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ORIGINAL ARTICLE

Precursor Lesions of the Vocal Cord: a Study on the Diagnostic Role of Histomorphology, Histometry and Ki-67 Proliferation

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Abstract
The precise typing of precursor lesions of squamous cell carcinoma of vocal cord is of vital importance since it determines the line of therapy and prognosis. The aim of the present study is to evaluate the possible value of the types of dyskeratosis, histometry and cell proliferation rate in discriminating these lesions. The present retrospective study was based on 145 patients, classified according to the updated 2017 WHO system and included: Low-grade dysplasia (24 cases), high -grade dysplasia (53 cases), carcinoma insitu (33 cases) and microinvasive carcinomas (35 cases). Cell proliferation was assessed by immunoreactivity to Ki-67. For histometry and quantitation of Ki-67 proliferation rate, an image analysis system was used (Leica LAS, Wetzlar, Germany). Epithelial pearls (cell nests) were commonly observed in microinvasive carcinoma (82.9%) than high-grade dysplasia (5.9%). The median epithelial thickness, as well as, proliferation rate showed a significant increase according to the grade of the lesion. It is concluded that dyskeratosis pattern, histometry and Ki-67proliferation rate are valuable parameters to characterize precursor lesions. The presence of epithelial pearls, thickness > 450 μm and Ki-67 > 40% denote high risk lesions that require adequate excision and/or radiotherapy.

Keywords Laryngeal cancer · Dysplasia · Dyskeratosis · Histometry · Ki-67 labeling index

Introduction

Carcinoma of the larynx is a rare malignant tumor, globally contributing only 2% of all cancers [1], and in Egypt an estimated incidence of 1.4% was registered [2]. About 70% arise from the vocal cord, and 95% are squamous carcinoma [3]. However, this tumor represents a major oncologic problem in view of the functional importance of the larynx, and the need of safe conservation treatment with preservation of the function.

Precursor lesions of squamous cell carcinoma of the vocal cord include: squamous dysplasia, carcinoma insitu and microinvasive carcinoma [4]. So far, there is a lack of international agreement on classification. The Bethesda system of dysplasia of uterine cervix [5] is inapplicable, since the squamous epithelium of larynx is a keratinizing type. Of the several classification systems proposed, only three main ones are in current use, namely: the World Health Organization (WHO) System [6, 7], the Squamous Intraepithelial Neoplasia (SIN) System [8] and the Ljubljana classification [9, 10], commonly used in Europe. Moreover, the diagnostic criteria of microinvasive carcinoma of the vocal cord also varied in different reports [11, 12]. All these classifications are based only on histopathologic morphologic features, hence are largely subjective.

The use of additional objective quantitative parameters, such as epithelial thickness and cell proliferation, may help in precise typing of these precursor lesions, as well as to determine their potential risk of progression to invasive malignancy. Few international studies are available on these subjects [13, 14] and none was reported from Egypt.

The present study was conducted to evaluate the possible diagnostic value of histomorphology, histometry and Ki-67 proliferation in characterizing the different precursor lesions of squamous carcinoma of the vocal cord.

Patients and Methods

The present investigation is a retrospective study on a total 145 precursor lesions of the vocal cord collected between the period 2009 and 2016 from both National Cancer Institute, Cairo University [68 cases] and private practice [77 cases]. An informed consent was obtained from all patients for the surgical procedure and the use of tissue for research. The

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approval of the ethical Institutional Review Board of NCI, Cairo University was also obtained (No. IRB00004025).

For the classification of dysplasia, the recently updated 2017 WHO classification was used [7]. In the three-tiered classification, the lesions were stratified as: low grade dysplasia (mild dysplasia), high grade (moderate and marked dysplasia) and carcinoma insitu. For microinvasive carcinoma, three criteria must be fulfilled, namely: size of the lesion ≤1.5 mm [11], mobile vocal cord and absence of lymphangioinvasion. For histologic classification of dyskeratosis, four subtypes are recognized, namely: hyperkeratosis, parakeratosis, individual cell keratinization and epithelial pearl formation (cell nests). For histometry, ten measurements were made for the lesion (in μm), with a distance of 100 μm interval (Fig. 1a), then the automated image analysis system (Leica LAS/Wetzlar, Germany) calculated the mean for each case. For statistical analysis and comparison, the median for each group of studied precursor lesions was used since the data were not normally distributed. In dysplastic lesions, thickness was measured from basement membrane

