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Original research article

Is immunohistochemical evaluation of p16 in oropharyngeal cancer enough to predict the HPV positivity?

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ABSTRACT

Aim: Our goal was to determine the expression levels of p16 in the cohort of the OPSCC patients and evaluation of the pathological and clinical differences between these two groups including patients' survival.
Background: HPV infection is the main causative factor of oropharyngeal cancer (OPSCC). Identification of HPV status in OPSCC requires positive evaluation of viral DNA integration into host cell however, p16 accumulation in the proliferating cell layers has been accepted as an alternative marker for HPV infection.
Material and Methods: The IHC staining for p16 has been performed in tumor tissue from 382 OPSCC patients. The sample was considered positive based on more than 70% of

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carcinoma tissue showing strong and diffused nuclear and cytoplasmic immunostaining. The clinicopathological characteristics of the patients including site, age, gender, tumor grade, tumor stage, the nodal status, smoking and survival have been analyzed when comparing p16 positive and p16 negative tumors.

Results: Out of our cohort in 38.2% cases positive staining for p16 has been recorded. Our analysis did not indicate significant differences in the distribution of the p16 positive patients and age of the patients, stage of the disease. Among the patients who have presented with the N+ neck, there were significantly more p16 positive tumors than in the group with N0 neck ($p = 0.0062$). There was highly significant correlation between the expression of p16 and smoking ($p < 0.0001$). The significant difference in survival ($p < 0.0001$) with more favorable prognosis in the p16 positive group has been observed.

Conclusions: Overexpression of p16 is accepted as a surrogate diagnostic marker for detecting HPV infection in oropharyngeal cancer. However, one should remember about existence of the small subgroups of p16 positive but HPV negative tumors, with relatively worse prognosis. Immunostaining for p16, however useful on everyday basis, should be complemented with other techniques in terms of reliable identification of the HPV infection.

1. Background

Despite great improvement in head and neck cancer early detection and more advanced treatment, the survival rates do not exceed 40–50% and have not significantly changed in the last decade. The tobacco smoking and alcohol consumption represent the highest environmental and behavioral risk factors for HNSCC development and progression, however, recently there is a great focus on the association between the infection of the oncogenic types of the human papilloma virus (HPV) and HNSCC often affecting non-smoking and non-drinking subjects.[1] Tumors arising from the oropharynx have been reported to be most likely developing in response to oncogenic HPV infection. Recently, the general risk of the HNSCC is slightly declining in the western world, probably in response to decreasing number of smoking individuals and the decline in hard liquor consumption. At the same time the increasing prevalence of the subgroup of the HPV related tumors of the oropharynx and oral cavity was observed. The profile of HPV-positive oropharyngeal carcinoma defined by epidemiological, clinical and genetic characteristics is markedly different than the HPV-negative.[2–4] Despite the same treatment strategies HPV positive tumors are associated with the more favorable prognosis.[5,6] There are multiple clinical studies toward improvement of the treatment approaches for the HPV-positive tumors in terms of the reduction of the site effects of the aggressive treatment. That is why sole identification of HPV virus in oropharyngeal cancer is not sufficient and requires the evidence for viral DNA integrating with affected cells DNA.[7] Most detection techniques based on polymerase chain reaction (PCR)-based provide high sensitivity, but this technique does not allow to distinguish the mere presence of HPV DNA of transient infection from biologically and clinically relevant for HNSCC development integration of the virus with host genome. Thus, some false negative results can be produced. Highly specific results can be obtained using in situ hybridization, however, this technique is known as technically demanding, expensive and unfortunately provide low sensitivity.[8]

The pRb signaling pathway is crucial in cell cycle regulation, as it is controlling the G1/S transition with cellular mechanisms involving the cyclin D1 and p16INK4A (short p16) proteins. There is evidence available that based on frequently observed inactivation of pRb pathway, it might represent one of the major mechanisms in HNSCC development. Importantly, the pRb acts at the transcriptional level negatively regulating the expression levels of p16.[9] Abnormal expression of E6 and E7 during proliferation of squamous epithelium cells might be related to virus mediated cellular transformation.[10] Binding of viral E7 oncoprotein to pRb protein leading to increase of p16 in the proliferating cell layers of the epithelium.[11,12] As a consequence, the p16 protein has been defined as an acceptable marker for the active oncogenic HPV infection.[13–16]

2. Aim

The aim of our study was the determination of the expression levels of p16 in the cohort of the oropharyngeal cancer patients and evaluation the pathological and clinical differences between these two groups including patients' survival.

