Immunohistochemical Analysis of Laryngeal Lesions and Clinical-Laryngoscopic Correlation

Caroline Fernandes Rimoli[1,2*], Rogério Hamerschmidt[3], Júlia Rodrigues Marcondes Dutra[4,5], Evaldo Dacheux de Macedo Filho[6], Henrique Furlan Pauna[7]

[1]MD, PhD in Clinical Surgery at the Federal University of Paraná (UFPR), Curitiba, PR, Brazil
[2]Department of Otolaryngology, Hospital Paranaense de Otorrinolaringologia, Curitiba, PR, Brazil
[3]MD, PhD in Clinical Surgery; Professor of the Postgraduate Program in Clinical Surgery at the Federal University of Paraná (UFPR), Curitiba, PR, Brazil
[4]Otolaryngologist, Fellowship program at the Hospital Paranaense de Otorrinolaringologia, Curitiba, PR, Brazil
[5]Department of Otorrinolaringology, Unimed Lorena and Santa Casa de Guaratinguetá, SP, Brazil;
[6]MD, PhD in Clinical Surgery; NEP, Hospital Paranaense de Otorrinolaringologia IPO, Curitiba, PR, Brazil
[7]PhD (FMRP-USP); Department of Otolaryngology, Hospital Paranaense de Otorrinolaringologia, Curitiba, PR, Brazil

Correspondence

Caroline Fernandes Rimoli, MD

Department of Otolaryngology, Hospital Paranaense de Otorrinolaringologia, Av. República Argentina, 2069, Água Verde, Curitiba, Paraná 80620010, Brazil

E-mail: carol@rimoli.med.br

Abstract

Introduction: The vocal fold is vulnerable to several types of lesions. Immunohistochemistry is a technique aimed to complement the routine histopathological investigation, showing the presence of different antigens in the tissue or cell by means of specific antibodies, thus allowing the assessment of the potential proliferation of neoplastic cells.

Objective: Detailed review of the results of vocal fold biopsies and correlation between histopathological data and clinical course among patients.

Methods: A descriptive retrospective study based on the analysis of medical records of patients undergoing laryngeal microsurgery due to benign diseases (polyps and nodules), whose biopsies required additional investigation using immunohistochemistry techniques. All biopsies were carried out by the same specialist, from January 2018 to December 2019 at the Hospital *Paranaense de Otorrinolaringologia*.

Results: Seven out of the 29 patients included in this study (26 male and 3 female patients) claimed to be tobacco users. All the 18 patients investigated for high-risk HPV scored negative in diagnostic immunohistochemistry. Three of the 19 patients with suspected laryngeal papillomatosis resulted in squamous cell carcinoma in the anatomopathological examination.

Conclusion: Due to similar histopathological aspects between the different types of vocal fold lesions, there is considerable doubt regarding the gold standard in histopathological examination. To determine the difference between benign lesions of the larynx, this paper reinforce the major role of performing immunohistochemistry examinations in the diagnosis of apparently benign lesions regarding early diagnosis and treatment.

Introduction

The vocal fold is a vibrating structure with peculiar anatomy, comprising a muscular layer, *lamina propria* and mucosa. The extracellular matrix of the *lamina propria* is rich in type-III collagen, elastin, hyaluronic acid, fibronectin, and fibroblasts [1,2]. The ultrastructure of the *lamina propria* and the basal membrane region contains sparse cellular material interposed to an extracellular matrix (allegedly responsible for the vocal fold's vibratory feature) [1,3,4]. The basal membrane visualized in electron microscopy studies was divided into *lamina lucida* (electron-lucent), *lamina densa* (electron-dense), and *lamina fibro-reticularis* (electron-lucent) [1,3].

