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Severe dysplasia can be distinguished from moderate and mild dysplasia of bronchial mucosa by changes in Ki-67 index

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Preneoplastic lesions on small bronchial biopsy specimens may cause a diagnostic dilemma. The aim of this study was to estimate karyometric variables and the Ki-67 index of preneoplastic bronchial lesions and squamous cell carcinoma of the lung. The study was performed on endoscopic samples of squamous cell carcinoma (n = 22), normal appearing mucosa surrounding carcinoma (n = 10), bronchial dysplasia of mild (n = 7), moderate (n = 6), and severe grade (n = 6), carcinoma *in situ* (n = 17), and normal mucosa from patients with chronic bronchitis (n = 26). Karyometric analysis was done using the image analyzer ImageJ 1.47q. Ki-67 activity was also quantified by ImageJ 1.47q with the plugin Cell Counter. The highest values of nuclear area were found in squamous cell carcinoma, and differences were statistically significant compared to normal mucosa, all grades of dysplasia and normal appearing mucosa surrounding carcinoma (p < 0.01). The Ki-67 index was significantly higher in squamous cell lung carcinoma compared to normal mucosa, mild and moderate dysplasia and normal appearing mucosa surrounding carcinoma (p < 0.01). The Ki-67 index was significantly higher in severe dysplasia than in mild and moderate dysplasia (p < 0.01). In conclusion, the Ki-67 index is a useful parameter for more objective grading and can be of prognostic value to determine the biological potential of preneoplastic bronchial lesions.

Key words: preneoplastic lesions, lung carcinoma, dysplasia, squamous cell carcinoma, Ki-67 index.

Introduction

Lung cancer is the most common fatal malignancy worldwide, and the number of cases continues to increase [1]. Lung cancer is the leading cancer site in males, comprising 17% of the total new cancer cases and 23% of the total cancer deaths [2]. Smoking is the primary cause in the great majority of these cases. The prognosis of lung cancer is still poor, with 5-year survival rates of approximately 10% in most countries [3].

Lung cancers, as with other epithelial malignancies, are preceded by a series of preneoplastic lesions. The World Health Organization (WHO) published a tumor classification system defining two different pre-neoplastic lesions of the bronchial epithelium which may be precursors to squamous cell carcinoma: squamous dysplasia and carcinoma *in situ* (CIS) [3]. Knowledge of these lesions will be crucial in the design and understanding of lung cancer screening, and molecular characteristics of these lesions will provide useful targets for detection and possibly even treatment [4].

The pathological and bronchoscopic diagnosis of preinvasive lesions remains difficult. These lesions may cause a diagnostic dilemma particularly on small biopsy specimens. Morphology is the gold standard in diagnosing premalignant squamous lesions, and no ancillary studies (e.g., immunohistochemistry) can be used as a diagnostic aid [5]. However, interobserv-

er and intraobserver variation in the histopathologic reporting of bronchial biopsy specimens exists even among experienced pathologists when using conventional histopathologic criteria [6-8]. The reproducibility for classifying preinvasive lesions showed intraobserver agreement of 0.71 and interobserver agreement was only 0.55 [6-8]. On the other hand, image analysis permits pathologists to obtain quantitative measurements on histologic preparations, so that visual impressions can be augmented by quantitative morphometry [9]. Karyometry presents some particular challenges to the development, evaluation, and application of classification procedures. An analysis of nuclear populations usually involves thousands of nuclei [10]. The addition of nuclear morphometry or molecular analysis to histopathologic grading allows more accurate classification of preinvasive lesions and better identification of lesions that are biologically more aggressive [11].

Deregulated cell proliferation is a hallmark of cancer, and Ki-67 immunostaining can be used to identify proliferating cells. Evaluation of cell proliferation may have utility as a biomarker of epithelial malignant transformation risk [12]. The proliferation index, as determined by a positive reaction to Ki-67, is an important factor differentiating the degrees of lesion development [13].

The aim of this study was to estimate karyometric variables and the Ki-67 index of preneoplastic bronchial lesions: squamous dysplasia with mild, moderate and severe grade, CIS and squamous cell carcinoma of the lung.

