancing stage at the time of diagnosis. Therefore, implementing screening programs in high-risk individuals in association with improving techniques in localizing and identifying early endobronchial lesions represent major challenges and areas of research in this field [3]. In this regard, improvements in autofluorescence bronchoscopy (AFB) techniques in association with the search for novel immunohistochemical carcinogenic markers carry the potential of improved detection of high-risk preneoplastic and early neoplastic lesions in central airways.

Lung carcinogenesis is a multistep process characterized by successive molecular abnormalities resulting in malignant transformation of epithelial cells [4]. Nevertheless, although the preneoplastic or preinvasive bronchial lesions may eventually progress to squamous cell carcinoma, the natural history of these lesions and their timing toward progression ar not well understood [5]. In early stages of carcinogenesis, up-regulation of cyclooxygenase (COX)-2, proliferating cell nuclear antigen (PCNA), and/or Ki67 may play a role by promoting the proliferation of tumoral cells and their resistance to apoptosis. In addition, stimulated angiogenesis may increase

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tumor cell invasion and speed up the metastatic process [6]. Indeed, several studies analysed the prognostic role of various proliferation and angiogenic markers in NSCLC. Nevertheless, owing to the heterogenity of data and immunostaining techniques, and to the changes in WHO/IASCL classification of bronchial neoplastic lesions, their results remain inconclusive. Increased expression of COX-2, Ki67 and PCNA in NSCLC was observed in some [7–10] but not all [11, 12] studies. Also, the analyses of the prognostic role of endothelial marker CD34, pericytal marker NG2, and of the intratumoral microvessel density (MVD) yielded inconsistent results [13, 15]. Therefore, the aim of the present study was to analyse the expression of proliferation (COX-2, Ki67, PCNA) and angiogenic markers (CD34, NG2) in preneoplastic lesions of bronchial epithelium detected by AFB and in invasive NSCLC specimen obtained from patients undergoing thoracic surgery.

Material and methods

Study population. The study was performed on a) bioptic samples of bronchial squamous dysplasia obtained during AFB from patients who were examined due to chronic cough with sputum production and had history of heavy smoking, and b) surgical specimen from well-differentiated invasive squamous cell carcinoma from patients who underwent thoracic surgery.

Autofluorescence bronchoscopy. AFB was performed using a prototype of the Diagnostic Autofluorescence Endoscopy (DAFE, Wolf, Germany). As an excitation light source, a conventional Xenon lamp (Wolf, Germany) equipped with a special filter eliminating the infrared light was used. In this set-up, only blue light of 420-480 nm is delivered through an excitation filter and transmitted through a light guide. The emitted autofluorescence is transmitted via an image guide which contains a fluorescence filter, image intensifier, and a TV camera, and is attached to the eyepiece of a standard Wolf bronchoscope and to the image video monitor in real time. The intensified autofluorescence of normal mucosa appears green, whereas abnormal areas show cold image associated with changes in the colour, relief and fine structure of the mucosal surface [16].

Sample preparation and selection. All biopsy samples obtained during AFB as well as all core biopsies of 1 mm in diameter taken from each lung cancer tissue obtained during thoracic surgery were fixed for minimum of 3 and maximum of 10 hours in 10% neutral buffered formalin, and subsequently embedded in paraffin. 4 μm sections were cut from each paraffin-embedded tissue block, and were deposited on SuperFrost Plus Slides (Menzel-Glaser, Germany). Haematoxylin- and eosin- stained lesions were classified by a pathologist according to the 1999 histological World Health Organisation (WHO)/ International Association for the Study of Lung Cancer (IASLC) criteria into: normal (NI), hyperplasia (H), metaplasia (M), mild dysplasia (MiD), moderate dysplasia (MoD), severe dysplasia (SD), carcinoma in situ (CIS) and invasive carcinoma (IC) [17]. Patients with invasive NSCLC were staged according to operating and pathological findings based on AJCC/UICC-TNM classification and stage grouping.

Samples were assessed independently by two pathologists experienced in IHC examination and classification of lung samples (AB and PK). Also, all immunostaining was scored by these two independent observers. The interobserver variation was between 5 and 12%.