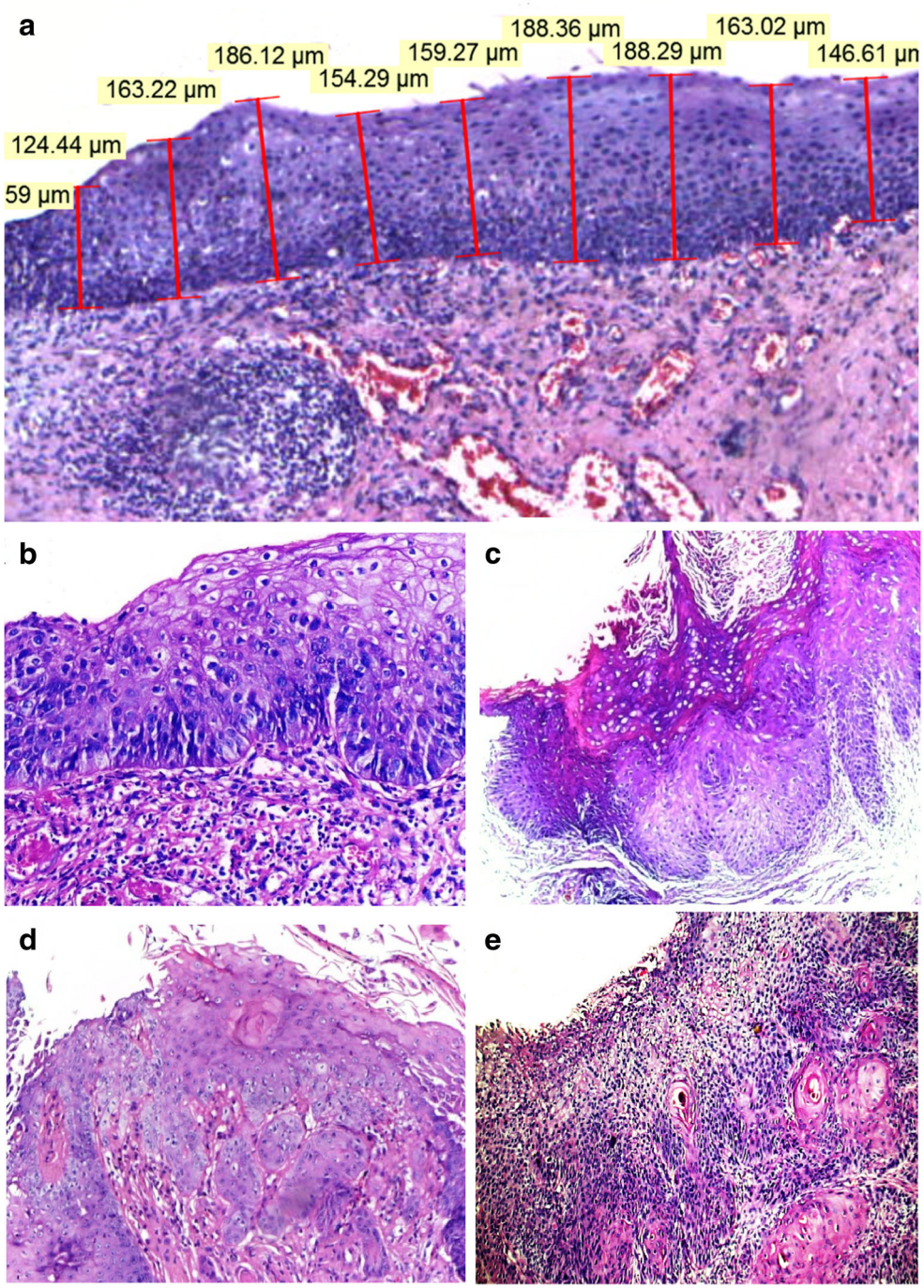

Fig. 1 Histomorphology of precursor lesions, H&E stain, objective ×20. (**a**) Low grade dysplasia, cellular atypia is limited to the lower half of epithelial thickness, also shown measurement method of epithelial thickness, (**b**) High grade dysplasia with epithelial atypia affecting more than lower half of epithelial thickness, (**c**) High grade dysplasia (carcinoma insitu) with extensive cellular atypia, loss of normal stratification, marked hyperkeratosis and parakeratosis, (**d**) Microinvasive carcinoma with invasion of the basement membrane and (**e**) Microinvasive carcinoma with keratotic cell nests

