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Correlations between HPV, p53 and p16 in malignancies involving the retromolar trigone–oropharynx junction

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Abstract

The aim of this study is to enhance knowledge regarding the behavior of human papilloma virus (HPV)-associated malignancies between two territories – maxillofacial and otorhinolaryngology. The HPV status and genotype, p16 and p53 expression were performed in 50 patients with malignancies located at the junction between the oropharynx and retromolar trigone alone or oropharynx spread to the junction. These were correlated with the treatment response, prognosis and survival of this kind of tumor located in oral posterior region, marking the transition between two territories (maxillofacial and otorhinolaryngology) of the selected cases. Results showed better treatment outcome and improved prognosis in HPV-positive compared to HPV-negative patients, and a strong link between HPV presence and p16 expression. Multimodal treatment including surgery, radiotherapy and chemotherapy provided the best results, although surgery was only an option in a limited number of cases, due to the advanced stage at presentation and extension in the surrounding tissues, considering the complex anatomy of the area. In the context of the increasing incidence of HPV-positive head and neck cancer, HPV testing together with molecular profiling for p16 and p53 tumor markers could help diagnose malignancies in the initial stages, and also provide important clues towards a targeted, more efficient treatment.

Keywords: HPV, squamous cell carcinoma, neoadjuvant therapy, prognosis, oropharynx.

Introduction

Cancers located at the junction between the retromolar trigone and the oropharynx have unique characteristics regarding local spread, with significant implications on the possibility of surgical treatment. Due to the posterior location and absence of obvious symptoms in the early stages, patients tend to present with locally advanced tumors. Surgical access, oncological safety and proper closure are difficult to ensure in extended tumors. For this reason, the oncological treatment becomes of outmost importance and should provide predictable positive results, and increased survival.

In the recent years, there has been increasing interest regarding the molecular profiling of head and neck cancers, in the attempt of reaching a more targeted, individualized oncological treatment that would lead to favorable outcomes and increased overall survival of patients [1].

Human papilloma virus (HPV)-related cancers are increasing in frequency and tend to involve younger patients. Increased knowledge of the mechanisms involved could not only improve diagnosis, treatment protocols, and prognosis, but it could also improve prophylaxis of the disease by the use of existing vaccines. Molecular profiling of HPV-positive head and neck cancer patients can provide explanations for the particular course of the disease and proving better prognosis of those patients. Certain tumor markers, as p53 and p16, are being increasingly studied for correlations between the behavior of HPV-positive head and neck cancer, treatment response and survival.

Being a cyclin-dependent kinase inhibitor molecule, p16 acts as a blocker of the cell cycle progression, arresting the cells in G0/G1 phase by inhibiting the retinoblastoma protein (pRb) phosphorylation [2]. On the other hand, Rb can be inactivated by E7, an HPV viral oncogene product. As a result, in HPV-associated carcinomas, p16 should be upregulated, thus overexpressed on immunohistochemistry test [3–5]. Hence, an immunohistochemical (IHC) overexpression of p16 may represent a useful marker when identifying those carcinomas associated with HPV infection, also cheaper considering that molecular detection methods of HPV are more expensive [6–10].

p53 is a transcription factor which regulates the deoxyribonucleic acid (DNA) damage and repair them. When the DNA damage is too severe and repair fails, p53 induces apoptosis. Unless mutated or inactivated, p53 is also implicated in cell cycle checkpoints. In HPV infection, E6 inhibits wild-type p53 resulting in tumor cells proliferation by deregulating cycle checkpoints [11–13]. Radiation activates p53, therefore increased levels of p53 seems to be associated with a better response to radiotherapy of HPV-positive oropharyngeal carcinomas [14–16].

The purpose of this study was to evaluate the IHC expression of p16 and p53 in carcinomas located at the junction between the retromolar trigone and the oropharynx, in correlation with prognosis and related to the treatment applied in the selected cases. The outcome of these studies could help the development of treatment protocols best suited for individual cases.

