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Research Article

Laryngeal Carcinoma Associated with Human Papilloma Virus: Place of "P16" Immunomarking as A Real Alternative to Virological Analysis

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Abstract

Introduction: Many HPV-related cell signaling pathways appear to be poorly elucidated in laryngeal carcinogenesis. The objective of this work was to study the expression of the P16 gene by immunostaining in laryngeal carcinomas in order to judge its necessity in place of a virological analysis of oncogenic HPV infection.

Material and Methods: This was a 10-year descriptive and transversal study carried out at the molecular pathology laboratory of the Faculty of Health Sciences in Brazzaville. The study material consisted mainly of biopsies of laryngeal carcinomas with histological proof. The samples excluded were those which had architectural modifications which did not allow immunological marking with anti-P16 monoclonal antibodies.

Results: Squamous cell carcinomas of the larynx represented 35% of ENT and head and neck cancers with a ratio of 6 in favor of men. In all cases, these were squamous cell carcinomas for which immunohistochemistry of the P16 gene had made it possible to identify two molecular profiles, namely: P16+ laryngeal carcinomas (33.3%) and P16 − laryngeal carcinomas (66.7%). Virologically, the molecular prevalence of HPV was approximately 33.3%, representing HPV-positive laryngeal carcinomas and correlated with overexpression of the P16 gene ($p = 0.004$). However, HPV-negative laryngeal carcinomas did not express the P16 gene on immunostaining and remain linked to other risk factors.

Conclusion: Overexpression of the P16 gene is a mutation which would be linked to persistent infection with oncogenic HPV on a squamous mucosa. The P16 gene thus becomes a diagnostic biomarker for HPV-positive laryngeal carcinomas, replacing virological analysis.

Keywords: Laryngeal Carcinomas; HPV; P16 Gene

Introduction

Laryngeal carcinomas are a group of malignant proliferations at the expense of the laryngeal mucosa [1]. They pose a real public health problem due to the increase in their frequency which represents approximately 10% of all cancers in the world [2]. If alcohol and tobacco intoxication and professions using the voice are known risk factors [3-5], it appears that certain mechanisms underlying carcinogenesis of the larynx are still poorly understood, like the cellular signaling pathways for which certain genes are involved during human papilloma virus (HPV) infection. However, little is known about the interactions of viral proteins E6 and E7 with certain cell cycle genes in the laryngeal mucosa. Are there cytoplasmic and nuclear factors involved in the development of laryngeal carcinomas? Some studies put forward the hypothesis of the role of the P16 gene in the development of epithelial cancers through mutations induced by HPV with a high oncogenic risk [11,12]. Indeed,

the P16 gene is a pro-oncogene whose main role is the control of the cell cycle during the G1-S phase in order to prevent certain genetic anomalies from passing onto daughter cells [13]. Thus, numerous studies report modifications in the expression of the P16 gene by immunostaining specifically in certain carcinomas of the oral cavity and oropharynx associated with HPV [11,14]. The laryngeal mucosa being close to the pharynx would also be a tropism for HPV and likely to carry the anomalies specific to it. It is in this context that this present work was carried out with the aim of studying the expression of the P16 gene by immunostaining in laryngeal carcinomas in order to judge its necessity in place of a virological analysis of the oncogenic HPV infection.

Patients and Methods

This was a 10-year descriptive cross-sectional study from January 1, 2013 to December 31, 2022 carried out at the molecular pathology laboratory of the Faculty of Health Sciences in Brazzaville and at the virology and molecular biology laboratory in Pointe-black (Congo). The study material consisted mainly of biopsies of laryngeal carcinomas with histological proof. These were the samples which allowed both the carrying out of immunostaining and amplification by PCR after extraction of the HPV viral DNA. The samples excluded from the study were those which initially met the inclusion criteria but during the technical management phase of the samples a problem arose which did not allow the extraction of HPV viral DNA. nor immunostaining. The technical management of the samples was carried out in three stages: treatment of the paraffin blocks, virological analysis and immunostaining.

Treatment of paraffin blocks

Each paraffin-embedded sample was cut by a microtome at 5 µm then mounted on slides and stained with hematoxylin-eosin. A final reading was taken to confirm the diagnosis of laryngeal carcinoma before virological analysis.

Virological analysis

The objective of this analysis was to distinguish HPV-positive and HPV-negative laryngeal carcinomas using Gen Xpert technology. It made it possible to carry out the extraction and quantification of HPV-DNA in ng/ml using the ReliaPrep gDNA Tissue Miniprep System extraction kit (Promega) and Qubit 3.0 fluorescence technology (Qubit® 3.0 Fluoremeter, life technology). Finally, genotyping was carried out by real-time PCR using an Xpert® HPV kit only suitable for oncogenic HPV.

Immunostaining

The molecular investigation consisted of searching for the expression of the P16 oncogene within groups of HPV-positive and HPV-negative laryngeal carcinomas. The marking technique on paraffin sections was carried out on super Frost + slides by applying 150 µl of anti-P16 monoclonal antibody (D25) for 30 minutes. Revelation was done by applying 150 µl of visualization reagent and 150 µl of DAB+ solution before counter-revelation using 150 µl of hematoxylin for 3 minutes. Therefore, the immunohistochemical study of the P16 gene led to the use of two necessary criteria: positivity and signal intensity from 50% of the marked tissues. This reading was made using an Olympus CX 31 brand microscope and the morphological diagnoses were reviewed according to the new WHO/IARC 2017 classification. The parameters studied were epidemiological (frequency), virological (molecular prevalence of HPV) and anatomopathological (immunohistochemical profile P16). Data entry and analysis were done using Epi info version 15.5 software. The comparison of quantitative variables was made using the student test and the significance threshold was set at $p < 0.05$.