Material and methods

Paraffin-embedded bronchoscopic biopsy samples were retrieved from pulmonary pathology archives at the Institute of Pathology, Medical Faculty, University of Niš, Serbia. The study was performed on endoscopic samples of squamous cell carcinoma (n = 22), normal appearing mucosa surrounding carcinoma (NAMSC) (n = 10), bronchial dysplasia with mild (n = 7), moderate (n = 6) and severe grade (n = 6), CIS (n = 17), and normal mucosa from patients with chronic bronchitis (n = 26). All biopsies were reviewed by two pathologists. Normal mucosa was represented by pseudostratified ciliated columnar epithelium. Dysplasia (mild, moderate and severe grade), CIS and squamous cell lung carcinoma were classified according to WHO criteria [3]. After formalin fixation and paraffin embedding, serial histologic sections of 4-5 μm thickness were routinely stained with hematoxylin end eosin.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor sections (4-5 μm) were made for immunohistochemical

analysis. Slides set aside for immunohistochemical evaluation after deparaffinization and endogenous peroxidase blocking (3% solution of H_2O_2 for 15 min) were submitted to microwave treatment (20 min at 620 W in 0.01 M citrate buffer, pH 6.0). MIB-1 monoclonal antibody for Ki-67, dilution 1 : 100 (DAKO, Glostrup, Denmark), was applied for 60 min at room temperature. Immunohistochemical staining was performed by the streptavidin-biotin method using an LSAB kit (DAKO, Glostrup, Denmark) according to the manufacturer's instructions (LSAB Kit, DAKO, Glostrup, Denmark). The chromogen was 3,3'-diaminobenzidine (DAB). Tissue sections were lightly counterstained with Mayer's hematoxylin (Merck, Germany). During the tissue staining, positive and negative control samples were simultaneously stained. All nuclei with brown nuclear staining were rated as positive for Ki-67.

Image analysis

Karyometric analysis was done using the image analyzer ImageJ 1.47q (Wayne Rasband, NIH, USA), on digital images (1024 × 760 pixels) obtained at objective 40× (NA = 0.75) with a BX50 microscope (Olympus, Tokyo, Japan). The images were manually edited. In each case 100 epithelial nuclei were measured. For each nucleus, the following morphometric parameters were analyzed: nuclear area, optical density (OD), perimeter, circularity, Feret's diameter and integrated optical density (IOD). Nuclear area was defined as the number of pixels. OD was the amount of light that passed through the object: OD (x,y)= -log (intensity (x,y) – black)/(incident light-black). Perimeter was the length of the outside boundary of the selection. Circularity was the derived shape measure, calculated from the area and perimeter (circularity = = 4p × area/perimeter ^{2}). Feret's diameter was the average distance between any two points on the contour of the nucleus. Integrated optical density was the sum of individual OD of each pixel in the area being measured. This was equivalent to the product of area and mean OD value.

Cell count morphometric analysis

Ki-67 activity was quantified by ImageJ 1.47q, with the plugin Cell Counter (Fig. 1), and assessing the labeling index from the ratio of the number of cells stained by Ki-67 to the total number of cells counted per section. A minimum of 200 cells in 10 different randomly selected areas using objective 40× (NA = 0.75) of the BX50 microscope were counted.

Statistical analysis

The results were statistically analyzed using descriptive and analytical statistical methods. Differences between groups were tested by MANOVA and
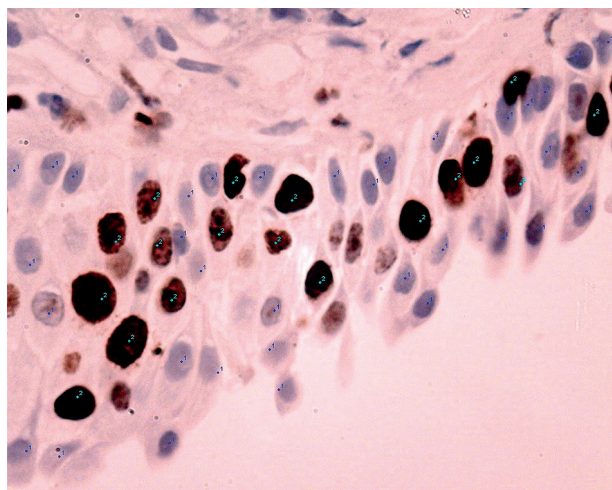
Fig. 1. Image analysis of Ki-67 activity in NAMSC