3. Materials and methods

3.1. *Patients and tissue samples*

The 382 oropharyngeal squamous cell cancer patients treated in 5 different hospitals: Greater Poland Cancer Center in Poznan, HolyCross Cancer Center in Kielce and University Hospitals in Bydgoszcz, Zabrze, Łódź and Lublin were enrolled in the study. The material comprised of archival primary tumor tissues embedded in paraffin blocks. Tissues were obtained during diagnostic biopsy or surgical resection between 2002 and 2014. The 204 cases represent primary tumors located in the palatine tonsil, 45 on the soft palate and 42 on the base of the tongue. In 87 cases more than one anatomical subsite was involved due to the extent of the disease. Eighty-nine patients

Table 1 – Clinicopathological characteristics of the patients.

Parameter	Overall
Age (mean)	59
Gender	
Male	(293) 78.8%
Female	(89) 23.9%
Differentiation	
I	(37) 10.1%
II	(244) 66.3%
III	(87) 23.6%
Subsite	
Tonsil	(204) 68.9%
Tongue	(43) 14.5%
Soft palate	(49) 16.6%
Lymph nodes	
Positive	(276/380) 72.6%
Negative	(104/380) 27.4%
Stage	
I–II	(61) 16.1%
III–IV	(318) 83.9%
Smoking[a]	
Yes	(89/320) 27.8%
No	(231/320) 72.2%

[a] Missing information for 62 patients.

were female and 293 male with the mean age of 58.9 ± 9.06 years at the diagnosis. Most of our cohort of patients have presented at the advanced stage of the disease, including 318 tumors classified as stage III or IV at diagnosis while only 61 represented stage I or II. The clinical and pathological data were gathered for included patients (Table 1).

3.2. *Immunohistochemistry*

The CINtec® Histology Kit (Ventana Medical Systems, Roche) was used for p16 immunohistochemistry, following manufacturer protocol. The evaluation was performed according to the previously published criteria consistent for clinical trial eligibility criteria.[5,17,18] The sample was considered positive, when greater than 70% of carcinoma tissue was detected with strong and diffuse nuclear and cytoplasmic immunostaining for p16. Lack of reactivity or faintly diffuse was counted as p16-negative (Fig. 1). Independent assessment of two investigators was used for staining intensity and the percentage of positively stained cells for each analyzed slide.

3.3. *Survival*

There was a survival data available for 347 patients. Patients, whose death was not related to the primary disease were excluded from the analysis. The observation time varied from 2 to 70 months with a mean of 50 months.

3.4. *Statistical analysis*

Statistical analysis was performed on SAS (SAS University Edition, SAS Institute Inc., Cary, NC, USA). Univariate logistic regression analysis was performed to estimate the effect and the odds ratio of p16 positive expression according to age, gender, differentiation, presence of metastasis in lymph nodes, stage, smoking and the survival. The GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA) software was used to perform survival analysis (Mantel–Cox) and to evaluate death rate after diagnosis (in months) in p16 positive and negative patients. A Fisher exact test was used to compare the expression of p16 between tumor sites (tonsil, tongue and palate). A p value ≤ 0.05 was considered statistical significant.

4. Results

4.1. *p16 immunohistochemical evaluation*

Out of our cohort in 146 (38.2%) cases positive staining for p16 has been recorded. Remaining 236 were considered negative according to the inclusion criteria discussed in Section 2.

Fig. 1 represents immunostaining of examples samples for p16 and grading: (A) 100% of the cells stained with high intensity in both nucleus and cytoplasm indicating p16 positive tumor, (B) less than 50% of the cells stained, low intensity – considered as p16 negative, (C) less than 50% of the cells stained, medium to high intensity – p16 negative, and (D) negative staining.

4.2. *Clinicopathological characteristics*

We have compared the clinical characteristics in the two groups, p16 positive and p16 negative (Table 2). Females represented greater p16 positive group when comparing with males ($p = 0.0015$). Tumor grading was another significant discriminating factor ($p = 0.049$). There was also observed poor differentiation in p16 positive tumors, while the majority of p16 negative tumors were well differentiated.

Our analysis indicated significant difference in the distribution of the p16 positive patients and stage of tumor. However, among the patients who have presented with the N+ neck, there were significantly more p16 positive tumors than in the group with N0 neck ($p = 0.0062$). The smoking data was available for 320 out of 382 patients. Our analysis also indicated highly significant correlation between smoking and the p16 expression. Among the patients who were smoking or quit smoking not earlier than 3 years, there were relatively more p16 positive cases than in the non-smokers ($p < 0.0001$).

The anatomical subsides within the oropharynx have significantly differed in the distribution of tumor classified as p16 positive. Much more p16 positive tumors were observed in the palatine tonsil (a), comparing to the other locations within the oropharynx (b) (Table 3).

