UDK: 616.22-006.6-074

KARYOMETRIC ANALYSIS OF LARYNGEAL PRECANCEROUS CHANGES

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Laryngeal precancerous lesions are squamous lesions with an increased risk/likelihood of progression to squamous cell carcinoma. Numerous classification schemes have evolved from this fundamental premise, but to date none have found universal acceptance.

Karyometric analysis of various reactive and precancerous changes of laryngeal epithelium.

Endoscopic laryngeal biopsies from patient with laryngeal polyps with normal mucosa (n = 22) and low-grade dysplasia (n = 17), patient with high-grade dysplasia (n = 21), and patients with squamous cell carcinoma (n = 37) were analyzed. Karyometric analysis was done using the image analyzer ImageJ 1.47q. Ki67 index was also quantified by ImageJ 1.47q with the plugin Cell Counter.

Nuclear size, IOD, and Ki67 index were significantly bigger in cancer cells than in normal mucosa and low grade dysplasia. Differences between squamous cell carcinomas and high grade dysplasia were not statistically significant.

Our results support classification schemes with two grades (low and high) of laryngeal dysplasia.

Keywords: *larynx, precancerous changes, Ki67 index*

KARIOMETRIJSKA ANALIZA PREKANCEROZNIH PROMENA LARINKSA

Prekancerozne lezije larinksa su skvamozne lezije sa povećanim rizikom/verovatnoćom progresije u karcinom skvamoznih ćelija. Brojne klasifikacione šeme su evoluirale iz ove osnovne premise, ali do danas nijedna nije naišla na univerzalno prihvatanje.

Kariometrijska analiza različitih reaktivnih i prekanceroznih promena epitela larinksa.

Endoskopske biopsije larinksa kod pacijenata sa polipima larinksa sa normalnom sluznicom (n = 22) i displazijom niskog stepena (n = 17), pacijenata sa displazijom visokog stepena (n = 21) i pacijenata sa karcinomom skvamoznih ćelija (n = 37) analizirani su. Kariometrijska analiza je urađena pomoću analizatora slike ImageJ 1.47k. Ki67 indeks je takođe kvantifikovan pomoću ImageJ 1.47k sa dodatkom Cell Counter.

Veličina jedra, IOD i Ki67 indeks bili su značajno veći u ćelijama raka nego u normalnoj sluzokoži i displaziji niskog stepena. Razlike između karcinoma skvamoznih ćelija i displazije visokog stepena nisu bile statistički značajne.

Naši rezultati podržavaju šeme klasifikacije sa dva stepena (niska i visoka) displazije larinksa.

Ključne reči: *larinks, prekancerozne promene, indeks Ki67*

Introduction

Laryngeal precancerous lesions are squamous lesions with an increased risk/likelihood of progression to squamous cell carcinoma. A constellation of architectural and cytologic features comprise dysplasia or laryngeal intraepithelial neoplasia, but these features are not uniformly accepted or interpreted, thereby leading to difficulties in intra - and interobserver differences of interpretation.

In 1952, Kleinsasser devised a classification for precancerous lesions of the laryngeal squamous epithelium: simple squamous cell hyperplasia, hyperplasia with atypia, and finally carcinoma *in situ* (CIS) (Kleinsasser, 1963). Numerous classification schemes have evolved from this fundamental premise, but to date none have found universal acceptance. Dysplasia is an alteration of surface epithelium, which is more than hyperplasia but less than carcinoma. It is wise to use the term atypia in the context of reactive, inflammatory, or reactive changes, while reserving dysplasia for the pre-malignant group of lesions. Whichever classification system is adopted, consistent application of the criteria will allow clinicians to correctly manage their patients (Thompson, 2006).

Precursor lesions are mostly seen in adult population and affect men more than women, especially pronounced after the sixth decade. Symptoms depend on the location and severity of the disease and are usually present for at list a few months before clinical attention. Endoscopically, these lesions have a varied appearance; can be circumscribed or diffuse, smooth or irregular, flat or exophytic.

To identify the earliest forms of dysplasia and to arbitrarily separate and rigidly divide the dysplasias into different categories is fraught with tremendous intra - and interobserver variability and an overall lack of reproducibility.

The aim of this study is karyometric analysis of various reactive and precancerous changes of laryngeal epithelium.

Material and methods

Patients

At Institute of Pathology, University of Niš, Serbia, endoscopic laryngeal biopsies from patient with laryngeal polyps with normal mucosa (n = 25) and low-grade dysplasia (n =17), patient with high-grade dysplasia (n = 21), and patients with squamous cell carcinoma (n = 37) were retrieved from archive. All patients were men, mean age 57 ± 9.8 years. After formalin fixation and paraffin embedding, 4 μm thick sections were routinely stained with hematoxilin and eosin (HE). Cases with moderate dysplasia were not analyzed because of low inter-observer agreement. All biopsies were reviewed by two pathologists (DM, and ZM).

Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor sections (4-5 μm) were made for immunohistochemical analysis. Slides set aside for immunehistochemical 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 1.47 q (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 istance 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 (Mijovic and Mihailovic, 2015).

Ki67 index

Ki-67 activity was quantified by ImageJ 1.47 q, with the plugin Cell Counter, 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 (Mijovic and Mihailovic, 2015).

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

In normal mucosa and mucosa with low grade dysplasia Ki67 positivity was found only in parabasal and basal cells (Figure 1). In mucosa with high grade dysplasia Ki67 positive cells were found in basal, parabasal and spinous layers (Figure 2). In squamous cell carcinoma tissue Ki67 positivity was irregular, and in all epithelial layers (Figure 3).

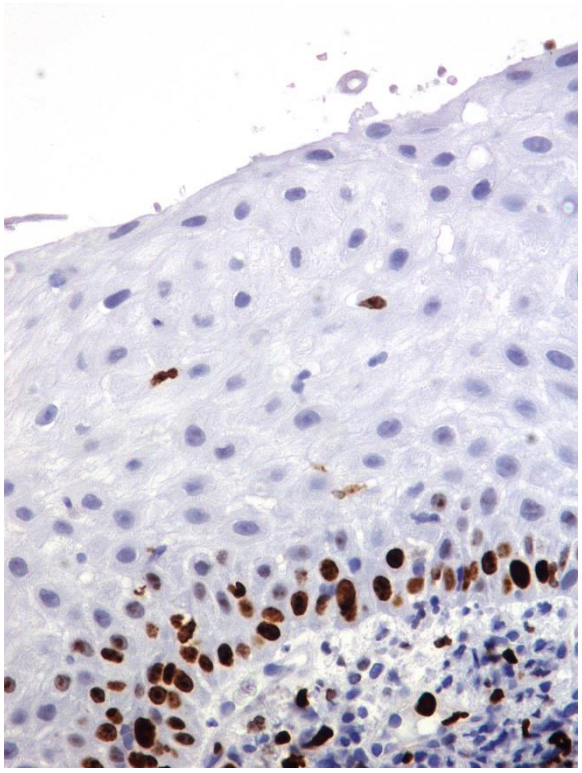

Figure 1. Low grade dysplasia of laryngeal mucosa. Ki67, obj.x40.

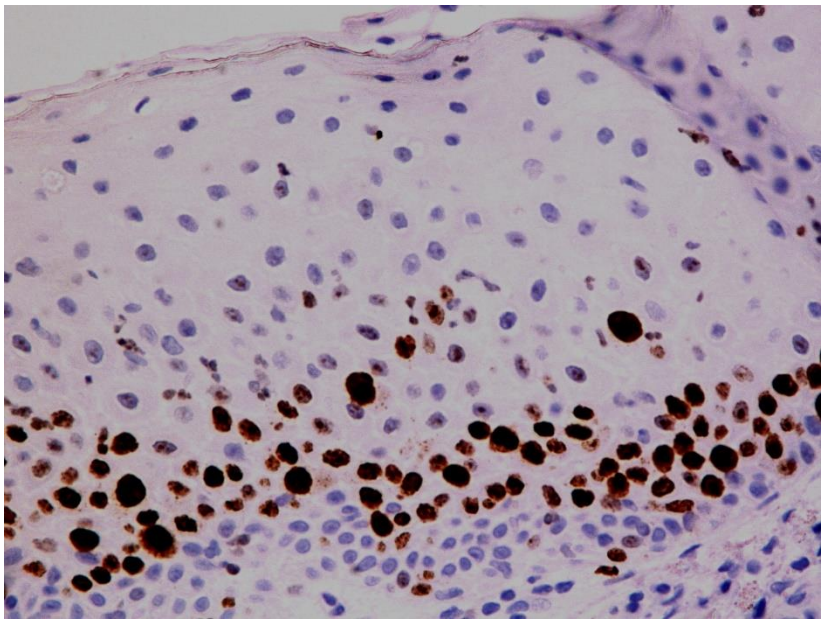

Figure 2. High grade of laryngeal mucosa. Ki67, obj.x40.