Fig. 2. Nuclear area in normal mucosa, bronchial preneoplastic lesions and squamous cell carcinoma (μm^2, mean ± SD)

Mann-Whitney test. P value less than 0.05 was considered to indicate statistical significance. Statistical analysis was performed using SPSS statistical software (version 12.0).

Results

The values of the nuclear variables which were assessed are listed in Table I and Figs. 2-6. The results are expressed as means ± standard deviation.

The highest values of nuclear size (nuclear area, Feret's diameter and perimeter) and of IOD were found in squamous cell carcinoma, and differences were statistically significant compared to normal mucosa, all grades of dysplasia and normal appearing mucosa surrounding carcinoma (NAMSC) (p < 0.01), except for Feret's diameter, perimeter and IOD in severe dysplasia (p > 0.05). No significant differences in nuclear area were found between var-
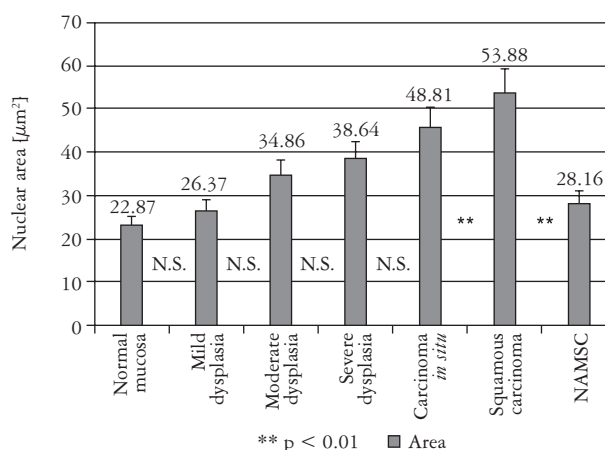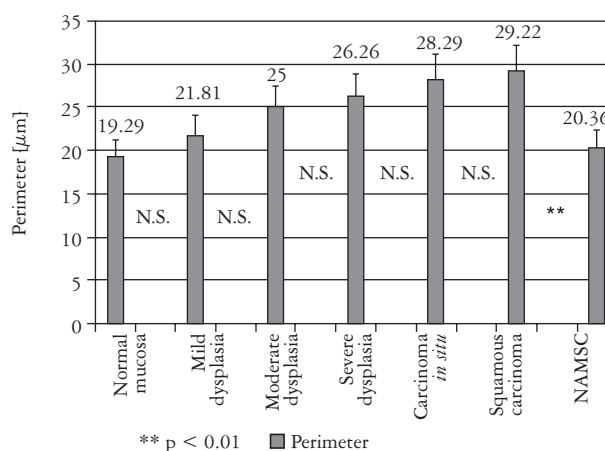

Fig. 3. Perimeter in normal mucosa, bronchial preneoplastic lesions and squamous cell carcinoma (μm, mean ± SD)

ious grades of dysplasia and between squamous cell carcinoma and CIS. Differences in nuclear size and IOD between normal appearing mucosa surround-

Table I. Karyometric variables in normal mucosa, bronchial preneoplastic lesions and squamous cell carcinoma (mean ± SD)