Patients, Materials and Methods

The study population consisted of 46 men and four women, aged between 39 and 78 years old, admitted in the Departments of Oral and Maxillo-Facial (OMF) Surgery and Ear, Nose and Throat (ENT – Otorhinolaryngology), in between 2011–2015. The written informed consent was obtained from all participants.

The inclusion criteria were the presence of histological confirmed oropharyngeal cancer or cancer located at the junction between the retromolar trigone and the oropharynx. Patients not able to provide informed consent due to medical comorbidities, and other types of oral and pharyngeal cancers were excluded from the study. The diagnosis and staging were established by a multidisciplinary team, including ENT, OMF surgery, pathology, radiology and oncology, on the basis of the clinical features, performed biopsy, presence of HPV and tumor markers, and the highlighting of the local spread assessed on computed tomography (CT) scanner. Each case was discussed in the Oncological Board and the treatment protocol was advised in accordance with the *National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology, Head and Neck Cancers*. HPV detection, p16 and p53 evaluation were performed in all patients.

HPV detection

The samples were collected using the HPV Screening kit from AID Diagnostika GmbH (Germany), by collecting in special tubes samples of epithelial cells from the oral cavity. For genotyping, we used the IVD kit (Opegen by Operon, Spain), allowing the genotyping of 19 HPV strains of medium and high risk.

We isolated the genomic DNA from the cytology product, which was then amplified by the multiplex polymerase chain reaction (PCR) technique, according to the Seeplex® HPV4A ACE Screening kit, or subsequently hybridized on strips from the High Papilloma Strip kit (Operon®), and the obtained product was migrated in agarose gel (2%). The HPV genotyping was performed by the qualitative (reverse blot) method and by the multiplex PCR qualitative method.

p53 and p16 immunostaining

The biopsy tissues were routinely processed by fixation in 10% neutral buffered formalin and then embedded in paraffin. Four μm thin sections were stained with Hematoxylin–Eosin (HE) for histopathological diagnosis. p53 and p16 were immunohistochemically evaluated using primary antibodies: anti-p53 monoclonal antibody (clone DO-7, Novocastra, Leica Biosystems, Newcastle-upon-Tyne, UK, 1:800 dilution, 30 minutes, at 25°C) and anti-p16 monoclonal antibody (clone G175-405, BD Pharmingen, 1:25 dilution, 60 minutes, at 25°C). The IHC technique included the following steps: deparaffinization, hydrating, exposing the antigenic sites, neutralizing the endogenous peroxidase, incubation with the primary antibody, visualization with Novolink™ Polymer Detection System, 3,3'-Diaminobenzidine (DAB) and chromogen counterstaining with Mayer's Hematoxylin. Positive [known cases of p53- and p16-positive squamous cell carcinomas (SCCs)] and negative (tonsil) controls were used. Cases were independently evaluated by three pathologists. For p16, positivity was considered when both nuclear and cytoplasmic staining was present.

This study was performed on the basis of obtained informed consent from each participant. It was approved by the Ethics Committee of "Sf. Spiridon" Hospital, Iaşi, Romania and of the "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi.

Statistical analysis

The data were analyzed with Statistical Package for the Social Sciences (SPSS) software version 24.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical tests specific to the type of categories were applied. The association of the variables was assessed on the basis of the Pearson's χ^2 (*chi*-square) or Yates *chi*-square test and the correlation between the studied aspects was based on the Spearman's rank *R*. The involvement of HPV, p53 and p16 tumor markers in retromolar trigone–oropharynx junction malignancies has been highlighted based on the results of multivariate analysis (logistic regression). The significance level for the final hypothesis decision was 0.05 (95% confidence interval).

Results

From the 50 tested patients (46 men and four women), 16 (32%) presented HPV infection (14 men and two women). The genotypes found among the detected patients were: HPV 16 (two cases), HPV 18 (one case), HPV 31 (one case), HPV 33 (two cases), HPV 51 (four cases), HPV 66 (six cases) (Figure 1).