COX-2 staining and scoring. Immunohistochemistry was performed using a standard ABC method [18] and a mouse monoclonal antibody directed against the human COX-2 sequence (amino acids 580-599; immunoglobulin IgG1) (Neomarkers, USA). The sections were dewaxed by xylene, and rehydrated in ethanol. Endogenous peroxidase activity was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature. The anti-COX-2 antibody diluted at 1:24 ratio (final titration 4.17 ng/μl) was incubated on the slide for 60 minutes at 37°C. The primary antigen-antibody complex was fixed using 0.05% glutaraldehyde in 0.9% NaCl. Consequently, incubation with a secondary biotinylated antibody for 8 min followed. The slides were stained using a aminoethylcarbasole detection kit (Sigma, Germany) and counterstained with haematoxyllin. Normal mouse serum was substituted for primary antibody as a negative control. For the assessment of COX-2 expression, only cytoplasmic staining was evaluated. The distribution of COX-2 was scored as: 1) no staining (0%), 2) focal staining (1-49%), and 3) diffuse stainig ($\geq$50% of the cells). The staining intensity was defined as: 0=no staining; 1=weak staining, intensity inferior or equal to those of the normal epithelial cells in the lung; 2=moderate staining, stronger intensity than that of normal lung epithelial cells; and 3=strong, very intense staining, stronger than that of endothelial cells, equal to the strongest staining seen in NSCLC cells [8].

Ki67 staining and scoring Formalin-fixed and paraffin-embedded tumor sections (4 μm) were stained with Ki67 (MIB-1) monoclonal antibody (Dako, Denmark) at a 1 in 150 dilution for 60 minutes, using the ABC method [18] and aminoethycarbasolein as chromogen. Primary antibody was omitted from negative controls. Sections were counterstained with haematoxylin. Ki67 immunostaining was scored counting 1000 cells in 10 different randomly selected areas at x400 magnification.

PCNA staining and scoring. Immunostaining was performed using the ABC method [18]. Sections from formalin-fixed and parafin-embedded tissues were dewaxed, rehydrated, and brought to phosphate-buffered solution. Endogenous peroxidase activity was blocked by incubation for 7 minutes in 3% H_2O_2. Monoclonal antibody against PCNA (PC10) (Dako, Denmark) diluted in 1 in 400 in PBS was incubated overnight. Normal mouse serum was substituted for primary antibody as a negative control. The sections were then treated for 30 minutes with a biotin-labelled second layer antibody and avidin-biotin-peroxidase complex (Immunotech, Beckman Coulter Comp., France) was added. Sections were

developed with diaminobenzidine for 10 minutes, counterstained with haematoxylin, and mounted in resin. PCNA (PC10) immunostaining was scored by counting 1000 tumour cells at x400 magnification from 10 representative fields; the fraction of positive cells was then determined.

Immunohistochemical staining of endothelial cells, pericytes and analysis of microvessel density. Slides were incubated for 30 minutes in 1:50 diluted monoclonal anti-CD34 (C QBEnd 10, Dako, Denmark) to identify endothelial cells, and by anti-NG2 proteoglycan (Immunotech, Beckman Coulter Comp., France) to identify pericytes. Biotinylated goat antimouse IgG were used as secondary antibodies. To evaluate microvessel density (MVD), individual microvessels were counted independently at high power magnification (x200; area 0.713 mm^2). Each score was obtained by adding the vessel counts of 4x200 fields, as previously described [19].

Statistical analysis. The Kolmogorov-Smirnov test of normality was applied. Continuous variables are shown as means ± SEM, non-normally distributed variables as median (25th, 75th percentile). Differences between groups in normally distributed variables were tested by Student's two-tailed unpaired t-test, and in non-normally distributed variables by non-parametric Mann-Whitney rank sum test. Chi-square test was used to compare the proportion of categoric variables between groups. Statistical analyses were performed using SPSS software version 14.0 (SPSS Inc., USA).