	AREA	**OD**	**PERIMETER**	**CIRCULARITY**	**FERET**	**IOD**	**KI-67 INDEX**
Normal mucosa	22.87 ±3.44	0.57 ±0.17	19.29 ±2.53	0.74 ±0.22	7.14 ±0.81	13.64 ±3	4.98 ±2.37
Mild dysplasia	26.37 ±1.23	0.65 ±0.13	21.81 ±1.11	0.7 ±0.05	7.42 ±0.38	16.96 ±3.24	22.69 ±8.74
Moderate dysplasia	34.86 ±5.14	0.47 ±0.08	25.0 ±1.87	0.7 ±0.05	8.71 ±0.6	16.35 ±4.3	33.22 ±9.84
Severe dysplasia	38.64 ±3.76	0.35 ±0.29	26.26 ±1.1	0.47 ±0.35	8.91 ±0.5	19.21 ±7.48	53.28 ±8.02
Carcinoma *in situ*	45.81 ±13.26	0.42 ±0.16	28.29 ±2.02	0.65 ±0.2	9.81 ±0.68	20.25 ±4.47	54.97 ±14.84
Squamous carcinoma	53.88 ±12.18	0.46 ±0.15	29.22 ±2.93	0.73 ±0.18	10.34 ±1.08	24.89 ±6.19	66.12 ±10.08
NAMSC	28.16 ±5.26	0.51 ±0.14	20.36 ±1.85	0.73 ±0.23	7.83 ±0.83	15.43 ±3.66	21.91 ±7.2

IOD – integrated optical density; NAMSC – normal appearing mucosa surrounding carcinoma

ing carcinoma (NAMSC) and other groups were not statistically significant, except for CIS and squamous cell carcinoma (p < 0.01) (Table I, Figs. 2-5).

The smallest value of the Ki-67 index was found in normal mucosa. Compared to other groups, differences were statistically significant. The Ki-67 index was significantly higher in squamous cell lung carcinoma compared to normal mucosa, mild and moderate dysplasia and normal appearing mucosa surrounding carcinoma (NAMSC) (p < 0.01). The Ki-67 index was significantly higher in severe dysplasia than in mild and moderate dysplasia (p < 0.01). No significant differences were found between mild versus moderate dysplasia, and carcinoma *in situ* versus squamous cell carcinoma. The Ki-67 index in normal appearing mucosa surrounding carcinoma (NAMSC) was significantly higher than in normal mucosa (p < 0.05), and lower than severe dysplasia, CIS and squamous cell carcinoma (p < 0.01) (Table I, Fig. 6).

Differences in other measured variables were not statistically significant (Table I).

Discussion

Lung carcinogenesis is a multistep process characterized by accumulation of successive molecular genetic and epigenetic abnormalities, resulting in epithelial cell malignant transformation. It is generally assumed that squamous cell cancer develops in a gradual and stepwise fashion according to the WHO grading of preneoplastic lesions from normal epithelium, hyperplasia, squamous metaplasia, dysplasia towards carcinoma *in situ* and microinvasive squamous cell carcinoma [14, 15].

Hyperplasia and metaplasia are thought to be reactive lesions, while dysplasia and CIS are considered as true preneoplastic lesions. Mild dysplasia exhibits only minimal architectural and cytological disturbance with disarray in the lower third of the epithelium and mild cytological atypia. Mitoses are absent or rare. Moderate dysplastic lesions are characterized by more cytological irregularity, disarray in the lower two thirds of epithelium and more significant cytological atypia. Mitotic figures are confined to the lower third. In severe dysplasia, the disarray extends into the upper third of the epithelium but does not reach the surface, and it is accompanied by cellular polymorphism. Mitoses are confined to the lower two thirds. CIS is associated with extension of the disarray to the epithelial surface with malignant cytological features and mitotic figures present through the full thickness. Atypical or malignant cytological features are characterized by variations in nuclear size, shape, hyperchromatism, multiplicity of nucleoli and irregularities of nuclear membrane [3].

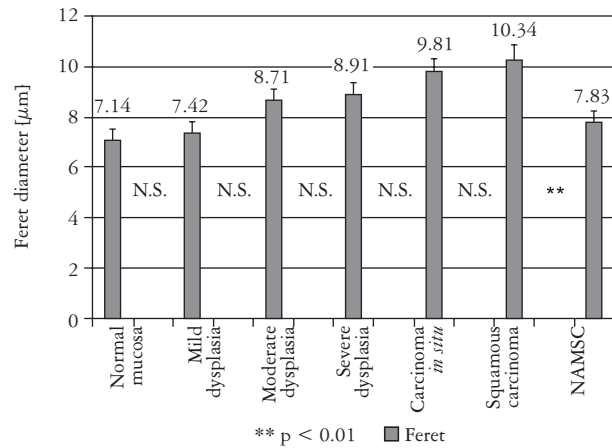In our study we performed image analysis of normal respiratory mucosa, preneoplastic lesions (squa-