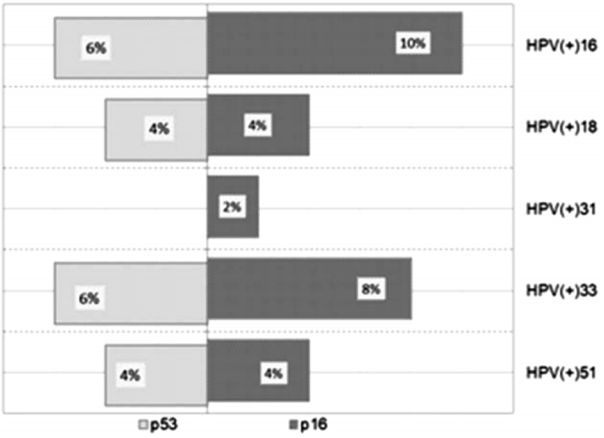

Figure 1 – ***Correlation between p53 and p16 status and HPV strands in HPV-positive cases. HPV: Human papilloma virus.***

In our study group, 32 (64%) carcinomas were p53 positive (28 men and four women) and 18 had a negative p53 status, p16 status was positive in 43 (86%) cases (39 men and four women) and negative in seven cases. All HPV-positive patients had p16-positive status, while only 11 were p53 positive.

Most SCCs (35, representing 70% of all cases) were moderately differentiated, only nine being well differentiated and six poorly differentiated (Figures 2–5).

Nine of the investigated patients underwent initial surgical treatment, of which seven were HPV-positive patients. All 50 patients underwent radiotherapy; in only 16 of them, radiotherapy was combined with chemotherapy.

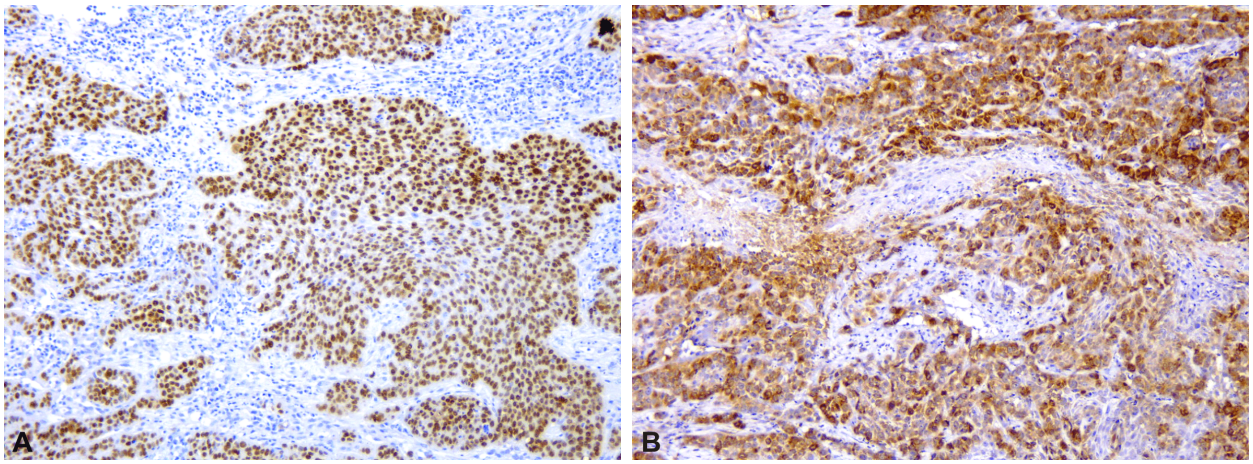

Figure 2 – ***HPV-positive moderately differentiated, invasive oral squamous cell carcinoma: (A) p53 intense and diffuse positivity in tumor cells (Anti-p53 antibody immunomarking, ×100); (B) p16 intense and diffuse positivity in tumor cells (Anti-p16 antibody immunomarking, ×100). HPV: Human papilloma virus.***