To obtain a better histological analysis of the samples, such as cell maturation index, severity of epithelial changes or signs of malignancy, the pathologist should be provided with a detailed medical record and diagnostic hypothesis [5]. Although formaldehyde is the gold standard fixative in routine histology, the immunohistochemistry technique has been used in multiple areas of biology, from the functional assessment of cells to characterize lesions [6]. It can complement the well-established routine of histopathological analysis but depending on the type of antigen to be detected. It has the additional benefit of showing the presence of different antigens in the tissue or cell by means of specific antibodies, thus allowing the assessment of the potential proliferation of neoplastic cells, as well as the tumor growth rate [6,7]. The goal of the immunohistochemistry method is to detect the greatest number of antigens possible with the lowest tissue content analyzed, to provide greater diagnostic sensitivity [6]. A common histological result is dysplasia, which is the

histopathological manifestation of mutations in regular epithelial cells, which may include structural and nuclear changes, as well as cell architecture changes. The main factors associated with the genesis and progression of laryngeal dysplasia are tobacco abuse (carcinogenesis directly related to irritation caused by tobacco) and alcohol abuse, and gastroesophageal reflux disease [8].

The association of human papillomavirus (HPV) with head and neck cancer in patients without tobacco or alcohol consumption history is recognized [9,10]. Laryngeal papillomatosis is a recurrent benign disease associated with HPV, with the possibility of causing airway obstruction [9]. HPV subtypes 6 and 11 are the most prevalent in laryngeal papillomatous lesions, even though associate with atypia or carcinomatosis, while subtypes 16 and 18 represent high risk for such association. Improved survival rates are observed in patients with HPV-positive tumors [10]. The hybrid capture test technique ensures rapid and safe detection of the infectivity rate of the sample by DNA polymerase chain reaction of HPV, thus showing the presence or absence of high- or low-risk subtypes for carcinomatosis [9-11].

Two main protein products make HPV oncogenic, E6 and E7. They play an important role in carcinogenesis by inhibiting agents with tumor suppressor functions by p53 and Retinoblastoma (Rb) respectively, evolving with overexpression of p16 [12]. The p16 is an important tumor suppressor by acting in cell cycle control, as well as in cell apoptosis [12]. Previous studies have shown that p16 acts as an independent prognostic factor for HPV, with the ability to inhibit tumor invasion. Tumors related to alcohol or tobacco consumption suggest that they are associated with downregulation of p16 and mutations of p53 gene, thus resulting in its overexpression [13,14]. The p53 gene corresponds to a proto-oncogene that helps regulate cell growth, promoting apoptosis of cells whose DNA has been altered or damaged. Expression of p53 in immunohistochemistry leads to increased protein expression or mutation.

The relationship between expression of p53 and the prognosis of different types of cancer is well known [15]. Ki-67 is a nuclear nonhistone protein, whose expression can be detected in actively proliferating or neoplastic cells, thus reflecting the fraction of tumor growth, and is currently seen as one of the most reliable proliferation markers [7]. Cell proliferation represents a key biological mechanism in oncogenesis. The literature supports an association with high degree of specificity in high levels of immunohistochemical expression of Ki-67 in laryngeal squamous cell carcinomas, as well as a worse progression of cases [15].

Furthermore, another benign vocal fold lesions are non-malignant growths of abnormal tissue, arising from the epithelium [16]. The common lesions of the vocal folds are nodule, polyps, polypoidal degeneration, and cysts [17]. In their study, involving 50 patients, Malik *et al*. [17] found that the benign lesions were most common among young patients (20-29 years). Single histologic features may not differentiate between different benign vocal fold lesions, but combinations make some diagnoses more likely than others [18].

Given the progress of histological analysis techniques associated with the progress of immunohistochemical techniques, the aim of this study is a detailed review of the results of vocal fold biopsies performed in an Otorhinolaryngology hospital, which required complementary investigation using immunohistochemistry.

Methods

The research project for this paper was approved by the Institutional Review Board of the *Hospital Paranaense de Otorrinolaringologia* (CAAE 31341020.7.0000.5529). The informed consent documentation was waived.

A descriptive retrospective study was performed, based on the analysis of medical records of patients who underwent laryngeal microsurgery from January 2018 to December 2019. The study included patients whose biopsy specimens were sent to anatomopathological analysis, and which required complementary investigation using immunohistochemistry for differential diagnosis of other types of injury. All biopsies were carried out at the Hospital Paranaense de Otorrinolaringologia by the same senior surgeon.

