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ORIGINAL ARTICLE

Tumor risk markers in recurrent respiratory papillomatosis

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Abstract
Objectives: This study aims to investigate the pattern of immunoexpression of proteins p16, p53 and Ki-67 in RRP, as well as to evaluate its influence on the number of surgeries that patients have undergone to date and to analyze the benefit of immunohistochemistry in this disease.
Methods: Clinical-demographic data and tumor samples were obtained from 33 patients with RRP. The expression of proteins p16, p53 and Ki-67 was analyzed by immunohistochemical method.
Results: Most patients had already undergone more than one surgery. The p16 marker was negative in 24.2% of the cases, with little positivity in 27.3% of the cases, moderate in 36.4% and intense in 12.1%. The p53 marker was positive in all cases, with little immunoexpression in 39.4% of cases, moderate in 36.4% and intense in 24.2%. The Ki-67 marker showed nuclear positivity in all lesions, although in varying degrees, with a mean proliferative index ± SD (standard deviation) of 51.7 ± 26.
Conclusions: The papillomatous lesions had varying degrees of immunoexpression of proteins p16, p53 and Ki-67, but no specific immunohistochemical pattern was observed. It was found, with statistical difference, that the number of surgeries was higher in cases with greater intensity of p53 expression, without correlation with the other markers. The benefit of immunohistochemistry in recurrent respiratory papillomatosis may lie in the prognostic assessment. However, further studies are needed to evaluate the use of this technique for this purpose.

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Level of evidence: : 4.

Introduction

Recurrent Respiratory Papillomatosis (RRP) is a histologically benign in general, with a high recurrence capacity. It is characterized by the formation of exophytic proliferative lesions in the connective tissue covered by epithelium, called papillomas.[1,2]

Although RRP can develop anywhere along the respiratory tract, there appears to be a strong predilection for the laryngeal tissue, being also called laryngeal or glottic papillomatosis.[1,3]

A bimodal age distribution is characteristic of RRP, with the disease affecting either young children or young adults.[1] However, its true incidence and prevalence remains unclear.[4]

The etiology is through infection with Human Papillomavirus (HPV), a Deoxyribonucleic Acid (DNA) virus that enters the host system through microabrasion.[4]

RRP is diagnosed through laryngoscopic examination, in which exophytic lesions are noted in the airways, associated with a variety of signs and symptoms, including hoarseness, chronic cough, dyspnea, recurrent upper airway infections, pneumonia, dysphagia, stridor and even respiratory failure.[1]

Nowadays surgery is used to ensure airway patency, to preserve nearby laryngeal tissues, and to maintain acceptable vocal quality. The advancement of surgical techniques for treatment, associated with adjuvant therapies, such as Cidofovir and, more recently, bevacizumab, has shown a better prognosis for the disease.[5]

Immunohistochemistry is a tissue analysis method that aims to identify molecular characteristics of diseases. It is based on specific antigen-antibody binding. It offers several applications, such as the diagnosis of inflammatory, infectious and neoplastic diseases. It is also important to determine prognostic and therapeutic factors and proliferative indices of some neoplasms.[6,7]

Some tumor markers allow the identification of cell cycle regulatory proteins, such as Ki-67, which is a cell proliferation marker, p53, whose product plays a role in inhibiting DNA synthesis, and p16, which indicates viral participation in the carcinogenesis of epithelial tumors.[8,9]

This study aimed at investigating the pattern of immunohistochemical expression of p16, p53 and Ki-67 proteins in recurrent respiratory papillomatosis, to evaluate the influence of immunohistochemical expression patterns of p16, p53 and Ki-67 proteins on the number of surgeries to which patients underwent and to analyze the benefit of immunohistochemical expression in recurrent respiratory papillomatosis.

Methods

The research project for this paper, under nº2.901.777/2018 (CAAE 95552318.1.0000.5529), was approved by the Human Research Ethics Committee (HREC) of *Hospital Paranaense de Otorrinolaringologia* (IPO). Free and Informed Consent Forms (FICF) were collected from all patients.