to the surface of epithelium always including the maximum thickness of the epithelium. However, in case of microinvasive carcinomas, measurement was made from the deepest point of invasion to the surface of epithelium or floor of the ulcer following the recent guidelines of AJCC [15]. No correction was made for the 20% shrinkage artifact due to Formalin fixation. For comparing cell proliferation, Ki-67 nuclear labeling rate was used. Standard Immunohistochemical methods were done using Dako Autostainer and reagents (Dako, Denmark), DAB (Diaminobenzidine) chromogen as a coloring agent and Hematoxylin as a counterstain. The automated image analysis system was used for the analysis of histometric studies (using microscopic objective ×20), as well as, for quantitation of Ki-67 nuclear labeling rate (using microscopic objective ×40) with measurement of at least 1000 cells in five microscopic fields. The automated system allowed for the separation of the crowded nuclei and excluding nuclei of stromal cells (size <5 μm).

Statistical analysis was performed using Statistical Package for Social Sciences, Version 23 (SPSS, Inc., Chicago, III, USA) for Windows. Categorical data were presented as number and percentage. Numerical data were presented as median and range since these data were not normally distributed. Only ages was expressed as mean and standard deviation since this variable showed a normal distribution. Comparison between multiple groups was done using Kruskal -Wallis H test. All tests were two tailed and a Probability (p value) equal or less than 0.05 was considered significant.

Results

The baseline clinicopathologic characteristics of the patients are presented in (Table 1). The mean age was 50 ± 13.7 years; ranging from 23 to 77 years. Males predominated with a male/female ratio of 5:1.

Table 1 Baseline demographic and pathological characteristics of the patients ($n = 145$)

Characterestics	N (%)
Age	
mean ± SD (years)	50 ± 13.7
Range	23–77
Sex	
Male	121(83.4)
Female	24(16.6)
Pathology	
Low grade dysplasia	24(16.6)
High grade dysplasia	53(36.6)
Carcinoma insitu	33(22.7)
Microinvasive carcinoma	35(24.1)

Based on the morphological criteria of the WHO 2017 three-tiered classification of laryngeal precursor lesions, low grade dysplasia contributed (16.6%), high grade dysplasia (36.6%) and carcinoma insitu (22.7%). The microinvasive carcinoma constituted 24.1%. The histomorphologic features of precursor lesions are demonstrated in (Fig. 1). Table 2 compares the frequency of keratin cell nests in microinvasive carcinoma and dysplasias of high grade including carcinoma insitu. Cell nests were common in microinvasive carcinoma (82.9%) than high grade dysplasia (5.8%), and the difference is statistically significant ($p < 0.001$). Dyskeratosis in microinvasive carcinoma is commonly in the form of cell nests (Fig. 1d, e), whereas, in dysplasia individual cell keratinization is usually observed (Fig. 1b, c).

Results of histometric measurements of the precursor lesions are demonstrated in (Table 3 and Fig. 2). There is a significant progressive increase in thickness ($p < 0.001$) from low grade dysplasia (median = 168.5 μm, ranging 100. 7–417.9 μm) over the high grade dysplasia (median = 270.3 μm, ranging 78. 9–571.6 μm), CIS (median 448.3 μm, ranging 223. 7–868.2 μm) and microinvasive carcinoma (median 774.4 μm, ranging 289–1123.6 μm).

Analysis of samples for proliferation rate using Ki-67 antibody showed a significant difference among the different histopathologic precursor lesions ($p < 0.001$) (Table 4). The percentage of ki-67 positive cells tends to increase progressively through the lesions (Figure 3) with a highest value in the microinvasive carcinoma (median 52.4%, ranging 25. 1–80.2%). Ki-67 staining in samples of low grade dysplasia shows a median of 21.2% positive epithelial cells (ranging 10. 1–27.3%), high grade dysplasia (median = 33.1%, ranging 18. 5–77.6%), and CIS (median 40.2%, ranging 22. 8–85.1%). The distribution pattern of Ki-67 positive cells also varied among precursor lesions. In low grade dysplasia, positive cells had a basal location unlike in high grade dysplasia, carcinoma insitu and microinvasive carcinoma, where the positive cells had a scattered pattern and involved more than half of the epithelium (Fig. 3).