Fig. 4. Feret diameter in normal mucosa, bronchial preneoplastic lesions and squamous cell carcinoma (μm, mean $\pm$ SD)
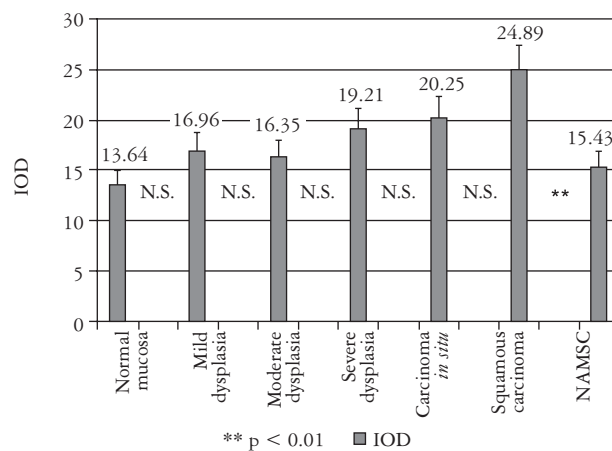

Fig. 5. Integrated optical density in normal mucosa, bronchial preneoplastic lesions and squamous cell carcinoma (mean $\pm$ SD).
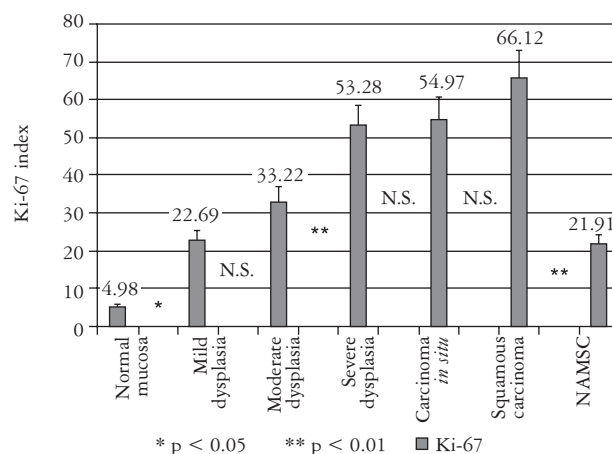

Fig. 6. Ki-67 index in normal mucosa, bronchial preneoplastic lesions and squamous cell carcinoma (mean $\pm$ SD)

mous dysplasia and CIS) and squamous cell carcinoma of the lung. The neoplastic process is a continuum of phenotypic changes driven by multiple genetic changes, and preneoplastic lesions may be morphological phenotypes of the different steps in this pro-

gression from normal to malignant tissue [16]. Based on our morphometric measurements, we found an increase of nuclear size and IOD from normal mucosa to preneoplastic lesions and squamous cell carcinoma. Accordingly, nuclear area was significantly larger in squamous cell carcinoma compared to mild, moderate and severe dysplasia, normal mucosa and normal appearing mucosa surrounding carcinoma. There were no significant differences in nuclear size between various grades of dysplasia (mild, moderate and severe), as well as between invasive carcinoma and CIS. The uncertainty in classification of the intermediate grades of dysplasia by visual examination, overlap between categories and our karyometric results support some investigators and clinicians who use only two divisions – low-grade versus high-grade dysplasia [16-21]. On the other hand, according to our results, the Ki-67 index was significantly higher in severe dysplasia than in mild and moderate dysplasias ($p < 0.01$).

Karyometric data of histologically normal appearing cells in areas that are peripheral to a malignant lesion (NAMSC) observed in our study were consistent with malignancy-associated changes (MAC) [22-24]. MAC are defined as subtle morphological alterations of mucosa surrounding carcinoma and invasive carcinoma or CIS. From the karyometric point of view, these subtle differences between normal appearing mucosa surrounding carcinoma and dysplastic lesions were not statistically significant.

