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 preneoplastic 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 biopsies 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 H₂O₂ 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: \[ OD(x,y) = \log\frac{\text{intensity}\,(x,y) - \text{black}}{\text{incident light-black}} \]. Perimeter was the length of the outside boundary of the selection. Circularity was the derived shape measure, calculated from the area and perimeter (\[ \text{circularity} = \frac{4\pi \times \text{area}}{\text{perimeter}^2} \]). 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
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 various grades of dysplasia and between squamous cell carcinoma and CIS. Differences in nuclear size and IOD between normal appearing mucosa surrounding squamous cell carcinoma and normal appearing mucosa surrounding dysplasia were statistically significant (p < 0.01).

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

<table>
<thead>
<tr>
<th></th>
<th>AREA (µm²)</th>
<th>OD</th>
<th>PERIMETER (µm)</th>
<th>CIRCULARITY</th>
<th>FERET (µm)</th>
<th>IOD (units)</th>
<th>Ki-67 index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>22.87 ± 3.44</td>
<td>0.57</td>
<td>19.29 ± 2.53</td>
<td>0.74</td>
<td>7.14</td>
<td>13.64</td>
<td>4.98</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>26.37 ± 1.23</td>
<td>0.65</td>
<td>21.81 ± 1.11</td>
<td>0.7</td>
<td>7.42</td>
<td>16.96</td>
<td>22.69</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>34.86 ± 5.14</td>
<td>0.47</td>
<td>25.00 ± 1.87</td>
<td>0.7</td>
<td>8.71</td>
<td>16.35</td>
<td>33.22</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>38.64 ± 3.76</td>
<td>0.35</td>
<td>26.26 ± 1.1</td>
<td>0.47</td>
<td>8.91</td>
<td>19.21</td>
<td>53.28</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>45.81 ± 13.26</td>
<td>0.42</td>
<td>28.29 ± 2.02</td>
<td>0.65</td>
<td>9.81</td>
<td>20.25</td>
<td>54.97</td>
</tr>
<tr>
<td>Squamous carcinoma</td>
<td>53.88 ± 12.18</td>
<td>0.46</td>
<td>29.22 ± 2.93</td>
<td>0.73</td>
<td>10.34</td>
<td>24.89</td>
<td>66.12</td>
</tr>
<tr>
<td>NAMSC</td>
<td>28.16 ± 5.26</td>
<td>0.51</td>
<td>20.36 ± 1.85</td>
<td>0.73</td>
<td>7.85</td>
<td>15.43</td>
<td>21.91</td>
</tr>
</tbody>
</table>

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-
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

Kì67 index and the dysplasia of bronchial epithelium


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