Results

Characteristics of patients. Thirty-six patients, all Caucasians (32 men and 4 women), were enrolled to the study at the tertiary referral teaching center. They were generally late middle-aged (mean age 59.8±1.9 years, range 31-81 years). The first group of patients consisted of 25 subjects (mean age 60.8±2.5 years; 2 women) in whom biopsies indicative of bronchial squamous dysplasia were obtained during AFB. Among these, low-grade preneoplastic changes (lesions up to moderate dysplasia) were observed in 13, whereas high-grade preneoplastic changes (severe dysplasia) in 12 patients. The second group consisted of 11 patients (mean age 57.7±2.2 years; 2 women) who underwent lung resection for their squamous cell carcinoma: 6 had Grade 2, and 5 Grade 3 NSCLC. No differences were observed between the two groups in mean age or the proportion of women (p=0.447; p=0.749, respectively).

COX-2, Ki67, and PCNA scores. Significant differences were observed in COX-2 and Ki67 staining between preneoplastic and NSCLC specimen. Lesions were positive for COX-2 in 12 out of 25 preneoplastic lesions, and in 10 out of 11 invasive carcinomas (p=0.025). Moreover, in preneoplastic lesions, the mean percentage of Ki67 positive cells was lower compared to invasive carcinomas (37.4±5.8 versus 58.6±8.4%, p=0.043). Representative examples of COX-2 and Ki67 staining in preneoplastic lesion and NSCLC are shown in Fig. 1 and 2, respectively. In contrast, no differences were observed in the mean percentage of PCNA positive cells between preneoplastic lesions and NSCLC specimen (75.6±3.2 versus 72.5±5.4%, p=0.617).

Microvessel staining and MVD. We noted significant differences in the intensity of MVD using antibodies against CD34 endothelial antigen between preneoplastic and NSCLC specimen [35.3 (25.9, 61.9) versus 22.1 (20.1, 32.6), p=0.016]. A representative example of CD34 staining in bronchial squamous dysplasia is illustrated in Fig. 3. In contrast, staining with the anti-NG2, antigen specific for pericytes, did not reveal differences between preneoplastic and NSCLC specimen (62.3±5.1 versus 52.6±11.5, p=0.375).

Discussion

By studying immunohistochemical markers of proliferation and vascularisation in preneoplastic bronchial lesions and invasive NSCLC specimen, the present study demonstrated higher COX-2 and Ki67 expressions in invasive squamous cell carcinoma compared to bronchial squamous dysplasia, with no differences in PCNA expression between the two groups. In addition, differences in intratumoral MVD were observed: endothelial anti-CD34 staining was higher in squamous dysplasia lesions compared to invasive squamous carcinomas. Previously, increases in COX-2, Ki67, PCNA, CD34 and NG2 in NSCLC were selectively documented in some but not all reports [7-15]. Most studies examined patients with invasive NSCLC, and did not analyze the differences in expression of proliferative and angiogenic antigens between preneoplastic lesions and invasive carcinomas. To our knowledge, our data are the first to examine concurrently three proliferative and two angiogenic markers within two well-defined set of specimen: preneoplastic bronchial lesions and invasive NSCLC.

Despite efforts towards improving early detection of bronchial malignancies with subsequent innovations of therapeutic interventions, lung cancer remains the leading cause of mortality among all malignancies worldwide [1]. Cigarette smoking is the single best-documented risk factor for lung cancer. Cigarette smoke contains a mixture of carcinogens which, upon metabolic activation with concomitant silencing of the competing detoxification pathways, increase cancer risk in a given individual [20]. Cyclooxygenase enzymes may play important role in the oxidation of carcinogens in the lung, and in the conversion of aromatic hydrocarbons to the ultimately carcinogenic diol epoxides [21]. Indeed, in the rat model of lung carcinoma, Ye et al. [20] have recently demonstrated increases in COX-2 expression in preneoplastic lesions. Nevertheless, although COX-2 was shown to take part in tumorigenesis, it was not involved in the proliferation processes. In addition, pretreatment with aspirin reduced the development of dysplasia, thus confirming the role of COX-2 in tumorigenesis in the animal model of lung cancer [20]. Importantly, studies analyzing expression of COX-2 in NSCLC in humans are in line with these experimental observations. Increased COX-2 expression was observed in precursor lesions of NSCLC [7] as well as in invasive cancer specimens [8, 22, 23]. Nevertheless,