This study was carried out from October 2018 to December 2019 with patients with laryngeal or recurrent respiratory papillomatosis, which were treated and underwent surgery at Hospital Paranaense de Otorrinolaringologia, in the city of Curitiba, state of Paraná. The clinical diagnoses were based on the visual impression of the lesions at the time of the videolaryngoscopic examination and confirmed with the anatomopathological exam report. Clinical and epidemiological data were collected from the patients. All surgeries were done 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 1 mg/kg) on the surgical bed, after removing the lesions. The papilloma specimens removed during the surgery were sent to Byori Human Pathology Laboratory, in the same city, for immunohistochemistry in order to analyze the expression of markers p16, p53 and Ki-67.

This is a prospective, cross-sectional observational study.

The study included patients with laryngeal or recurrent respiratory papillomatosis, who were treated and underwent surgery at the IPO Hospital in the city of Curitiba, with histopathological confirmation, from October 2018 to December 2019, and they all agreed to the consent forms for the study.

There are no exclusion criteria defined.

Tumor tissue specimens previously fixed in 10% neutral buffered formalin were subjected to histological processing and dehydrated in alcohol baths in increasing concentration, clarified in xylol and paraffin embedded.

Subsequently, the sections were included in paraffin wax and sectioned in microtomy with a thickness of 4 μm. The sections containing tumor tissue were mounted on silanized slides and placed in an oven at 60°C for 60 min. Before starting the immunohistochemical processing, they were dewaxed with xylene and rehydrated in sequential ethanol baths with decreasing concentrations and then washed in distilled water.

Primary anti-p16 monoclonal antibody (INK4 protein, clone 1D7D2, Thermo Fisher) 1:200 dilution and anti-p53 antibody, mouse monoclonal (clone DO-7, ready to use, Agilient) and Ki-67 (clone MIB1, ready for use, Agilent) were processed, incubated and visualized according to spe-

cific pre-programmed protocols of the Dako Autostainer and EnVision™ platform, from Agilent Dako. Then, the slides were counterstained with Harris' hematoxylin (EasyPath®), covered with a coverslip and mounted using mounting medium (Entellan® – Merck Millipore).

All specimens were reviewed and classified independently, by two experienced pathologists. Differing cases were reviewed and a consensus for classification was reached.

Regardless of the immunohistochemical expression intensity, cells whose nuclei and/or cytoplasms were stained brown by antibody anti-p16 (INK4, clone 1D7D2) were regarded as positive. The gradation of immunohistochemical expression was determined by means of the semiquantification of the staining intensity in the nucleus or cytoplasm (0 – negative; 1 – weak; 2 – moderate; 3 – strong) and percentage of positively stained epithelial cells (1 – <30%; 2 – Between 30% and 60%; 3 – >60%).

For the evaluation of p53 expression, those epithelial cells whose nucleus were stained reddish-brown by the anti p53 antibody (clone DO-7) were regarded as positive, regardless of the cytoplasmic staining. The semiquantitative scoring system for the nuclear staining used was: 0 – No staining; 1 – 1% to 24% tumor cells positive; 2 – 25%–74% tumor cells positive and 3 – $\geq$75% tumor cells positive.

In turn, cells whose nuclei were stained by antibody anti-Ki-67 (MIB-1), 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. The count was carried out in the so-called ''hotspot'' area – a place where, at the lowest increase, scores the highest number of stained cells.

The results obtained in the study were described by means, standard deviations, medians, minimum and maximum values (quantitative variables) or by frequencies and percentages (categorical variables). In order to compare the scoring of the markers as compared to the number of surgeries, the nonparametric Mann-Whitney test and Kruskal-Wallis test were used; $p < 0.05$ values suggest statistical significance. For multiple comparisons of two-by-two ratings, Bonferroni corrections for *p*-values were employed. Data were analyzed using computer program IBM SPSS Statistics v.20.0. Armonk, NY: IBM Corp.

Results

The analysis shown below was performed with 33 patients who fulfilled the inclusion criteria for the study.