Integrated optical density reflects the quantity of DNA and other nuclear constituents. Studies of the DNA content of cell nuclei, as a measure of chromosomal gain or loss, are used to assess the degree of nuclear aberration in a malignant cell population. Studies in squamous cell metaplasia and dysplasia have shown progressive, increased aneuploidy with increasing atypia [16]. In our study, the greatest value of IOD was found in the nuclei of squamous cell lung carcinoma, probably reflecting marked aneuploidy.

Uncontrolled cellular proliferation is a hallmark of cancer, and Ki-67 immunostaining can be used to identify proliferating cells. The Ki-67 antigen is localized to the nucleus, and is expressed in all phases of the cell cycle except for G0 [25]. In the available literature, no data from morphometric evaluation of nuclei in preneoplastic bronchial lesions in endoscopic samples in relation to the expression of Ki-67 were found. To our knowledge, our data are the first to examine concurrently the Ki-67 proliferative index and karyometric variables within two well-defined sets of specimens: bronchial preneoplastic lesions and squamous cell carcinoma. The smallest value of the Ki-67 index was found in normal mucosa. The Ki-67 index was significantly higher in squamous cell lung carcinoma compared to normal mucosa, mild

and moderate dysplasia and normal appearing mucosa surrounding carcinoma. The Ki-67 index was significantly higher in severe dysplasia than in mild and moderate dysplasia. Similarly, Meert et al. [26] reported that the expression of Ki-67 depends on the development level of the preneoplastic lesion and grows significantly from low dysplasia to CIS. It clearly shows that proliferation activity during the development of squamous carcinoma is directly related to the increase of cell atypia [13]. In the present study, differences between mild and moderate or between severe dysplasia and CIS were not statistically significant. Our results are consistent with the findings of Meert et al. [26], who concluded that severe dysplasia behaved more like CIS than mild or moderate dysplasia. Also, Cavarga et al. [27] and Hoshino et al. [28] indicated that increases in Ki-67 expression in preneoplastic lesions might be associated with the development of bronchogenic carcinomas and possibly with acquisition of an invasive phenotype. Ki-67 appears to correlate with the progression of the malignant processes from the preneoplastic to the invasive stage [29], and may be useful in predicting prognosis in patients with non small-cell lung cancer [30]. However, further studies are needed to explore this concept.

Conclusions

The Ki-67 index significantly differentiated severe dysplasia from mild and moderate dysplasias, and NAMSC from normal mucosa. Moreover, the Ki-67 index can be of prognostic value to determine the biological potential of preneoplastic lesions which should be carefully followed up. Even though our karyometric results represent only a small sample, they suggest that nuclear morphometry is a useful method for objective distinction between dysplasia and squamous cell carcinoma of the lung in routine bronchoscopic biopsies, particularly in difficult cases.

The authors declare no conflict of interest.

References

1. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55: 74-108.
2. Jemal A, Bray F, Center M, et al. Global Cancer Statistics. CA Cancer J Clin 2011; 61: 69-90.
3. Pathology and genetics of tumours of the lung, pleura, thymus and heart. Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC (ed.). IARC Press, Lyon 2004.
4. Kerr KM. Pulmonary preinvasive neoplasia. J Clin Pathol 2001; 54: 257-271.
5. Dacic S. Pulmonary Preneoplasia. Arch Pathol Lab Med 2008; 132: 1073-1078.
6. Sutedja G. New techniques for early detection of lung cancer. Eur Respir J 2003; 21: Suppl. 39: 57s-66s.

7. Venmans BJ, van der Linden HC, Elbers HR, et al. Observer variability in histopathologic reporting of bronchial biopsy specimens. Influence on the results of autofluorescence bronchoscopy in detection of preinvasive bronchial neoplasia. J Bronchol 2000; 7: 210-214.

8. Nicholson AG, Perry LJ, Cury PM, et al. Reproducibility of the WHO/IASLC grading system for pre-invasive squamous lesions of the bronchus: a study of inter-observer and intra-observer variation. Histopathology 2001; 38: 202-208.