The clinical diagnoses were based on the visual impression of the lesions at the time of the laryngoscopic examination and confirmed with the anatomopathological exam report after the biopsy. Clinical and epidemiological data were collected from the patients. All surgeries were performed in the operating room under general anesthesia, using the traditional technique with cold instruments or with a shaver, with or without the application of *Cidofovir* (75 mg/mL, with a maximum dose of 1mg/ kg) on the surgical bed, immediately after removing the lesions. Before removing the lesions, liquid-based cytology was collected with a brush. The papilloma specimens removed during the surgery, along with the brushes containing the liquid-based cytology collected, were sent to the anatomopathological analysis laboratory in the same city, for immunohistochemistry, to assess the expression of markers p16 and Ki-67, and to perform the hybrid capture to identify HPV, whether it is low or high risk. Patients with insufficient material for histological analysis or with incomplete medical records were excluded.

The characteristics of the lesions described in the reports of the laryngoscopy exams performed prior to the surgery were analyzed, to carry out an anatomopathological diagnostic comparison. The type of marker and expression levels of the lesions that underwent diagnostic immunohistochemistry were evaluated.

Diagnostic immunochemistry

Regardless of the immunohistochemical staining intensity, cells whose nuclei and/or cytoplasm were stained brown by antibody Ab-7 (anti-p16) were regarded as positive. Additional staining was considered for antibody p63, cytokeratin 5/6, antibody p40, and TP 53. The classification of the immunohistochemical expression was determined by means of the semi-quantitative scoring of the staining intensity (0 = negative; 1 = weak; 2 = mild; and 3 = strong) and percentage of the positively stained cells (1 ≤ 30%; 2 = 30 to 60%; and 3 ≥ 60%). The final score of each tumor was calculated by the sum of the intensity and percentage scores [19].

In turn, cells whose nuclei were stained by antibody MIB-1 (anti-Ki-67), regardless of cytoplasmic staining, were regarded as positive. The classification of the immunohistochemical expression was determined by percentage of positive cells in 1,000 cells counted.

Analysis of hybrid capture

The Hybrid Capture 2 (HC2) technique from Digene® (Life Technologies Inc, USA) was used for high- and low-risk DNA/

HPV detection. The presence of HPV was determined by quantification of the light emission and expressed in relative light unit (RLU), following the manufacturer's guidelines. Specimens with RLU < 1 pg/ml were considered negative. Results with RLU ≥ 1 pg/ml were considered positive.

Statistical analysis

The results obtained in the study were described by means, standard deviations, minimum and maximum values (quantitative variables) or by frequencies and percentages (categorical variables). Data were analyzed using computer program Stata/SE v. 14. 1. StataCorp LP, USA..

Results

This study included 29 patients; 26 (89.7%) of which were male and three (10.3%) females. The average age among the patients in the study was 52.9±16.8 years (ranging from 23 to 79 years old). As for tobacco consumption, nine medical records presented information on the abuse of such substance and seven patients (77.8%) declared to be smokers (Table 1).

Table 1. Description of the specimen

Variable	Total	Classification	Result*	
Age (years)	29		52.9 ± 16.8 (23 – 79)	
Sex	29	Male	26	89.70%
		Female	3	10.30%
Smoker	9	No	2	22.20%
		Yes	7	77.80%
Low risk HPV	7	Negative	3	42.90%
		Positive (Sub-type 6)	4	57.10%
High risk HPV	18	Negative	18	100%
Ac P16	11	Negative	8	72.70%
		Positive	3	27.30%
Ac P63	3	Positive	3	100%
Ac Ki-67	12		0.37 ± 0,32 (0.05 – 0.99)	
Cytoker-tatin 5/6	3	Positive	3	100
Ac P40	1	Positive	1	-
TP53	4	Positive	4	100

*Described by mean ± standard deviation (minimum – maximum) or by frequency and percentage

From the laryngoscopy exam results, the main lesion described was papillomatous in 65% of cases, followed by lesions that suggest malignancy (carcinoma) in 13.8% of them. Leukoplasia was described in 10.3% of the cases, followed by polypoid lesion in 6.9% and granulomatous lesion in 3.4% of the cases.