At surgery, their mean age was 41.4 years $\pm$ standard deviation of 12.7 years. Male gender was predominant, with 29 patients (87.9%). 20 patients (60.6%) received the drug Cidofovir on the surgical bed. When asked about HPV vaccination, 18 patients (54.5%) had completed the vaccination schedule and 45% had not been vaccinated yet. Most patients (66.6%) had already undergone more than one surgery, with a minimum of two surgeries and a maximum of 34.

Table 1 shows the result of the immunohistochemistry analysis of the 33 patients and Figs. 1, 2, 3 and 4 show the immunohistochemical expression.

Table 1 Immunohistochemistry analysis.

Variable	n valid	Classification	Result[a]
p16	33	Negative	8 (24.2)
		+/+++	9 (27.3)
		++/+++	12 (36.4)
		+++/+++	4 (12.1)
p53	33	+/+++	13 (39.4)
		++/+++	12 (36.4)
		+++/+++	8 (24.2)
Ki-67 (%)	33		51.7 $\pm$ 26; 50 (5–95)

Source: The author (2021).
[a] Described by mean $\pm$ standard deviation; median (minimum – maximum) or by frequency (percentage).

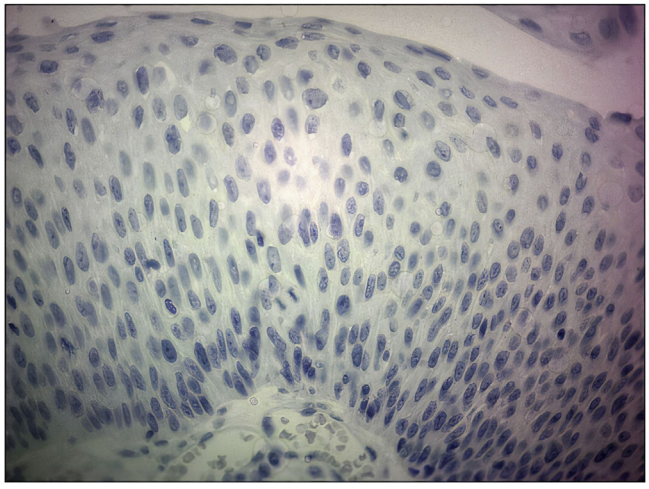

Figure 1 Photomicrograph illustrating immunohistochemical reaction revealing squamous epithelium without p16 Protein expression (p16, $\times$400). Source: The author (2021).

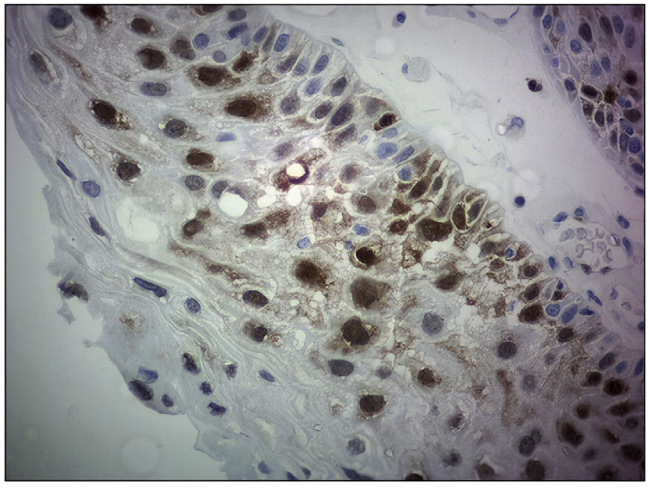

Figure 2 Photomicrograph illustrating immunohistochemical reaction revealing squamous epithelium with nuclear positivity for p16 protein (p16, $\times$400). Source: The author (2021).

Table 2 Correlation between the immunohistochemical expression with p16 and the number of surgeries to which patients were submitted.

p16	n	Nº Surgery		p[a]
		Mean ± standard deviation	Median (minimum – maximum)	
Negative	8	4.9 ± 7.8	2 (1–24)	
+/+++	9	4.4 ± 3.1	5 (1–9)	
++/+++	12	7.9 ± 9.7	4.5 (1–34)	
+++/+++	4	13.3 ± 14.2	11 (1–30)	0.672

Source: The author (2021).
[a] Kruskal–Wallis non-parametric test, $p < 0.05$.