9. Berman JJ, Moore GW. Image analysis software for the detection of preneoplastic and early neoplastic lesions. Cancer Lett 1994; 77: 103-109.

10. Bartels PH, Bartels HG. Classification in karyometry. Performance testing and prediction error. Anal Quant Cytopathol Histopathol 2013; 35: 181-188.

11. Ishizumi T, McWilliams A, MacAulay C, et al. Natural history of bronchial preinvasive lesions.Cancer Metastasis Rev 2010; 29: 5-14.

12. Khojasteh M, Buys TP, LeRiche J, et al. A framework for quantitative assessment of Ki 67 distribution in preneoplastic bronchial epithelial lesions. Anal Quant Cytol Histol 2012; 34: 120-138.

13. Pankiewicz W, Minarowski L, Niklinska W, et al. Immunohistochemical markers of cancerogenesis in the lung. Folia Histochem Cytobiol 2007; 45: 65-74.

14. Brambilla E, Travis WD, Colby TV, et al. The new WHO classification of lung tumours. Eur Respir J 2001; 18: 1059-1068.

15. Saccomanno G, Archer VE, Auerbach O, et al. Development of carcinoma of the lung as reflected in exfoliated cells. Cancer 1974; 33: 256-270.

16. Greenberg AK, Herman Yee H, Rom WN. Preoplastic lesions of the lung. Respir Res 2002; 3: 20.

17. Moro-Sibilot D, Fievet F, Jeanmart M, et al. Clinical prognostic indicators of high-grade pre-invasive bronchial lesions. Eur Respir J 2004; 24: 24-29.

18. Jeanmart M, Lantuejoul S, Fievet F, et al. Value of immunohistochemical markers in preinvasive bronchial lesions in risk assessment of lung cancer. Clin Cancer Res 2003; 9: 2195-2203.

19. Breuer RH, Pasic A, Smit EF, et al. The natural course of preneoplastic lesions in bronchial epithelium. Clin Cancer Res 2005; 11 (2 Pt 1): 537-543.

20. Lantuejoul S, Soria JC, Morat L, et al. Telomere shortening and telomerase reverse transcriptase expression in preinvasive bronchial lesions. Clin Cancer Res 2005; 11: 2074-2082.

21. Wang GF, Lai MD, Yang RR, et al. Histological types and significance of bronchial epithelial dysplasia. Mod Pathol 2006; 19: 429-437.

22. Palcic B. Nuclear texture: Can it be used as a surrogate endpoint biomarker? J Cell Biochem 1994; 19: 40-46.

23. MacAulay C, Lam S, Payne PW, et al. Malignancy-associated changes in bronchial epithelial cells in biopsy specimens. Anal Quant Cytol Histol 1995; 17: 55-61.

24. Ikeda N, MacAulay C, Lam S, et al. Malignancy associated changes in bronchial epithelial cells and clinical applications as a biomarker. Lung Cancer 1998; 19: 161-166.

25. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000; 182: 311-322.

26. Meert AP, Feoli F, Martin B, et al. Ki 67 expression in bronchial preneoplastic lesions and carcinoma in situ defined according to the new 1999 WHO/IASLC criteria: a preliminary study. Histopathology 2004; 44: 47-53.

27. Cavarga I, Kocan P, Boor A, et al. Immunohistochemical markers of proliferation and vascularisation in preneoplastic bronchial lesions and invasive non-small cell lung cancer. Neoplasma 2009; 56: 414-421.

28. Hoshino H, Shibuya K, Chiyo M, et al. Biological features of bronchial squamous dysplasia followed up by autofluorescence bronchoscopy. Lung Cancer 2004; 46: 187-196.

29. Sousa V, Santo JE, Silva M, et al. EGFR/erB-1, HER2/erB-2, CK7, LP34, Ki67 and P53 expression in preneoplastic lesions of bronchial epithelium: an immunohistochemical and genetic study. Virchows Arch 2011; 458: 571-581.

30. Ciancio N, Galasso MG, Campisi R, et al. Prognostic value of p53 and Ki67 expression in fiberoptic bronchial biopsies of patients with non small cell lung cancer. Multidiscip Respir Med 2012; 7: 29.

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