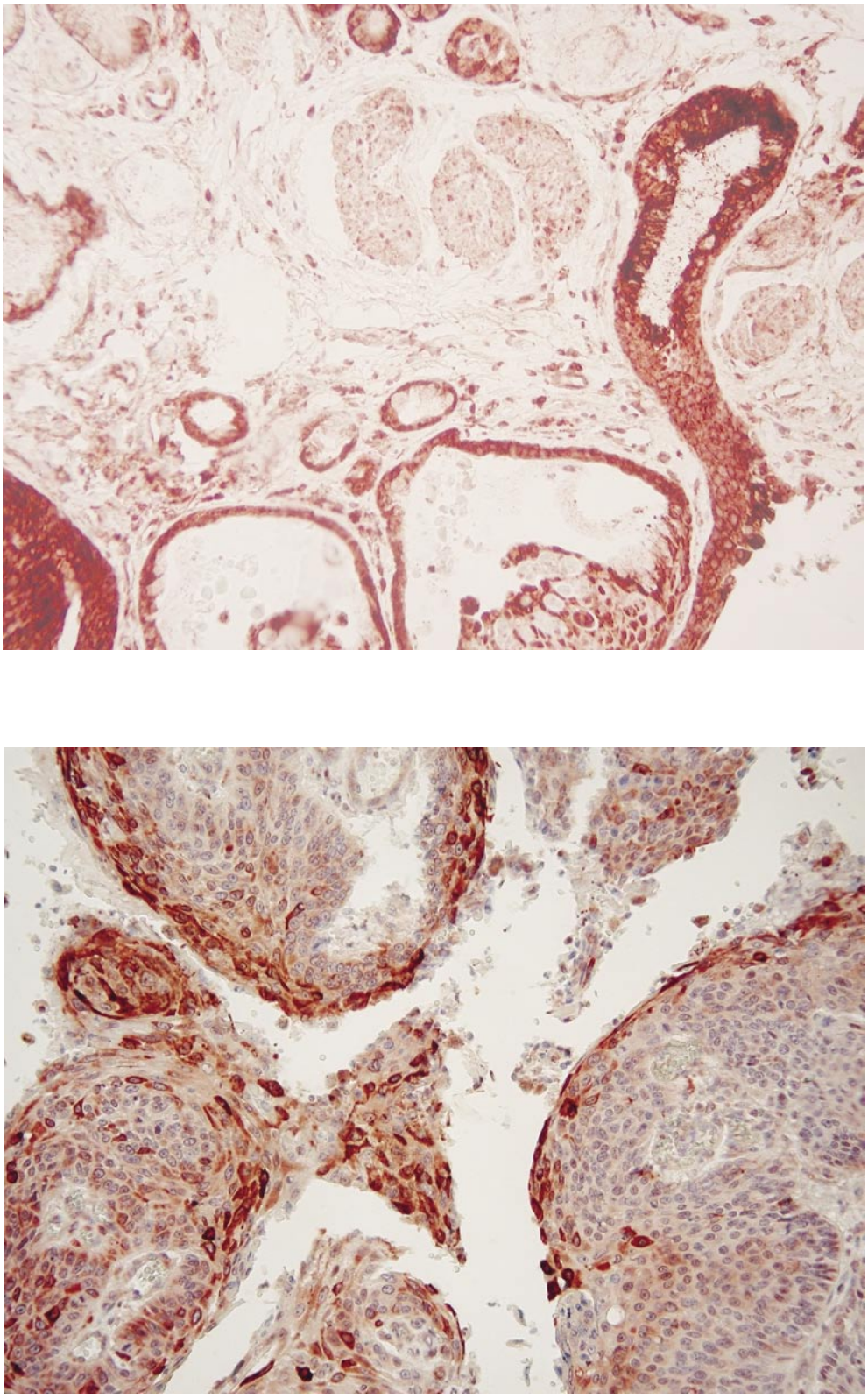

Figure 1. COX-2 immunostaining in (A) bronchial squamous dysplasia, (B) bronchial squamous cell carcinoma (x 200 magnification).