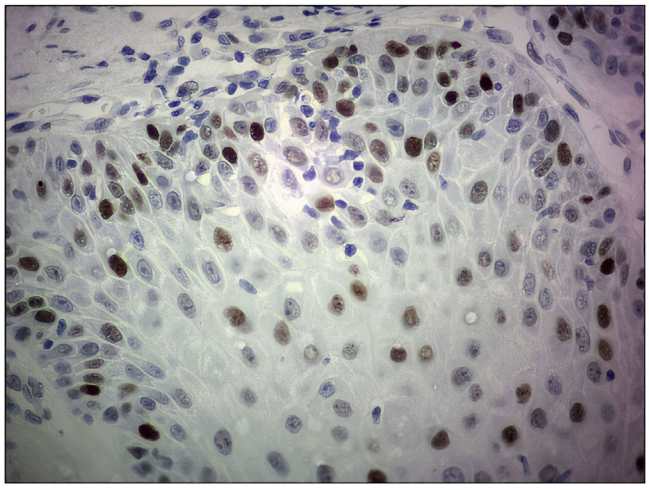

Figure 3 Photomicrograph illustrating immunohistochemical reaction revealing squamous epithelium with nuclear positivity for p53 protein (p53, ×400). Source: The author (2021).

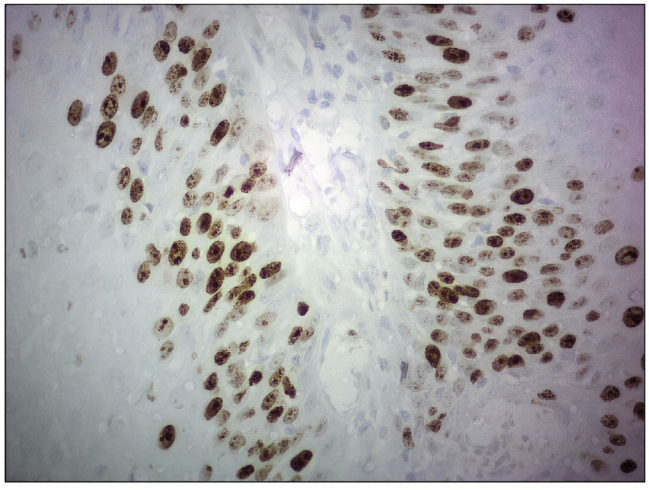

Figure 4 Photomicrograph illustrating immunohistochemical reaction revealing squamous epithelium with nuclear positivity for MIB-1 – Ki-67 proliferative index (MIB-1, ×400). Source: The author (2021).

p16 marker

We tested the null hypothesis that the number of surgeries is the same for all marker classifications, versus the alternative hypothesis that the number of surgeries is not all the same for the marker classifications. Table 2 shows the results considering the four p16 classifications.

No significant difference was found between p16 classifications regarding the number of surgeries. Therefore, no significant association was observed between p16 and the number of surgeries.

p53 marker

We tested the null hypothesis that the number of surgeries is the same for all marker classifications, versus the alternative hypothesis that the number of surgeries are not all the same for the marker classifications. Table 3 presents descriptive statistics for the number of surgeries, according to marker classifications and *p*-values of the statistical tests.

Considering that a significant difference was found between the p53 classifications in relation to the number of surgeries, these were compared two by two. Table 4 shows the *p*-values of such comparisons.

A significant difference was found between p53 classifications regarding the number of surgeries ($p = 0.038$). When comparing the classifications two by two, a significant difference was found between the classifications +/+++ and +++/+++ ($p = 0.030$). The number of surgeries is greater in cases with higher intensity of p53 expression.

Ki-67 marker

We tested the null hypothesis that the correlation coefficient between the number of surgeries and the Ki-67 expression equals zero (there is no correlation between the two variables), versus the alternative hypothesis that the correlation coefficient is different from zero (there is correlation). The estimated Spearman's rank correlation coefficient was 0.12, and the *p*-value of the statistical test was 0.524. No significant correlation was observed between the number of surgeries and the p16 and the Ki-67 expression.