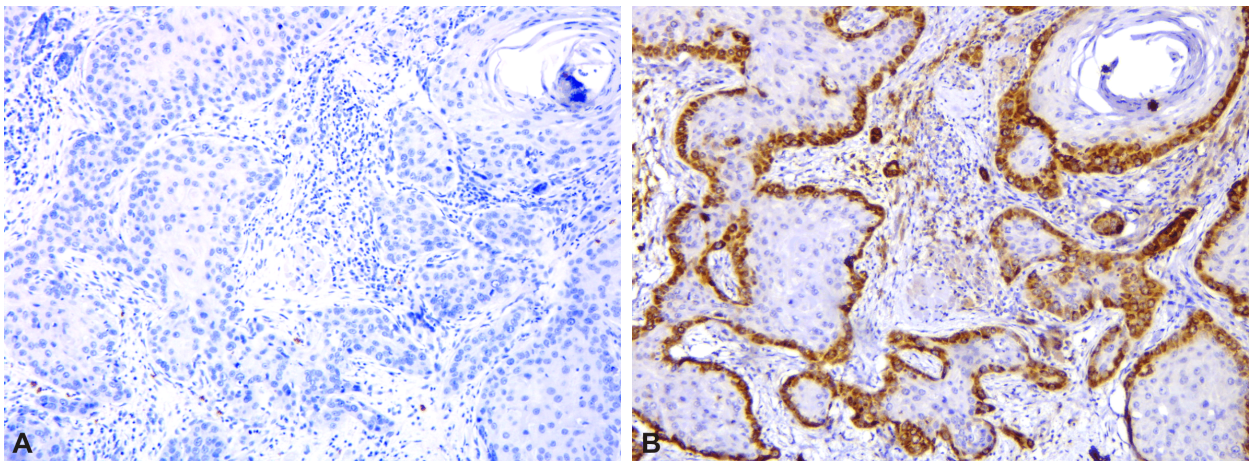

Figure 3 – ***HPV-negative well-differentiated, invasive oral squamous cell carcinoma: (A) p53 negative in tumor cells (Anti-p53 antibody immunomarking, ×100); (B) p16 intense positivity in tumor cells (Anti-p16 antibody immunomarking, ×100). HPV: Human papilloma virus.***

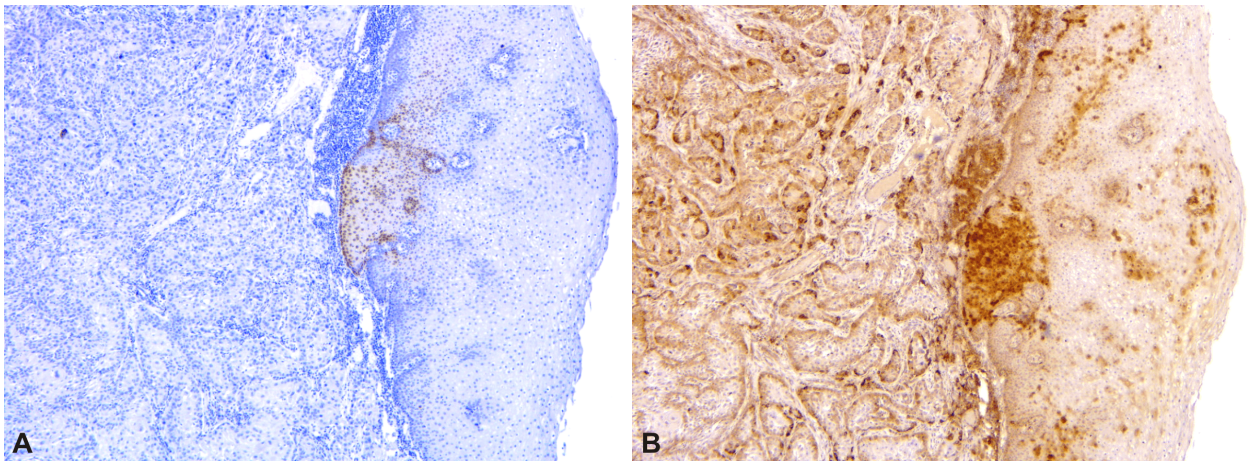

Figure 4 – ***HPV-positive well-differentiated, invasive oral squamous cell carcinoma: (A) p53 negative in tumor cells, focal positivity in basal layer of surface epithelium (Anti-p53 antibody immunomarking, ×50); (B) p16 diffuse positivity in tumor cells and basal layer of mucosa (Anti-p16 antibody immunomarking, ×50). HPV: Human papilloma virus.***