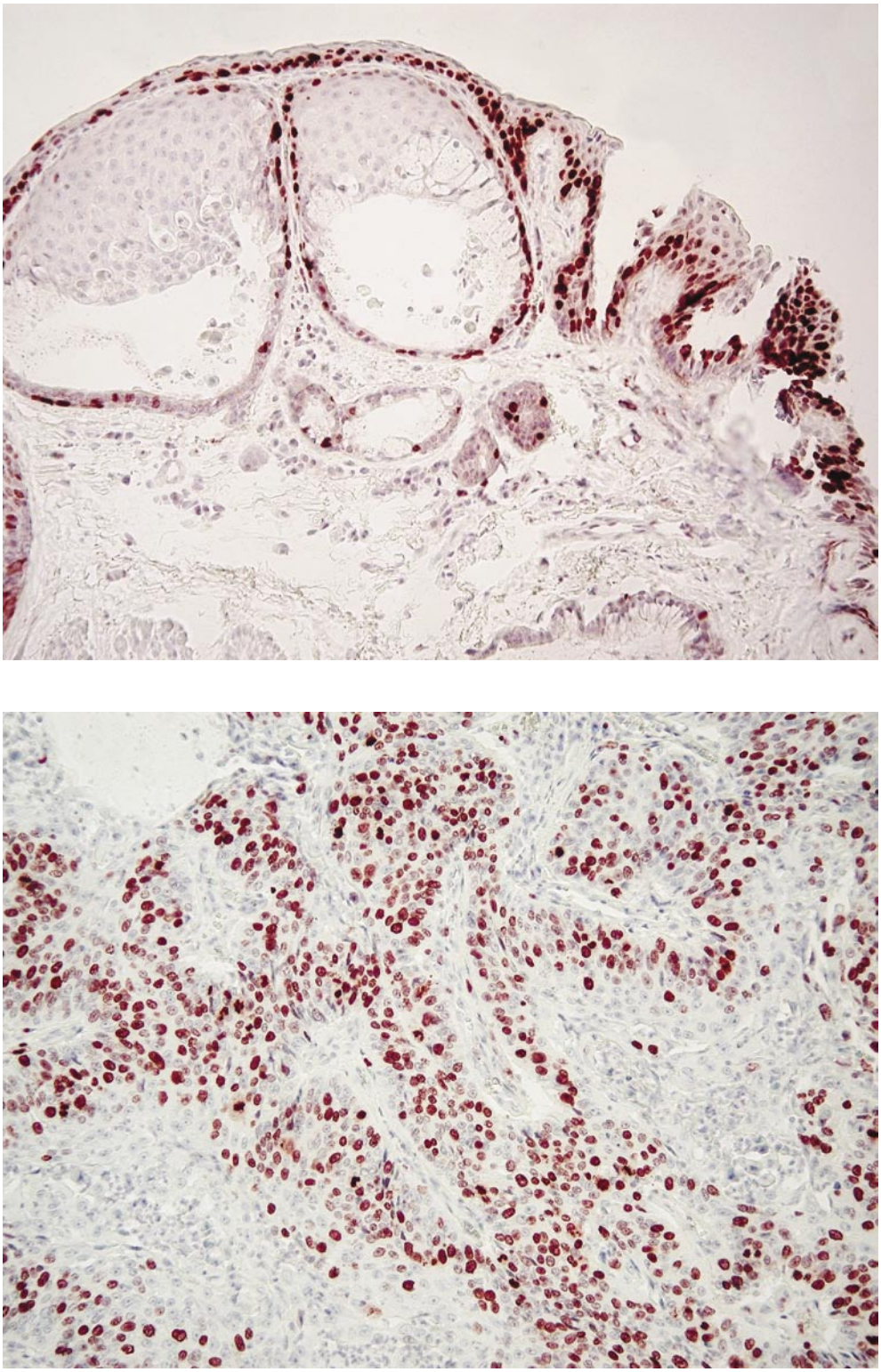

Figure 2. Ki67 immunostaining stained in (A) bronchial squamous dysplasia, (B) bronchial squamous cell carcinoma (x 200 magnification).