Table 2 Frequency of keratin cell nests in microinvasive carcinoma and higher grades of dysplasia ($n = 121$ cases)

Precursor lesions		Intraepithelial Keratin pearls		P value
	No.	Absent (%)	Present (%)	
High grade dysplasia	86	81 (94.2%)	5 (5.8%)	<0.001
Microinvasive carcinoma	35	6 (17.1%)	29 (82.9%)	
Total	121			

Table 3 Histometric values of the precursor lesions (n = 145)

Vocal cord lesion	Thickness/μm			
	Median	Minimum	Maximum	*P* value
Low grade dysplasia	168.5	100.7	417.9	<0.001
High grade dysplasia	270.4	78.9	571.6	
Carcinoma insitu	448.3	223.7	868.2	
Microinvasive carcinoma	774.4	289.0	1123.6	

Table 4 Ki-67 labeling index in precursor lesions (n = 145)

Lesions	KI67 labeling index (%)			
	Median	Minimum	Maximum	*P* value
Low grade dysplasia	21.2	10.1	27.3	<0.001
High grade dysplasia	33.1	18.5	77.6	
Carcinoma insitu	40.2	22.8	85.1	
Microinvasive carcinoma	52.4	25.1	80.2	

Discussion

Malignant laryngeal tumors are mostly of squamous cell carcinoma type, representing more than 95% of total laryngeal tumors, 70% of which arise in vocal cord [3]. Most of Vocal cord carcinomas arise from precursor lesions. The increased rate of malignant transformation is associated with the increased grade of dysplasia [9], hence early detection of precursor lesions, as well as, proper assessment of the degree of their dysplasia are important for both preventing cancer development and avoiding major or mutilating treatment. A meta-analysis of 9 studies done by Weller et al. found a significant difference in the risk of malignant progression between lower grades of dysplasia (10.6%) as compared to high -grade dysplasia and carcinoma insitu (30.4%) [16].

The main method for establishing the malignant potential of vocal cord precursor lesion is the conventional microscopic examination of H&E-stained laryngoscopic biopsies. Three classification methods were reported namely: squamous intraepithelial neoplasia (SIN), Ljubljana classification of squamous intraepithelial lesion (SIL) and 2005 World Health Organisation (WHO) [6–10]. However, these studies were not reproducible and suffered a high inter-observer variation [17–19]. For these reasons, the usefulness of morphology alone as the primary mean of directing therapeutic strategies is still limited. Consequently, the use of additional objective quantitative parameters, such as epithelial thickness and cell proliferation, may help in precise typing of these precursor lesions. Few international studies are available on this subject [13, 14].

Recently, the WHO 2017 classification system was introduced, classifying precursor lesions into low grade dysplasia and high grade dysplasia [7]. Furthermore for treatment purpose, the high grade dysplasia is divided into high grade dysplasia and carcinoma insitu (CIS) based on morphologic features namely: cellular dysplasia and stratification pattern [7]. This system was confirmed to have a better inter-observer agreement than previous studies [9]. In the present study we used histologic criteria based on recent three-tier WHO 2017 grading system [7].

Histopathologic examination revealed 24 cases (16.6%) of low grade dysplasia, 53 cases (36.6%) of high grade dysplasia, 33 cases (22.7%) of carcinoma insitu and 35 cases (24.1%) of microinvasive carcinoma. In our study, presence of keratin pearl is significantly associated with carcinoma invasiveness ($p < 0.001$). The high frequency of epithelial pearls in invasive squamous cell carcinoma is explained by the disturbed natural process of cell exfoliation. Thus, in normal and dysplastic lesions, maturation occurs from deep to superficial layers and apoptotic cells are lost at the surface. With invasive