From the total patients analyzed, the main lesion found was papilloma, which accounted for 65.5% of the cases, followed by squamous cell carcinomas (24.1%). Other lesions were found in only one individual each: granuloma, polyp, and nodule (Table 2).

Table 2. Distribution of cases according to anatomopathological diagnosis

Histological diagnosis (grouping)	n	%
Papilloma	19	65.5
SCC	7	24.1
Granuloma	1	3.4
Polyp	1	3.4
Nodule	1	3.4
Total	29	100

Using the hybrid capture method, 57.1% of the patients investigated for low risk tested positive for subtype 6; in the high-risk investigation, 100% of the specimens tested negative.

Only 27% of patients analyzed for immunohistochemical expression of p16 in biopsies tested positive. 75% of these patients with positive results were compatible with the diagnosis of squamous cell carcinoma (SCC) and 25% compatible with papilloma. Unlike those tested for p63, in which 100% were diagnosed with SCC.

In immunohistochemical expression of Ki-67, the mean percentage was 37% (5-99), being the highest values expressed in the most undifferentiated carcinomas.

Only four patients resulted positive for P53, two of which were diagnosed with squamous cell carcinoma, one diagnosed with a laryngeal nodule and one patient diagnosed with papilloma (Table 3).

Table 3. Distribution of cases according to videolaryngoscopy diagnosis

Diagnosis	n	%
Papilloma	19	65.5
SCC	4	13.8
Leukoplasia	3	10.3
Polyp	2	6.9
Granuloma	1	3.4
Total	29	100

Based on the laryngoscopy exam report, those that suggested papilloma were correct in 84.2% of the cases, the remainder of which being the result of squamous cell carcinoma (SCC) biopsy. While in 75% of those that suggested SCC there was a true correlation, and 25% had a histological diagnosis of papilloma.

The suspected leukoplasia seen at the laryngoscopy exam was histologically classified as papilloma in 66.7% of the cases and as a nodule in 33.3% of the cases of vocal fold leukoplasia.

A polypoid lesion of benign aspect and its recurrence were sent for diagnostic immunohistochemistry after two laryngeal microsurgeries, where the hypothesis was confirmed in the first microsurgery, and the second revealed the diagnosis of malignancy (SCC). Vocal fold granuloma showed 100% positive correlation between image and diagnostic immunohistochemistry. Table 4 shows the Comparison between videolaryngoscopy and anatomopathological diagnosis.

Table 4. Comparison between videolaryngoscopy and anatomopathological diagnosis

Histological diagnosis	Videolaryngoscopy diagnosis				
	Papilloma	SCC	Leukoplasia	Polyp	Granuloma
Papilloma	16	1	2	-	-
	84.2%	25.0%	66.7%		
SCC	3	3	-	1	-
	15.8%	75.0%		50%	
Granuloma	-	-	-	-	1
					100%
Polyp	-	-	-	1	-
				50%	
Nodule	-	-	1	-	-
			33.3%		
Total	19	4	3	2	1

Discussion

Dikkers & Nikkles [18] analyzed 74 biopsies of the three benign lesions using optical microscopy, electron microscopy and immunohistochemistry. Approximately 75% of polyp biopsies and Reinke's edema showed edematous lakes, whose diagnostic immunohistochemistry was negative for antibodies to factor VII antigen. The presence of macrophages containing fibrin and iron and recent bleeding/thrombosis signs was almost exclusive to vocal polyps. Neves et al. [1] used optical microscopy for analysis of the vocal fold epithelium of 26 patients with inflammatory laryngeal lesions and compared it with regular vocal folds from cadavers. They performed immunohistochemical staining techniques for laminin and type IV collagen and showed greater immunohistochemical expression of these substances in the basement membrane of vocal nodules when compared to polyps or epithelium from regular vocal folds. We found two cases that were suspicious for polyp at videolaryngoscopy but the histology confirmed just one case.