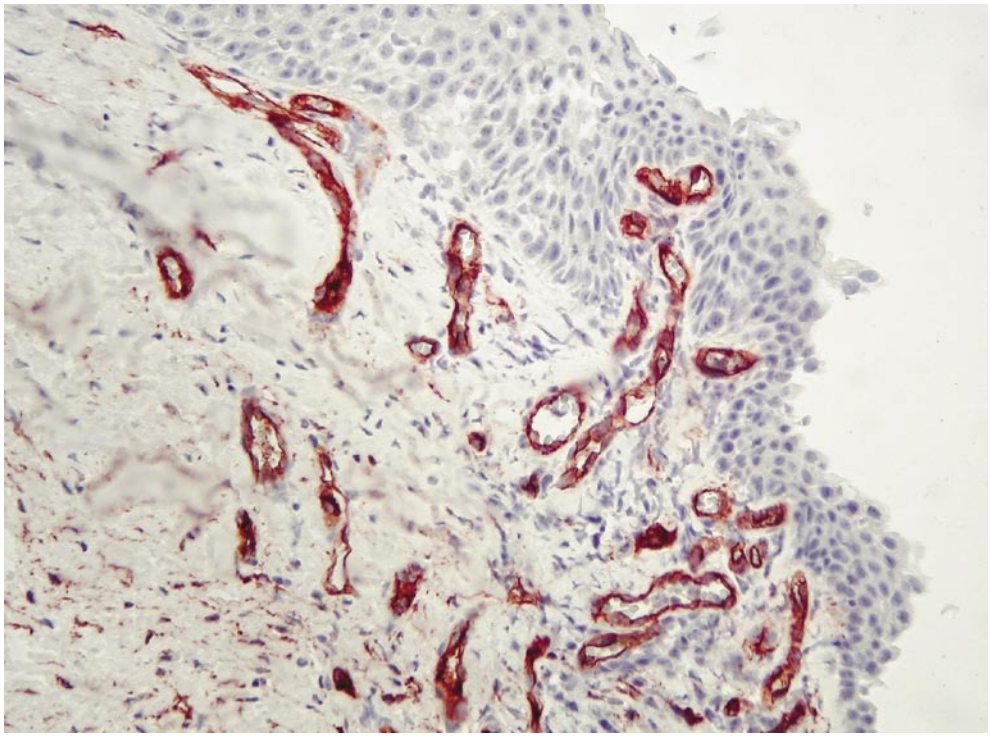

Figure 3. Anti CD34 staining in bronchial squamous dysplasia (x 40 magnification).

data on the comparison between COX-2 expression in premalignant and invasive lesions are scarse. Recently, Mascaux et al. [8] observed positive COX-2 lesions in eight out of 14 severe dysplasia patients, eight out of 14 in situ carcinomas and five out of eight invasive carcinomas. Data of the present study extend these observations further: although COX-2 plays a key role in early stages of carcinogenesis by promoting the proliferation of tumoral cells and their resistance to apoptosis and angiogenesis [24], the proportion of patients with COX-2 positive lesions is higher among NSCLC specimen compared to preneoplastic bronchial lesions.