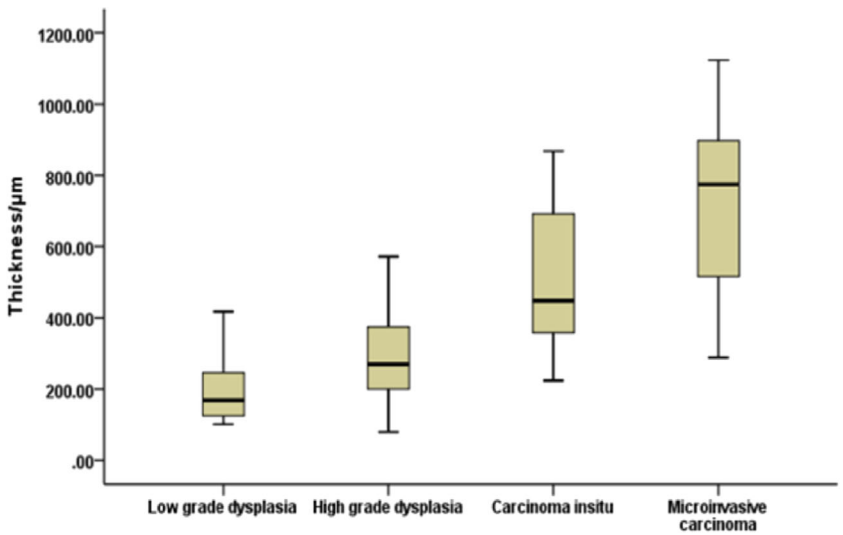

Fig. 2 Statistical box-and whisker diagram of histometric measurments of precursor lesions. The lines inside the boxes represent the median, and the lines outside the boxes indicate variations outside the upper and lower quartile. There is a progressive significant increase of thickness with increase of lesions' grades (*P* value <0.001)

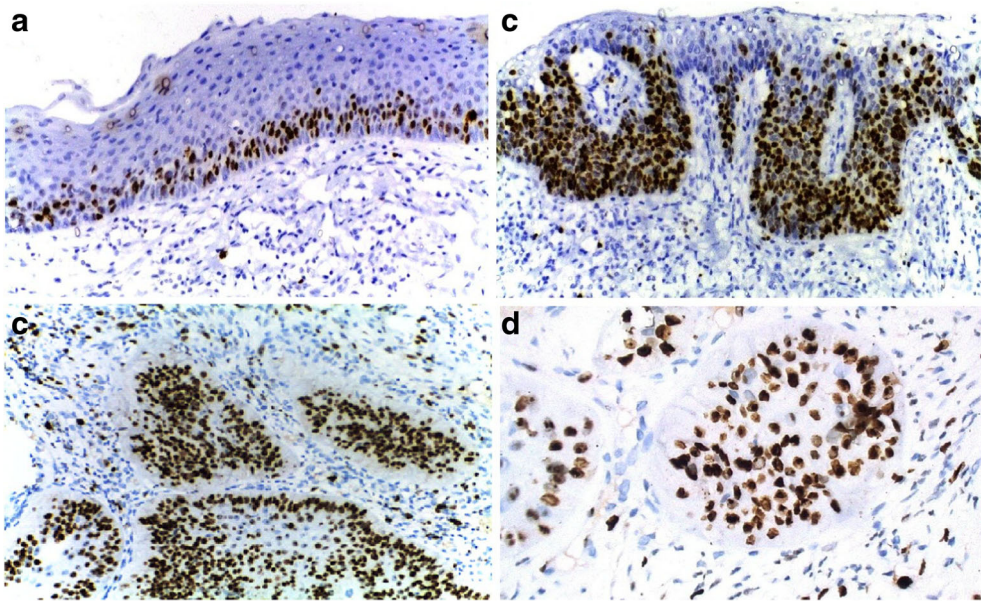

Fig. 3 Ki-67 distribution pattern and nuclear labeling rate. (**a**) Low grade dysplasia showing basal distribution of Ki-67 reactivity, objective ×20, (**b**) High grade dysplasia with Ki-67 labeling involving more than half of the epithelium using objective ×20, (**c**) Microinvasive carcinoma showing high nuclear labeling with a scattered pattern, involving full epithelial thickness and the microinvasive groups, objective ×20. (**d**) Microinvasive carcinoma, high magnification using objective ×40 showing high Ki-67 labeling index

cancer, no surface is available for exfoliation; hence apoptotic cells will form epithelial pearls.