Discussion

RRP is an important disease to be studied, due to its relevant morbidity and the great challenge posed for its control. Despite the fact that we have some prognostic factors mentioned before, patients with similar lesions do not behave in

Table 3 Correlation between the immunohistochemical expression with p53 and the number of surgeries to which patients were submitted.

p53	n	Nº Surgery		p^a
		Mean ± standard deviation	Median (minimum–maximum)	
+/+++	13	3.7 ± 6.2	2 (1–24)	
++/+++	12	5.9 ± 5.3	6 (1–18)	
+++/+++	8	13.5 ± 12.8	7.5 (1–34)	0.038

Source: The author (2021).
[a] Kruskal–Wallis non-parametric test, $p < 0.05$.

Table 4 Correlation between immunohistochemical expression results with p53.

Compared p53 classifications	p^a
+/+++ × ++/+++	0.422
+/+++ × +++/+++	0.030
++/+++ × +++/+++	0.533

Source: The author (2021).
[a] Extension of the Kruskal–Wallis test, $p < 0.05$ (Bonferroni corrections for p-values).

the same way. It is important to investigate new variables related to the disease.

With the RRP, we observe the usual need for repeated surgeries within a short period of time, thus causing great emotional damage to patients and a high economic cost.[4] In this study, most participants (66.6%) had already been submitted to more than one surgery, with cases of more than 20 procedures throughout life.

The treatment aims at keeping airway patency and providing fair vocal quality. Hence, disease control takes into account the lower demand for surgeries and a longer interval between them. A poor prognosis is related to a high number of procedures, not to mention the malignancy of the lesions.

The incidence of HPV-positive head and neck cancer has increased in recent years, especially the oropharyngeal squamous cell carcinoma.[10] Although the involvement of factors such as alcohol and tobacco are still wide in these cases, HPV is now the primary factor in this type of cancer in some regions of the world.[11,12]

As the action of each organism differs, having palpable parameters is extremely important for a correct prognostic assessment of HPV manifestations, especially of RRP. The markers used in this study were chosen based on general immunohistochemistry knowledge, in particular as regards malignant head and neck tumors, especially laryngeal ones, since there is no specific protocol on immunohistochemistry in RRP.

p16 protein is an inhibitor of cyclin-dependent kinases and an important cell regulator. It is routinely overexpressed in SCC and adenocarcinoma cases.[13] In this study, it was negative in 24.2% of cases, with little positivity in 27.3% of cases, moderate in 36.4% and intense in only 12%.

Clinical studies of head and neck cancer revealed longer survival rate for HPV-positive patients, tumors with detectable E6/E7 mRNA, and p16 overexpression. p16 overexpression also shows high correlation with expression of HPV E6/E7 mRNA, an active HPV infection marker. In addition, studies suggest that p16 overexpression results in a more favorable outcome.[13,14]

In cases of head and neck cancer, p16 can be a prognostic marker and possible predictive marker for therapeutic decision-making and predicting the treatment outcome.[13,15] In this study, no significant association between p16 and the number of surgeries was observed.

In turn, p53 gene is a tumor suppressor phosphoprotein and a key regulator of cell cycle progression and division. Its mutations are the most common changes in human cancer and may be part of the pathogenesis and growth of the tumor.[16,17] The immunohistochemical expression of p53 is very suggestive for the presence of a laryngeal carcinoma. Moreover, high-risk p53 mutations were associated with low survival rate.[18]

In this study, p53 marker was positive in all cases, with scarce immunohistochemical expression in 39.4% of cases, moderate in 36.4% and intense in 24.2%. A study with laryngeal SCC analyzed the immunohistochemical expression of p53 in 76 patients and found a statistically significant association with histological grades. Therefore, the authors suggest that carcinogenesis, as well as prognosis, may be associated with p53 expression, as it may play an important role in the early stage of malignant transformation of laryngeal tumors.[19]

In another study that included 30 patients with laryngeal SCC, p53 antibody was positive in 50% of cases. Total 70% of these were high-grade histological tumors and 60% had regional metastasis, also suggesting that, perhaps, p53 immunohistochemical expression in patients with laryngeal SCC is related to poor prognosis.[20] In addition, Vielba et al.[21] showed a statistically significant correlation between the presence of p53 and lower survival rates in patients with laryngeal SCC.