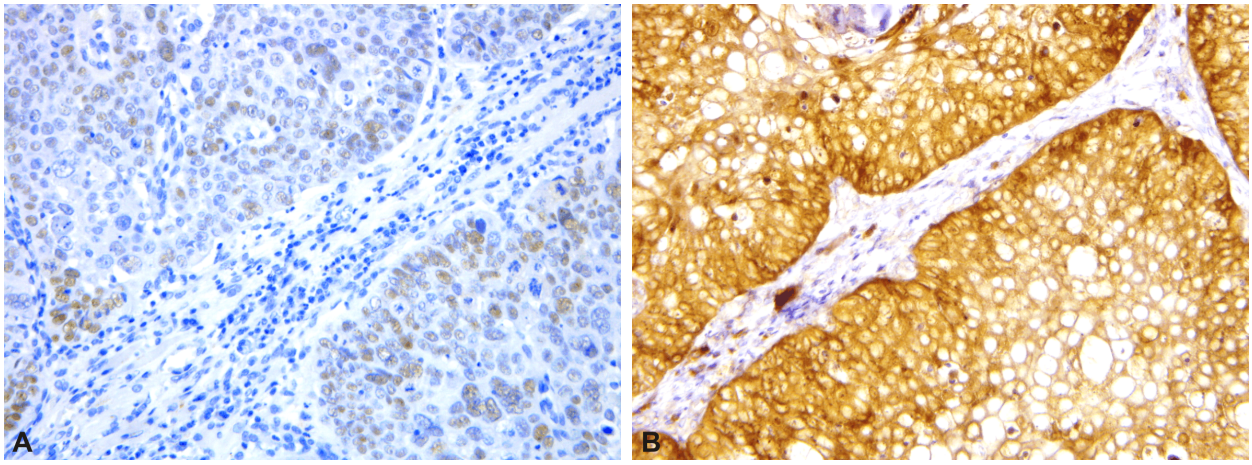

Figure 5 – ***HPV-positive moderately differentiated, invasive oral squamous cell carcinoma: (A) p53 weak and diffuse positivity in tumor cells (Anti-p53 antibody immunomarking, ×200); (B) p16 intense and diffuse positivity in tumor cells (Anti-p16 antibody immunomarking, ×200). HPV: Human papilloma virus.***

A good result was noticed in 28 cases, of which 15 were HPV-positive patients. From the whole cohort, 16 (32%) cases relapsed (one HPV-positive) and six (12%) patients died (HPV-negative) during the study interval (Table 1).

Table 1 – ***Evolution of the selected patients with cancer located at the junction between the retromolar trigone and the oropharynx or oropharynx***

Surg + RT + CHT	Good	4
	Recurrence	–
	Death	–
RT + CHT	Good	9
	Recurrence	3
	Death	–
Surg + RT	Good	5
	Recurrence	–
	Death	–
RT alone	Good	12
	Recurrence	9
	Death	8

CHT: Chemotherapy; RT: Radiotherapy; Surg: Surgery.

Best results were noticed in patients undergoing surgical treatment followed by radiotherapy or radio-chemotherapy. Patients who underwent surgical treatment had better survival rates than patients who did not. The cases treated by chemotherapy alone had lower survival rates than the ones benefiting from associated adjuvant chemotherapy and radiotherapy. Most cases with good results had moderately differentiated carcinomas and underwent either radiotherapy or chemo-radiotherapy. Recurrences and deaths were recorded in patients who underwent radiotherapy alone (Table 2).

Results following treatment were overall superior for the HPV-positive group, including postoperative outcomes. Most HPV-positive patients had good results following treatment and increased survival, as opposed to HPV-negative cases, in which recurrences and deaths had the highest frequency.

p53-positive patients (Table 3), as well as p16-positive patients (Table 4) (r=0.452, p=0.03539), had better overall results. Most p53 cases (46%) that had good results, underwent either surgery associated with radiotherapy or surgery followed by radiotherapy and chemotherapy (r=0.617, p=0.01411).