4.3. *Survival*

The Cox regression model analysis has showed a that p16 status significantly correlate with the survival of the patients affected by head and neck cancer (<0.0001) (Table 4). The analysis indicated more favorable prognosis and higher chances for survival in patients affected by p16 positive tumors (Fig. 2).

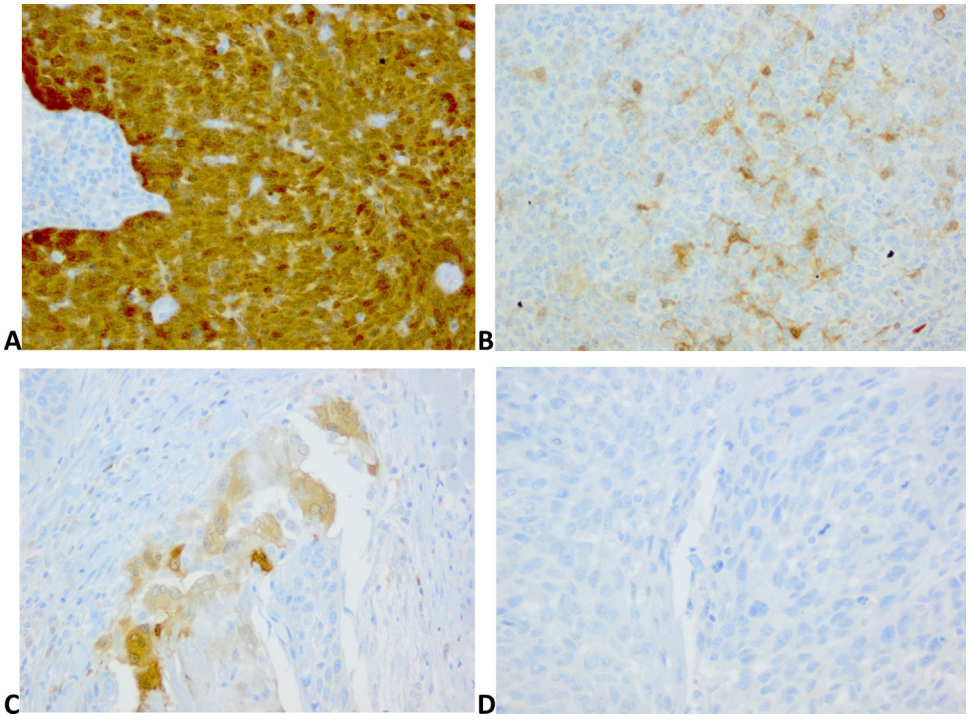

Fig. 1 – Representative examples of p16 immunostaining on 1 tumor p16 positive (A –100% of the cells stained with high intensity in both nucleus and cytoplasm) and 3 p16 negative tumors (B – less than 50% of the cells stained, low intensity, C – less than 50% of the cells stained, medium to high intensity), D – no staining.

5. Discussion

Human papilloma virus positive oropharynx tumors constitute a group of the most increasing incidence among the head and neck. They have been proved to be different from both clinical and pathological perspective and the HPV status is one of the most important predictive factor for prognosis.[1,5,12] That is why, the application of the reliable techniques allowing to stratify the OPSCC patients in terms of the tumor etiology is essential. Moreover, it affects the decision making process in treatment and follow up. The immunohistochemical analysis of p16 protein in HNSCC has been accepted as a representative marker for the HPV related cancer development and an alternative to the other laborious and expensive techniques not always available for the clinical application.[16]

In our study we performed the immunohistochemical staining for the archival material of 382 OPSCC patients treated in 5 different medical centers. The staining was performed in

Table 2 – Comparison of p16 positive and p16 negative tumors.

Parameter	p16–	p16+	*p*-Value	Odds ratio (CI)
Age	59	59	0.61	1.00 (0.98–1.03)
Gender				
Male	82.2% (194)	67.8% (99)	0.0015	2.18 (1.34–3.53)
Female	17.8% (42)	32.2% (47)		
Tumor grade (G)				
I	12.3% (28)	6.4% (9)	0.049	1.45 (1.00–2.10)
II	66.1% (150)	66.8% (94)		
III	21.6% (49)	27.0% (38)		
Lymph nodes				
N+	67.7% (159/235)	80.7% (117/145)	0.0062	1.99 (1.22–3.27)
Staging (S)				
I–II	17.6% (41)	13.7% (20)	0.32	0.75 (0.42–1.33)
III–IV	82.4% (192)	86.3% (126)		
Smoking[a]				
Yes	17.0% (34/200)	45.8% (55/120)	<0.0001	4.10 (2.45–6.87)

[a] Missing information for 62 patients.

Table 3 – The p16 expression in the subsites of the oropharynx.