Immunohistochemistry is a tissue analysis method using a microscope, to identify molecular characteristics of diseases. It is based on specific antigen-antibody binding and offers several applications, such as the diagnosis of inflammatory, infectious, and neoplastic diseases. It is also very important to determine predictive and prognostic factors in patients with cancer [20]. Immunohistochemical studies have been playing a prominent role in research involving tumors and, since 1996, immunohistochemistry has been described to help diagnosis of benign laryngeal lesions [1,3,21]. Tumor markers have been used as prognostic indicators, and some of them allow the identification of cell cycle regulatory proteins, such as Ki-67, which is a cell proliferation marker, and p16, which indicates viral participation in the carcinogenesis of epithelial tumors [22]. Our study found three patients diagnosed with leukoplasia correlated with SCC. Yet, immunohistochemical expression of Ki-67 was positive in 37% of the cases, being the highest values expressed in the most undifferentiated carcinomas.

Additionally, expression of p16 also correlates with HPV, which is considered a prognostic factor for disease-free survival among patients with laryngeal SCC history [12]. The specificity of p16 for HPV status in the Barrueco et al. [12] study was 0.88 (95% CI = 0.47-0.99), combined with a sensitivity of 0.63 (95% CI = 0.44-0.80), a negative predictive value of 0.39 (95% CI = 0.17-0.64) and a positive predictive value of 0.95 (95% CI = 0.75-0.99). Although a cut-off points greater than 50% for positive p16 results was applied, all positive cases showed 80% to 100% expression in tumor cells, which is in accordance with the known pattern of p16 in SCC. In the present study, we found a positive classification with low risk for HPV in 57.1% of patients, and a negative classification with high risk for HPV in 100% of patients. To this date, our findings support the evidence collectively suggesting that, as opposed to the etiologic role and well-established prognostic of HPV in oropharyngeal neoplasms, its role in laryngeal carcinoma is comparatively limited.

The reported frequency of anti-p53 autoantibodies in individual cancer studies varies significantly, due to the small specimen sizes (in general, a greater proportion of advanced stage tumors were included, and different detection methods were used). In a historical review, results were compiled from 80 studies of anti-p53 autoantibodies in 18 cancers, over a 20-year period. The mean serum seropositivity rate in all cancer types was 16.9%, as compared to 1.45% in controls, thus demonstrating remarkable specificity (98%), but low sensitivity [23].

A gradual increase in the combined expression of EGFR, cyclin D1, p53 and Ki-67 proteins was observed as dysplasia progression. In addition, other studies have showed a significant correlation between the expression of EGFR protein and different levels of atypia in hyperplastic lesions. The results also showed a significant difference in staining with cyclin D1, p53 and Ki-67 of high-grade dysplastic lesions as compared to simple hyperplasia and low-grade dysplasia, thus confirming the need for caution and different treatment of such lesions with potential malignancy. Although the immunohistochemical analysis of different markers such as EGFR and p53 still lacks standardization, which sometimes results in difficulty to compare results from different laboratories and authors, our

results agree with the literature data. Immunohistochemistry techniques are more accessible to laboratories and may contribute to greater diagnostic accuracy of laryngeal lesions.

One of the main limitations of our study is the lack of information on tobacco and alcohol use, which are the dominant risk factors for laryngeal cancer. Presumably, most laryngeal cancers in this study were related to these exposures. The results of our study suggest that any etiologic role of HPV is limited to just a fraction of laryngeal cancers.

Conclusion

By means of a detailed review of the results of vocal fold biopsies that required complementary investigation using immunohistochemistry, we concluded that such technique was important for the accurate diagnosis of certain lesions, mainly those malignant lesions that, visually, may have a benign appearance.

References

1. Neves BMJ, Neto JG, Pontes P. Diferenciação histopatológica e imunoistoquímica das alterações epiteliais no nódulo vocal em relação aos pólipos e ao edema de laringe. Rev Bras Otorrinolaringol. 2004; 70(4):439-48.

2. Vahabzadeh-Hagh AM, Zhang Z, Chhetri DK. Hirano's cover-body model and its unique laryngeal postures revisited. Laryngoscope. 2018;128(6):1412-1418.

3. Courey MS, Shohet JA, Scott MA, Ossoff RH. Immunohistochemical characterization of benign laryngeal lesions. Ann Otol Rhinol Laryngol. 1996;105(7):525-531.