Among the methods used to evaluate the proliferative activity of neoplastic tissue, reactivity with the monoclonal antibodies Ki67 and PCNA are widely accepted. Ki67 protein is present in nuclei of cells in all phases of the cycle except G_0. Ki67 expression increases as a cell progresses through the cell cycle, with highest expression seen in G_2/M phase [25]. PCNA is a nuclear protein associated with DNA polymerase delta. In contrast to Ki67, because of its relatively long half-life, PCNA may also persist in non-cycling cells. Although correlations between Ki67 and/or PCNA score and histological grade, tumour stage, and prognosis have been reported in a wide variety of malignancies such as pancreatic cancer [26], bladder [27] and colon carcinoma [28], several studies failed to confirm these associations [29, 30]. Interestingly, controversies regarding the relationships between histological grade, prognostic role and Ki67 and PCNA expression in NSCLC parallel those observed in other cancers. On one hand, some data suggest that high expression of Ki67 is a poor prognostic factor in invasive NSCLC [9, 10]. On the other hand, however, Ki67 immunoreactivity and ploidy did not significantly differ according to degree of differentiation, nodal status and stage grouping in other studies [11, 12]. In the present study, Ki67 did whereas PCNA immunostaining did not differ between preneoplastic lesions and invasive NSCLC specimen. This novel finding extends the previous ones that had indicated that immunostainings of PCNA and Ki67 appear to be equally valuable for assessing the proliferative activity in invasive NSCLC [31]. Our results suggest that while Ki67 and PCNA may have similar activities in invasive NSCLC, Ki67 appears to correlate better with the progression of the malignant processes from the preneoplastic to the invasive stage. Further studies are needed to explore this concept in more details.

Intratumoral MVD has been identified as an independent prognostic factor in predicting both disease-free and overall survival in various malignancies including NSCLC [19, 32, 33]. Nevertheless, most of these studies examined the relationships between the clinical outcomes and MVD in patients with invasive carcinomas. The natural course of MVD, and the differences in MVD between preneoplastic lesions and invasive tumors remained unanswered. One has to bear in

mind that the structural and functional abnormalities in blood vessels during tumorigenesis are unusually dynamic, and naturally undergo sprouting, proliferation, remodeling but also regression [34]. Immunostainig with anti-CD34 antibodies has been repeatedly used to assess the MVD in different tumours including NSCLC [13, 14]. Anti-CD34 antibodies predominantly stain the luminal endothelial membrane in normal resting tissue, and endothelial abluminal microprocesses in tumoral tissue [35]. In contrast, pericytes are visualised using antibodies to NG2, an accessible pericyte surface proteoglycan [15]. A question arises whether changes in tumour vasculature such as proliferation or regression of arteries are necessarily accompanied by changes in CD34 and NG2 immunostaining paralleling each other. In the present study, MVD assessed from CD34 immunostaining scores was significantly higher in preneoplastic lesions compared to invasive NSCLC, whereas NG2 immunostaining did not differ between the two groups. These findings are in line with others that have suggested that changes in proliferative activity of endothelial cells are not necessarily accompanied by simultanous changes in pericytes, and that differences in immunostaining for CD34 and NG2 do not necessarily follow each other [34].

Differentiating the potentially more malignant preneoplastic lesions among the many preneoplastic lesions present in the bronchial mucosa remains a challenge. Recently, Breuer et al. [36] observed 54% regression rate of all preneoplastic lesions, and 26% to 39% progression rate of lower-grade or severe dysplasia to carcinoma in situ or invasive NSCLC. The authors concluded that the current classification of any preneoplastic lesion cannot be reliably used to accurate risk assessment in lung carcinogenesis [36]. From this perspective, the assessment of MVD within preneoplastic lesions might represent an important additional measure for the assessment of the malignant potential of any given lesion. In the present study, we have observed higher MVD within the squamous dysplasia lesions compared to bronchial squamous carcinoma specimen. This interesting finding paralells recent observations of Regina et al. [13] who demonstrated lower MVD in the inner part of lung tumors than in the adjacent non-affected lung tissue. Detailed studies on the assessment of MVD in bronchial preneoplastic lesions and on the relationships between the MVD and natural course of the lesions are highly warranted.

In conclusion, findings of the present study indicate that increases in COX-2 and Ki67 expression may be associated with the development of bronchogenic carcinomas and possibly with acquisition of an invasive phenotype. In contrast, increased CD34 expression in preneoplastic lesions suggests that increased MVD appears to represent an early marker of lung carcinogenesis. Further studies on the molecular mechanisms underlying the pathogenetic processes of lung cancer are highly warranted as they may potentially open new horizonts for the search of innovative NSCLC therapies.

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