Parameter	Total	p16−	p16+
Tonsil	267	52.8% (141)	47.2% (126)[a]
Tongue	55	76.4% (42)	23.6% (13)[b]
Palate	59	89.8% (53)	10.2% (6)[b]

Much more p16 positive tumors were observed in the palatine tonsil (a), comparing to the other locations within the oropharynx (b).

Table 4 – p16 and survival.

Parameter	Death rate	*p*-Value	Odds ratio (CI)
p16			
Positive	15.9% (21/132)	<0.0001	3.44 (1.99–5.94)
Negative	38.6% (83/215)		

one institution and evaluated by the experienced pathologists according to the well-established criteria.[5] Then, we investigated whether the p16 immunohistochemical status would allow to stratify the oropharyngeal cancer patients according to the clinicopathological findings such as age of the patients, gender, histological grading, the status of the neck lymph nodes, smoking the anatomical site and overall survival.

There is general trend indicating that generally younger individuals are affected by HPV positive cancer, while the pick of incidence of HPV negative cancer is in 6th decade of life.[19] In our cohort however we did not discern a significant difference in the patients' age in p16 positive and negative groups.

Most of the analyzed group of patients comprised of men 78.8%. However, the gender distribution was significantly different in p16 negative and p16 positive group. The group of female patients was relatively more numerous in p16 positive group. This finding is oppose to most of the available literature reporting that the males are more likely to be affected by p16 positive tumor when comparing with females.[20,21] The HPV positive tumors are reported to have different histological appearance than HPV negative. They are usually less differentiated with a basaloid appearance.[22–24] In our group the histological grade was found to be a significant distinctive factor ($p = 0.049$). There is available data, suggesting that the viral etiology of the OPCC may be more predictive than the TNM classification.[25] We analyzed the p16 expression status

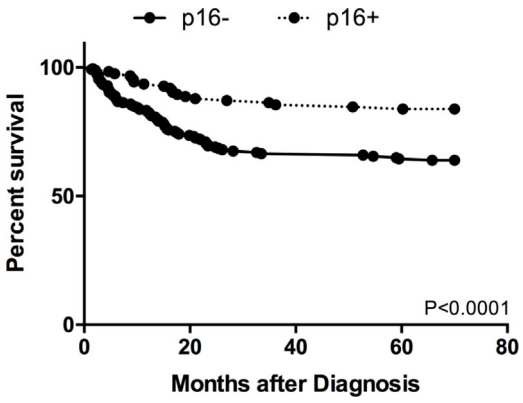

Fig. 2 – Statistical analysis Kaplan–Meier curve showing the overall survival in relation to the p16 protein expression status.

of the patients and the stage of tumor at the diagnosis, but we did not reported any essential difference. However, we found the significant difference regarding the lymph node metastases. There were significantly more patients with the N+ neck at diagnosis in the p16 positive group. The presence of the early metastases to the neck lymph nodes is commonly described to be representative for HPV positive tumors.[26,27] Metastatic mass in the neck with the unknown primary is also of more frequent prevalence in HPV positive OPSCC.[28] Due to its anatomy, the palatine tonsil is the most likely subsite to develop the HPV positive cancer in head and neck.[17,28] In patients enrolled to our study the tumors arising from palatine tonsils represented significantly greater frequency in the p16 positive group than tumors located in other sites of the oropharynx which can clearly indicate the causative role of the HPV infection.

p16 overexpression has been identified as an independent marker for prognosis in patients affected by oropharyngeal cancer by many studies.[5,29,30] We have observed the significant difference in overall survival, where the group with the p16 expression had markedly more favorable survival rates ($p < 0.0001$). This finding is in the line with the well established better prognosis of the HPV positive tumors. On the other hand there are studies clearly indicating that there is a large number of patients who develop HPV negative OPSCC with high activity of p16 (p16-positive).[16,31,32] The number of such cases varies from 15 to 20%.[33] So there is a probability that such patients are also present in our cohort and might have an impact on the survival analysis. In their studies, Rietbergen[34] and Perrone[31] have indicated that the survival rate of the HPV negative OPSCC cases with high activity of p16 positive does not differ from HPV negative patients. Interestingly Saito and colleagues analyzing the cohort of Japanese patients, proved that the p16 expression may be the independent factor for survival irrespectively from the HPV status.[35]

6. Conclusions

Overexpression of p16 is accepted as a surrogate diagnostic marker for detecting HPV infection in oropharyngeal cancer. It is easy to perform, cheap and reproducible test. However, there are several limitations including a subgroup of HPV negative tumors representing false positive outcomes in terms of p16 staining. Another group represent p16 negative but HPV positive tumors characterized by relatively worse prognosis. That is why we do believe, that the p16 immunohistochemical staining, however useful on everyday basis, should be complemented with other techniques in terms of reliable identification of the HPV infection.

Conflict of interest

None declared.

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