Normally, the vocal cord epithelium shows a thickness ranging from 100 to 200 μm [20]. Arens et al. also reported a mean of 147 μm for the normal epithelial thickness [13]. There is a disagreement so far on the acceptable size of microcarcinoma of the vocal cord, as well as, method of its measurement. Most previous reports measured only the invasive component of the carcinoma in the stroma, namely from basement membrane to the deepest spread of the tumor. The sizes reported varied between 0.5 mm to 2 mm [11, 12]. In the present study, tumors 1.5 mm or less were included. Depth of invasion was measured from its deepest part to the surface of epithelium following the recent guidelines of AJCC [15]. Inclusion of overlying epithelial thickness in the measurement gives more realistic value of tumor size and allows comparability with the thickness of other precursor lesions. All cases had mobile cords and not associated with lymphangioinvasion.

Our morphometric analysis showed a double increase of the epithelium thickness in high grade dysplasia as compared to low grade dysplasia (median = 270.3 μm), thus nearly double the size of the previously reported normal vocal cord thickness (147 μm) [13]. CIS and microinvasive carcinoma thickness in our study showed a progressing increase up to four and seven times, respectively, that of normal epithelial thickness (CIS median = 448.3 μm, microinvasive carcinoma median = 774.4 μm). This increase in epithelial thickness is explained by an imbalance between cell proliferation and cell loss among these lesions.

Our results are in agreement with previous international studies on morphometric measurement of vocal cord precursor lesions. However, these studies used the older grading classification systems. In 1985, Kalter et al. noticed a continuous increase in epithelial thickening as a function of the grade of laryngeal dysplasia [21]. CÖr et al. described a mean epithelial thickness of 350 μm for carcinoma in situ [22]. On the other hand, Arens et al. showed progressing thickening of precursor lesions, where in moderate dysplasia there was a double increase in thickness, carcinoma in situ a triple increase and early invasive carcinoma achieved even a sixfold increase of the mean epithelial thickness compared to normal laryngeal mucosa [13]. They reported a mean CIS epithelial thickness of 444.8 μm.

Our study revealed that Ki67 expression has a definite role in predicting the biological behavior of the vocal cord precancerous lesions. We found a significant difference in Ki-67-proliferation rate between vocal cord dysplastic lesions, CIS and microinvasive carcinoma. Additionally, the percentage of ki-67 positive cells tends to increase progressively through the lesions. Low grade dysplasia on average showed 21.2% positive epithelial cells, high grade dysplasia 33.1%, CIS 40.2% and microinvasive carcinoma 52.4%. Previous reports also stated a similar significant difference between vocal cord precursor lesions [14, 23–25]. Of these studies, Mondal et al. reported an increase in Ki-67 rate with progressive increase of dysplastic lesions grades and carcinoma. They also found that distribution of Ki67 staining in precursor lesions and carcinoma was related to the level of cell differentiation [14]. Ki-67 index was reported by Ashraf et al. to be lower in 94% of normal to low grade dysplasia, while higher in 95% of high grade dysplasia [23]. Engiz et al. reported a significant difference in Ki-67 expression between all categories of epithelial precursor lesions except atypical hyperplasia and in situ carcinoma [24]. Although Mondal et al. and Pavlovic et al., reported lower average Ki67 labeling index in CIS than our findings [25.6%] [14, 25], other authors found higher average Ki-67 percentage for CIS [55.72%] [24]. So far, there is no

agreement on the clear-cut level of Ki-67 that distinguishes prognostically important groups, this is due to the continuous increase in epithelial cells proliferative activity.

Conclusions

It is concluded from the present study that histomorphology, histometry and Ki-67 proliferation rate are valuable diagnostic features in vocal cord precursor lesions. Thus, lesions more than 450 μm in size, proliferation rate more than 40% and presence of epithelial pearls denote a high-risk lesion which needs intervention by surgery and/or irradiation.