Table 2 – ***Evolution of patients according to HPV status***

		HPV							Total
		Negative	***16***	***18***	***31***	***33***	***51***	***66***	
Evolution	Good	13	2	0	1	2	4	6	28
	Recurrence	15	0	1	0	0	0	0	16
	Death	6	0	0	0	0	0	0	6
Total		*34*	*2*	*1*	*1*	*2*	*4*	*6*	*50*

HPV: Human papilloma virus.

Table 3 – ***Evolution of patients following treatment according to p53 status***

p53	Treatment	Evolution						*n* (subtotal)	Total
		Good		***Recurrence***		***Death***			
Positive	Surg + RT + CHT	3	100%	0	0%	0	0%	3	35
	RT + CHT	6	60%	4	40%	0	0%	10	
	Surg + RT	4	100%	0	0%	0	0%	4	
	RT alone	10	55.6%	6	33.3%	2	11.1%	18	
Negative	Surg + RT + CHT	0	0%	1	0%	0	0%	1	15
	RT + CHT	2	100%	0	0%	0	0%	2	
	Surg + RT	2	100%	0	0%	0	0%	2	
	RT alone	2	20%	2	20%	6	60%	10	

CHT: Chemotherapy; RT: Radiotherapy; Surg: Surgery; *n*: No. of cases; Yates *chi*-square: χ^2=12.642, p=0.049*; Spearman's rank R: r=0.617, p=0.01411*; **p*-value <0.05 – statistically significant.

Table 4 – ***Evolution of patients following treatment according to p16 status***

p16	Treatment	Evolution						*n* (subtotal)	Total
		Good		*Recurrence*		*Death*			
Positive	Surg + RT + CHT	3	100%	0	0%	0	0%	3	43
	RT + CHT	7	63.64%	4	36.36%	0	0%	11	
	Surg + RT	6	100%	0	0%	0	0%	6	
	RT alone	11	47.83%	7	30.43%	5	21.74%	23	
Negative	Surg + RT + CHT	0	0%	1	100%	0	0%	1	7
	RT + CHT	1	100%	0	0%	0	0%	1	
	Surg + RT	0	0%	0	0%	0	0%	0	
	RT alone	1	20%	1	20%	3	60%	5	

CHT: Chemotherapy; RT: Radiotherapy; Surg: Surgery; *n*: No. of cases; Pearson's *chi*-square: χ^2=10.167, *p*=0.01177*; Spearman's rank *R*: *r*=0.452, *p*=0.03539*; **p*-value <0.05 – statistically significant.

Discussions

HPV-positive tumor cells overexpress the p16 protein in a diffuse manner. This overexpression is directly related to the molecular process involved in the carcinogenesis induced by the major HPV oncogenes (E6 and E7). The p16 protein is both a key element of the negative feedback mechanism of mitosis, which is mainly aimed at promoting the inhibition by Rb of the transition to the cell cycle, and a regulator of cell growth factor [1, 17–19]. Genetic or epigenetic changes by inactivating p16, cause cancer cell growth in HPV-negative oropharyngeal cancers [20].

The involvement of HPV in oropharyngeal carcinogenesis, epidemiology of HPV and specificity of tumor localization, HPV genome expression and p16 protein expression correlates significantly.

For this reason, the IHC staining of p16 may be a surrogate marker for the presence of the HPV genome [21]. This was also true regarding our study since we found all HPV-positive cases were also p16 positive, while not all HPV-positive cases were also p53 positive.

Given the high rate of false negative results of PCR and the low sensitivity of *in situ* hybridization techniques, it is important to perform HPV testing in oropharyngeal cancers by using at least two different techniques. Therefore, it is recommended to use both the viral genome detection techniques, and techniques that show the overexpression of the p16 protein [21]. Considering the increasing number of HPV-positive head and neck cancers, also called a recent "epidemic", HPV testing should be performed routinely and screening programs should be developed for high-risk patients.

The inactivation, degradation or mutation of the *p53* gene can result in the disturbance of its functions, resulting in cellular proliferation, accumulation of defective DNA and prolonged survival of affected cells. Still, the loss of p53 function is not enough for the development of cancer. Other cytogenetic alterations are necessary to carry out the malignant transformation [22, 23]. p53 mutations with high molecular expression are involved in malignancies found in chronic smokers [18, 21, 22].