4. Carvalho EGB, Pauna HF, Machado AJ Junior, Nicola EMD, Altemani AMAM, Crespo AN. Evaluation of the Grafted Fascia in the Vocal Fold of Dogs: A Histologic Study. J Voice. 2017;31(5):605-609.

5. Braut T, Krstulja M, Marijić B, et al. Immunohistochemical analysis of vocal cord polyps applying markers of squamous cell carcinogenesis. Pathol Res Pract. 2019;215(1):144-150.

6. Gautier J-C. Drug safety evaluation. Methods in molecular biology. 2017. 1182 p.

7. 7.Gioacchini FM, Alicandri-Ciufelli M, Magliulo G, Rubini C, Presutti L, Re M. The clinical relevance of Ki-67 expression in laryngeal squamous cell carcinoma. Eur Arch Otorhinolaryngol. 2015;272(7):1569-1576.

8. Ali SA, Smith JD, Hogikyan ND. The White Lesion, Hyperkeratosis, and Dysplasia. Otolaryngol Clin North Am. 2019;52(4):703-712.

9. Moore CE, Wiatrak BJ, McClatchey KD, et al. High-risk human papillomavirus types and squamous cell carcinoma in patients with respiratory papillomas. Otolaryngol Head Neck Surg. 1999;120(5):698-705.

10. 10.Lee WT, Tubbs RR, Teker AM, et al. Use of in situ hybridization to detect human papillomavirus in head and neck squamous cell carcinoma patients without a history of alcohol or tobacco use. Arch Pathol Lab Med. 2008;132(10):1653-1656.

11. Orita Y, Gion Y, Tachibana T, et al. Laryngeal squamous cell papilloma is highly associated with human papillomavirus. Jpn J Clin Oncol. 2018;48(4):350–355.

12. Sánchez Barrueco A, González Galán F, Villacampa Aubá JM, et al. p16 Influence on Laryngeal Squamous Cell Carcinoma Relapse and Survival. Otolaryngol Head Neck Surg. 2019;160(6):1042-1047.

13. Fayette J. Head and neck squamous cell carcinomas. Anticancer Drugs. 2011;22(7):585.

14. Meshman J, Wang PC, Chin R, et al. Prognostic significance of p16 in squamous cell carcinoma of the larynx and hypopharynx. Am J Otolaryngol. 2017;38(1):31-37.

15. RODRIGUES, R. B., da Ros Motta R, dos Santos Machado SM, et al. Valor prognóstico da correlação imunoistoquímica do Ki-67 e p53 em carcinomas epidermóides da laringe. Braz J Otorhinolaryngol. 2008; 74(6):855-859.

16. Pickhard A, Reiter R. Benigne Stimmlippentumore [Benign vocal fold lesions]. Laryngorhinootologie. 2013;92(5):304-312.

17. Malik P, Yadav SPS, Sen R, et al. The Clinicopathological Study of Benign Lesions of Vocal Cords. Indian J Otolaryngol Head Neck Surg. 2019;71(Suppl 1):212-220.

18. Dikkers FG, Nikkels PG. Benign lesions of the vocal folds: histopathology and phonotrauma. Ann Otol Rhinol Laryngol. 1995;104(9 Pt 1):698-703.

19. Prowse DM, Ktori EN, Chandrasekaran D, Prapa A, Baithun S. Human papillomavirus-associated increase in p16INK4A expression in penile lichen sclerosus and squamous cell carcinoma. Br J Dermatol. 2008;158(2):261-265.

20. Ramos-Vara JA. Technical aspects of immunohistochemistry. Vet Pathol. 2005;42(4):405-426.

21. Martins RH, Domingues MA, Fabro AT, Dias NH, Santana MF. Reinkes' edema: immunoexpression study of fibronectin, laminin and collagen IV in 60 cases by immunohistochemical techniques. Braz J Otorhinolaryngol. 2009;75(6):821-825.

22. Alves ÂV, Ribeiro DR, Lima SO, et al. Expression of Ki-67 and P16 INK4a in chemically-induced perioral squamous cell carcinomas in mice. Rev Col Bras Cir. 2016;43(2):72-79.

23. Soussi T. p53 alterations in human cancer: more questions than answers. Oncogene. 2007;26(15):2145-2156.