Results

Epidemiological characteristics

During the study period we collected a total of 120 samples of various ENT cancers in the pathological anatomy and cytology laboratory. Among these samples, 42 concerned laryngeal squamous cell carcinomas which met the selection criteria favorable to a molecular biology analysis and representing 35% of head and neck and head and neck cancers. The average age was 53.33 years ± 15.17 (range: 38-79 years) for a ratio of 6 in favor of men.

Virological characteristics

The extraction of oncogenic AND-HPV was successfully carried out on 14 samples (table 1) and the laryngeal squamous cell carcinomas were thus presented in two groups according to the molecular prevalence of HPV: HPV-positive carcinomas (33.3%) and carcinomas. HPV-negative (66.7%).

P16 immunostaining

The immunohistochemistry technique on paraffin sections revealed overexpression of the P16 gene in 33.3% of laryngeal carcinomas, thus defining two groups of carcinomas: P16+ carcinomas and P16 carcinomas (Table 2). The correlation between P16+ carcinomas and oncogenic HPV infection was statistically significant ($p = 0.04$) as presented in Table 3.

HPV infection	Effective	Percentage (%)
HPV-positives carcinomas	14	33,3
HPV-negatives carcinomas	28	66,7
Total	42	100

Table 1: Molecular prevalence of HPV in laryngeal carcinomas.

Phénotypes	Effectives	Percentage (%)
P16 +	14	33,3
P16 −	28	66,7
Total	42	100

Table 2: P16 phenotypes identified after immunostaining.

Virological characteristics	Phénotypes		Total	P-value
	P16 + n (%)	P16 − n (%)		
HPV-positives LC	14 (33,3)	0	14 (33,3)	0,004
HPV-negatives LC	0	28 (66,7)	28 (66,7)	
Total	14 (33,3)	28 (66,7)	42 (100)	

Table 3: P16 phenotype and virological characteristics.

n: Number; %: Percentage; LC: Laryngeal Carcinomas.

Discussion

Immunostaining is a technique used in molecular biology and using monoclonal antibodies directed against an antigenic epitope for diagnostic purposes. In the context of HPV-associated laryngeal carcinoma, the P16 protein becomes a specific and determining oncogene for which immunostaining will make it possible to identify the expression. This is why many authors consider the P16 protein as a diagnostic biomarker for oral and pharyngeal carcinomas; which would explain its strong immunoreactivity in epithelial malignant tumors [15-17]. Therefore, it seemed necessary to us to carry out P16 immunostaining in order to study the specificity of this antigenic phenotype with respect to HPV and to admit a new simple and effective diagnostic possibility. The samples used were each intended for virological tests (HPV-DNA extraction, genotyping) and immunostaining then separated into two groups according to HPV status on one side and P16 immuno-reactivity on the other. This double biological investigation led us to correlate P16 immunoreactivity with the molecular prevalence of oncogenic HPV within laryngeal carcinomas. It appears from this study that the P16+ and P16– phenotypic profiles characterize the diagnosis of laryngeal carcinomas. If in this study P16+ laryngeal carcinomas represent approximately 33.3%, however LARSEN C., *et al*. [18] report an overexpression of the P16 gene in the order of 20% much lower than ours.

According to the International Union Against Cancer (UICC) in its eighth and latest edition, the classification of oral and pharyngeal carcinomas according to oncogenic HPV infection should be based simply on the positivity of P16/ki67 marking [19]. According to this new classification, squamous cell carcinomas associated with HPV would be P16+ while those which are not associated with HPV would be P16 - thus simplifying the diagnostic method by simple immunostaining instead of virological tests such as DNA extraction - HPV and antigen amplification by PCR. In the present study the molecular prevalence of oncogenic HPV was 33.3% of all laryngeal carcinomas. This proportion of HPV-associated laryngeal carcinomas was also specifically linked to overexpression of the P16 gene ($p = 0.004$), making P16 immunostaining a simple and indirect diagnostic tool for HPV-induced laryngeal carcinomas. Indeed, when a human cell is infected with HPV, the cytoplasmic and nuclear expression of the P16 gene is very strong and diffuse within the tumor tissue [20]. Therefore, the P16 protein is considered a surrogate biomarker of persistent infection by HPV with a high oncogenic risk [21].

Conclusion

Laryngeal carcinomas associated with HPV remain common in Brazzaville. Overexpression of the P16 gene detectable by the immunostaining technique on paraffin sections is linked to persistent infection by oncogenic HPVs. The P16 gene can thus be considered as a specific diagnostic biomarker for HPV-positive laryngeal carcinomas.

Conflicts of Interest

The authors declare no conflict of interest.

Participation

- Title and introduction: Otouana Dzon HB
- Patients, methods and results: Otouana Dzon HB, Moukassa D
- Discussion and references: Otouana Dzon HB, Ondzotto GW, Ngouoni GC, Itiéré-Odzili FA.

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