Molecular profiling can be a useful tool in determining elements relating to treatment response and prognosis with implications in treatment decision. Consistent with the outcomes of our study, HPV-positive cancer patients with a greater expression of p16 and lower p53 expression respond better to treatment and have an improved prognosis.

The most common subtype of HPV detected according to the literature is HPV 16, consisting about 90% of all HPV-positive SCCs of the head and neck [22, 24, 25]. This is not consistent with our study. Out of the 16 HPV-positive patients, only two were HPV 16 subtype. This could plead for a geographical distribution of HPV subtypes, which could help improve prophylaxis by the help of geographically specific vaccines. Further studies with larger number of cases are needed for rendering a more relevant statistical analysis regarding all factors.

In oropharyngeal squamous cell tumors, determination of baseline HPV status, demonstrated by IHC expression of $p16^{INK4a}$, and detection of HPV DNA by PCR can be used as prognostic indicators. Smoking and chronic alcohol consumption are well known, universally accepted risk factors for SCCs of the head and neck, that frequently associate to the HPV status [26, 27]. Further studies are needed to determine the role of additional risk factors in the appearance of HPV-induced tumors.

Traditionally, most cases of oropharyngeal cancer were associated with smoking and alcohol abuse. This leads to the loss of *p16* and *p53* gene mutations. The decrease in tobacco consumption coupled with an increase in HPV-positive patients changed the frequency pattern of oropharyngeal cancers, now appearing more often in non-smokers.

Additionally, patients with HPV-positive cancers tend to be younger than those with HPV-negative tumors, raising the presumption of dysfunctional sexual habits [28]. Most HPV-positive patients in our study were under the age of 60 at the time of diagnosis with the youngest patient aged 39.

HPV-positive cancers are associated with a very good survival, despite an advanced tumor stage. This is particularly important since the posterior location of oropharyngeal–retromolar trigone malignancies can lead to presentation in more advanced stages, the main reason for the reduced number of patients included in our study that underwent initial surgical resection. Best outcomes were obtained by association of surgery, radiotherapy and chemotherapy. Most HPV-positive patients had good results following treatment, including postoperative and increased survival, as opposed to HPV-negative cases in which recurrences and deaths had the highest frequency [25, 29, 30].

The risk of nodal metastasis increases with the tumor stage. Patients with p16-positive oropharyngeal tumors have been stated less likely to have persisting lymph node

metastases following chemo-radiotherapy and it was hypothesized that a cervical neck dissection can therefore be avoided [19, 20]. During the study, there was only one recurrence in an HPV-positive, p16-positive patient. This underlines the importance of molecular profiling in head and neck cancer for providing the best treatment strategy. The inclusion of high-risk HPV-positive patients into screening programs could help the diagnosis of the disease in an incipient stage with increased chances of achieving prolonged survival.

Although the number of patients included in the study was reduced, there was a clear prevalence of improved outcomes of HPV-positive subjects and good results related to the expression of p16 protein. This is consistent with data obtained from other studies [24, 31]. The current system of staging for oropharyngeal cancer should be amended to better reflect the prognosis regarding HPV status leading also to a more defined guide to the targeted treatment of these cases [31, 32].

Conclusions

In our study, we proved the involvement of HPV in the genesis of cancers located at the junction between the retromolar trigone and the oropharynx, a particular area due to its location at the border of two territories, OMF and ENT. Additionally, we found implications of HPV status, p16 and p53 expression regarding prognosis and related to the treatment applied in the selected cases. The correlations found were mostly similar to those of other studies. Subsequent research is needed for translating the information regarding molecular profiling and prognosis into relevant treatment protocols. Overall, it can be stated that the involvement of HPV in oropharyngeal malignancies showed favorable response to treatment and a good overall prognosis by the expression of p16, which can be considered a marker for identifying HPV-positive malignancies in the region of the head and neck, a common finding with other studies in this regard.

Conflict of interests

The authors have stated explicitly that there is no conflict of interests connected to this article.

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