This study found, with a significant difference, that the number of surgeries is greater in cases with higher intensity of p53 expression, another factor that may indicate a poor prognosis in the case of RRP – high tumor recurrence.

Etiology of malignant transformation derives from a gradual molecular change. One study showed that p53 protooncogene mutation was associated with the integration of HPV-11 DNA into the host genome in samples of histologically malignant lesions derived from RRP.[22]

On the other hand, Ki-67 is a monoclonal antibody that identifies cell proliferation by recognizing the nuclear antigen expressed during the cell cycle phases (G1, S, M and

G2), not recognizing the G0 phase, in which the cell is quiescent.[23]

In this study, Ki-67 marker showed nuclear positivity in all lesions, despite the varying degrees, with a mean proliferative index ± SD (Standard Deviation) of 51.7 ± 26. In the study carried out by Rodrigues et al.[20] 76% of the laryngeal SCC showed immunohistochemical expression for Ki-67. The immunohistochemical expression of this marker was evident in 73.3% of patients with metastatic lymph nodes and in 75% of high-grade tumors. That suggests, with statistical relevance, that Ki-67 may be related to poor prognostic factors, which was also corroborated by other authors.[24]

It is necessary to continue the study as a long-term cohort, in order to monitor patients throughout the evolution of RRP, correlating immunohistochemistry with the HPV subtype, so as to better assess the prognosis and whether there are changes in these aspects between surgeries performed throughout the patient's life. Also, a possible correlation between the evolution of the disease and HPV vaccination can be investigated.

As a future perspective, we may consider the use of the expression of immunohistochemical markers not only for prognostic assessment, but also as an auxiliary data for a more individualized therapy.

Conclusion

The papillomatous lesions showed different degrees of immunohistochemical expression of p16, p53 and Ki-67 proteins, but no specific immunohistochemical pattern was observed.

The study found, with statistical difference, that the number of surgeries was higher in cases with higher intensity of p53 expression, without correlation with the other markers.

The benefit of immunohistochemistry in recurrent respiratory papillomatosis may rest in the prognostic assessment; however, further studies are required to use the technique for such purpose.

Authors' contributions

Caroline Fernandes Rimoli: Conceived and designed the analysis, collected the data, performed the analysis, wrote the paper.

Rogerio Hamerschmidt: Conceived and designed the analysis, performed the analysis, wrote the paper.

Evaldo Dacheux de Macedo Filho: Conceived and designed the analysis, performed the analysis, wrote the paper.

Vanessa Mazanek Santos: Contributed analysis tools, performed the analysis.

Lucas Resende Lucinda Mangia: Contributed analysis tools, performed the analysis.

Bettina Carvalho: Contributed analysis tools, performed the analysis.

Locations

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Federal University of Paraná (UFPR), Curitiba, PR, Brazil;

Byori Human Pathology Laboratory, Curitiba, PR, Brazil;

Presentation

This manuscript was not previously presented. It was accepted as a poster presentation at the 34th International Papillomavirus Conference (IPVC) in Toronto, wich will be from November 15–19, 2021.

Availability of data and material

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The research project for this paper, under nº 2.901.777/2018 (CAAE 95552318.1.0000.5529), was approved by the Human Research Ethics Committee (HREC) of the Hospital Paranaense de Otorrinolaringologia (IPO). Free and Informed Consent Forms (FICF) were collected from all patients.

Conflicts of interest

The authors declare no have conflicts of interest.

Acknowledgements

Danielle Giacometti Sakamoto – Degree: MD, Pathologist. Role: Immunohistochemical Analysis.

Márcia Olandoski – Degree: PhD, Statistic; Role: Statistical Analysis.

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