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Compliance with Ethical Standards

Conflict of Interest The authors have no conflict of interests to declare.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136:E359–E386
2. Globocan (2012) Estimated Cancer incidence, mortality and prevalence worldwide: Sources, methods and major patterns. http://globocan.iarc.fr/Pages/fact_sheets_population.aspx?country=818
3. Kumar V, Abbas A, Aster J (2018) Robbins basic pathology, 10th edn. Elsevier Inc, Philadelphia, pp 546–547
4. Fletcher C (ed) (2013) Diagnostic histopathology of tumors, 4th edn. Elsevier, Saunders, Philadelphia, p 171
5. Nayar R, Wilbur D (eds) (2015) The Bethesda system for Reporting cervical cytology, ed 3. Definitions, criteria, and explanatory notes. Springer, New York
6. Barnes L, Eveson IW, Reichart P, Sidransky D (eds) (2005) WHO pathology and genetics, head and neck tumors, 3rd edition, p120, IARC. Lyon, France
7. El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ (2017) WHO classification of head and neck tumors, 4th edition, p 91–93. IARC, Lyon
8. Gnepp DR (ed) (2009) Diagnostic surgical pathology of head and neck, 2nd edn. Saunders Elsevier, Philadelphia
9. Gale N, Michaels L, Luzar B, Poljak M, Zidar N, Fischinger J, Cardesa A (2009) Current review on squamous intraepithelial lesions of the larynx. Histopathology 54:639–656
10. Gale N, Blagus R, El-Mofty S et al (2014) Evaluation of a new grading system for laryngeal squamous intraepithelial lesions—a proposed unified classification. Histopathology 65:456–464
11. Grissman JD, Zarbo RJ, Drozdowicz S (1988) Carcinoma in situ and microinvasive squamous carcinoma of the laryngeal glottis. Arch Otolaryngol Head Neck Surg 114:299–307
12. Barnes L (ed) (2009) Surgical pathology of head and neck, 3rd edn. Informa Health care, New York
13. Arens C, Glanz H, Wonckhaus J, Hersemeyer K, Kraft M (2007) Histologic assessment of epithelial thickness in early laryngeal cancer or precursor lesions and its impact on endoscopic imaging. Eur Arch Otorhinolaryngol 264:645–649
14. Mondal D, Saha K, Datta C, Chatterjee U, Sengupta A (2013) Ki-67, p27, p53 expression in squamous epithelial lesions of larynx. Indian J Otolaryngol Head Neck Surg 65:126–133
15. Amin MB et al (2017) AJCC cancer staging manual. Springer-Verlag, New York
16. Weller MD, Nankivell PC, McConkey C, Paleri V, Mehanna HM (2012) The risk and interval to malignancy of patients with laryngeal dysplasia; a systematic review of case series and meta-analysis. Clin Otolaryngol 35(5):364–372
17. Fleskens SA, Bergshoeff VE, Voogd AC et al (2011) Interobserver variability of laryngeal mucosal premalignant lesions: a histopathological evaluation. Mod Pathol 24:892–898
18. Sarioglu S, Cakalagaoglu F, Elagoz S et al (2010) Inter-observer agreement in laryngeal pre-neoplastic lesions. Head Neck Pathol 4: 276–280
19. McLaren KM, Burnett RA, Goodlad JR et al (2000) Consistency of histopathological reporting of laryngeal dysplasia. Histopathology 37:460–463
20. Hirano M (1974) Morphological structure of the vocal cord as a vibrator and its variants as a vibrator and its variations. Folia Phoniatr (Basel) 26:89–94
21. Kalter OP, Delemarre JFM, Alons CL, Veldhuizen RW, Meyer C, Snow GB (1985) Quantitative morphometry of squamous cell hyperplasia of the larynx. J Clin Pathol 38:489–495
22. CÖr A, Gale N, Kambic V (1997) Quantitative pathology of laryngeal epithelial hyperplastic lesions. Acta Otolaryngol Suppl 527: 57–61
23. Ashraf MJ, Maghbul M, Azapira N et al (2010) Expression of Ki-67 and p53 in primary squamous cell carcinoma of the larynx. Indian J Pathol Microbiol 53:661–665
24. Sengiz S, Pabuccuoglu U, Sarioglu S (2004) Immunohistological comparison of the World Health Organisation (WHO) and Ljubljana classifications on the grading of preneoplastic lesions of the larynx. Pathol Res Pract 200:181–188
25. Pavlovic B, Djukic V, Milovanovic J, Tomanovic N, Milovanovic A, Trivic A (2013) Morphometric analysis of Ki-67 and p16 expression in laryngeal precursor lesions. Eur Arch Otorhinolaryngol 270:1405–1410