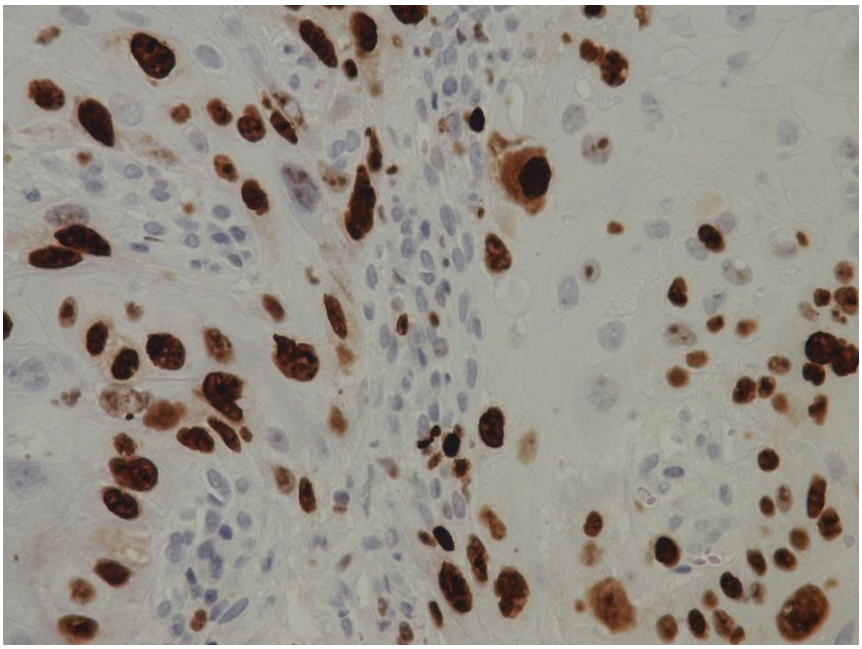

Figure 3. Squamous cell laryngeal mucosa. . Ki67, obj.x40.

Nuclear size of epithelial cell (nuclear area, perimeter and Feret diameter) and integrated optical density (IOD) were significantly larger in cancer cells than in normal mucosa and low grade dysplasia ($p < 0.01$). Diffferences in mean optical density were not statistically significant. Differences between squamous cell carcinomas and high grade dysplasia were not statistically significant ($p > 0.05$). Circularity was significantly lower in high grade dysplasia than in other groups of patients ($p < 0.05$) (Table 1).

Ki67 index was significantly larger in cancer tissue than in normal mucosa and low grade dysplasia ($p < 0.01$). Differences between squamous cell carcinomas and high grade dysplasia were not statistically significant ($p > 0.05$) (Table 1).

Table 1. Nuclear size, optical density, and Ki67 index in laryngeal mucosa and squamous cell laryngeal carcinoma

	Area (μm^2)	Mean optical density (a.u.)	Mode optical density (a.u.)	Perimeter (μm)	Circularity	Feret diameter (μm)	IOD	Ki67 index (%)
Normal mucosa	31.26±7.69	0.53±0.08	0.53	21.39±2.23	0.84±0.04	7.88±0.69	15.95±1.34	
Low grade dysplasia	44.52±7.02	0.45±0.09	0.44	25.22±1.96	0.87±0.03	9.38±0.73	19.59±1.97	
High grade dysplasia	50.12±8.94	0.56±0.11	0.6	28.66±2.94	0.77±0.03	11.15±0.33	27.31±5.59	
Squamos cell carcinoma	60.05±11.93	0.47±0.11	0.48	29.91±2.83	0.83±0.04	11.0±1.16	28.36±6.44	

Discussion

Histologically, lesions that do not fulfill the criteria for frank malignancy are referred to as dysplastic. Lesions are dysplastic when there is evident cytological and tissue architectural atypia without invasion. CIS lies at the extreme end of the dysplastic spectrum, where morphological features of malignancy are displayed by an epithelial lesion that has not yet breached the underlying basement membrane (Eversole, 2009).

Dysplastic epithelium represents an increased risk of malignant transformation compared with nondysplastic epithelium. However, the term dysplasia covers a broad range of morphological atypia that do not always lie neatly on a spectrum from normal epithelium to CIS (Gale et al, 2005).

The two major grading systems for laryngeal dysplasia in use are the World Health Organization and the Ljubljana classifications. The WHO classification has three dysplastic categories: mild, moderate, and severe. Histological features found in dysplasia, which suggest malignant potential, include abnormal mitotic figures, in terms of both morphology and numbers, nuclear pleomorphism, and stromal inflammation. Mild, moderate, and severe dysplasia represent a spatial increase in dysplastic epithelium, with cytological and architectural disturbance limited to the lower one-third (basal/parabasal), the lower two-thirds, and extending throughout the epithelium, respectively. The degree of cytological atypia also increases with increasing grade. By these definitions, the distinction between severe dysplasia and CIS is somewhat blurred and many pathologists and clinicians manage these entities similarly.

In our study various nuclear parameters were analyzed: nuclear size (area, perimeter, and Feret diameter), nuclear shape (circularity), optical and integrated optical density (IOD), and proliferation (Ki67) index were analyzed. According to our results, differences in nuclear size, integrated optical density, and Ki67 index were significantly larger in high grade dysplasia than in

low grade dysplasia. Differences in mean optical density were not statistically significant.

From the morphological point of view, in low grade dysplasia Ki67 positive cells were found in basal and parabasal layers only. In contrast, in high grade dysplasia Ki67 positive cells were found in spinous layer, too.

Conclusion

Our results support classification schemes with two grades (low and high) of laryngeal dysplasia.

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