Clinical Research

ENT Updates 2018;8(1):13–18
doi:10.2399/jmu.2018001007

ENT updates
www.entupdates.org

Detection of human papilloma virus in normal and tumoral oropharyngeal tissue using HPV DNA in situ hybridization and p16 expression and its clinicopathologic importance

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Abstract

Objective: The rise in the number of cancer cases with human papilloma virus (HPV)-positive squamous carcinoma of the oropharynx makes the detection of HPV clinically important. We aimed to investigate the HPV positivity in our patients who have oropharyngeal cancer and compare the two different HPV detection methods, which are HPV in situ hybridization (ISH) and p16 immunohistochemistry (IHC), and show the staining patterns.

Methods: Twenty-three specimens of oropharyngeal cancer patients and ten tonsillectomy specimens that revealed no cancerous tissue (control group) were collected from retrospective file analysis. All specimens were evaluated by both p16 IHC and HPV ISH on paraffin blocks.

Results: Seven of 23 cases showed p16 expression. Of all these 7 cases that showed p16 expression, six showed high p16 expression and one showed low p16 expression. All six cases that showed high p16 expression were HPV ISH (+). One case that showed low expression of p16 was HPV ISH (-). All cases that were p16 (+) showed diffuse p16 expression and none of the cases showed focal p16 expression.

Conclusion: High p16 expression (>70%) is a reliable marker of HPV positivity. Combining p16 IHC with HPV ISH will further improve its specificity. All p16 positive cases showed diffuse p16 expression, thus did not show tumor heterogeneity, suggesting that even a biopsy specimen showing diffuse p16 expression shows p16 positivity of the whole tumoral tissue.

Keywords: Human papilloma virus, oropharyngeal cancer, p16 immunohistochemistry, in situ hybridization.

Özet: Normal ve tümöral orofaringeal dokuda in situ hibridizasyon ve p16 ekspresyonu ile human papilloma virüsü varlığının değerlendirilmesi ve klinikopatolojik önemi

Amaç: Human papilloma virüs (HPV) pozitif orofaringeal hücreli kanser olgularında son yıllarda görülen artış, bu virüsün tespitinin klinik önemini artırmaktadır. Bu çalışmada amacımız orofaringeal kanser hastalarımızın HPV pozitiflik oranlarını bulmak, farklı HPV tespit yöntemleri olan p16 immünohistokimya (IHC) ve in situ hibridizasyonunun (ISH) etkinliğini karşılaştırarak boyanma paternlerini göstermektir.

Yöntem: Retrospektif dosya taraması ile bulunan 23 hasta ve 10 kontrol çalışmaya dahil edilerek hastaların patoloji arşivinden bulunan parafin bloklarında p16 IHC ve HPV ISH çalışıldı.

Bulgular: Yirmi üç olgunun 7'si p16 pozitif idi. Bunların altısı yüksek p16 ekspresyonu gösterirken biri düşük p16 ekspresyonu göstermekteydi. 23 olgunun altısı ISH pozitif idi. Yüksek p16 ekspresyonu gösteren tüm olgular HPV ISH pozitif iken düşük ekspresyon gösteren bir olgu HPV ISH negatif idi. Tüm p16 pozitif olgular diffüz p16 ekspresyonu göstermekteydi, dolayısıyla tümör heterojenitesi göstermemekteydi.

Sonuç: Yüksek p16 ekspresyonu (>%70) HPV pozitifliğinin güvenilir bir göstergesidir ve p16 IHC ile kombine etmek spesifitesini artırmaktadır. Olgular tümör heterojenitesi göstermemekte, dolayısıyla alınan az miktarda biyopsi parçasında bile p16 ekspresyonunun gözlenmesi bize tüm tümöral dokuda p16 ekspresyonu olduğunu göstermektedir.

Anahtar sözcükler: Human papilloma virüs, orofaringeal kanser, p16 immunohistokimya, in situ hibridizasyon.

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Received: February 27, 2018; **Accepted:** March 16, 2018

Online available at:
www.entupdates.org
doi:10.2399/jmu.2018001007
QR code:

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Cancer of the head and neck is the sixth most common cancer diagnosed worldwide, with tobacco and alcohol abuse being the most established risk factors.[1,2] However, human papilloma virus (HPV) has also been shown to have a role in development of cancer of head and neck, especially in the oropharynx.[1,3–5] The number of head and neck cancers has shown a steady decrease over the recent years, whereas the number of oropharyngeal squamous cell carcinoma has shown an increase, which may be attributed to increased rates of HPV infection.[6,7] The HPV-positive squamous cell carcinoma (SCC) is different from HPV-negative SCC in that it commonly occurs in younger patients with multiple sex partners and higher exposure to oral sex, and is less related to alcohol and tobacco consumption when compared to HPV-negative SCC.[5,8] There is also data suggesting that patients who have HPV-positive oropharyngeal cancer differ from the HPV patients regarding the prognosis and survival.[9,10] Therefore, it has become even more important to detect HPV infection.

There are a couple of methods used for detection of HPV. However, the best method for detection of HPV is still controversial. HPV-positive oropharyngeal cancers have been increasing, making it increasingly important to identify the HPV status in oropharyngeal SCC. The current study aimed to examine the HPV positivity in tissues of oropharyngeal cancer and normal oropharyngeal mucosa, compare two different methods of HPV detection that were HPV in situ hybridization (ISH) and p16 immunohistochemistry (IHC), and study the relationship between the existence of HPV DNA and p16 expression in normal and cancerous oropharyngeal tissues.

Materials and Methods

Twenty-three specimens of oropharyngeal cancer patients and ten tonsillectomy specimens that revealed no cancerous tissue were retrieved from the paraffin block archives in the Department of Pathology, Hacettepe University from 1990 to 2014. The formalin-fixed paraffin-embedded tissue specimens and Hematoxylin and Eosin stained slides of tumors for each case were retrieved. The patients who had their pathologic specimens obtained in another hospital and were referred to our hospital for further treatment, patients with inconsistent or missing data, patients with any previous treatments, patients whose paraffin block was not available in the pathology archives or whose specimen was limited to do additional research were excluded. All slides of cases were re-examined for confirmation the diagnosis, and the paraffin block which included adequate tumor tissue was selected for DNA isolation. Demographics of the patients, clinical findings and the pathological characteristics of the tumor including histopathological differentiation, tumor extension, and nodal status were recorded. Ten cases in the control group were selected from the patients who had tonsillectomy for reasons other than malignancy (i.e. chronic tonsillitis, obstructive sleep apnea syndrome). The study protocol was approved by the Ethics Committee of the University and was conducted in accordance with the Declaration of Helsinki.

p16 immunohistochemistry

All specimens were examined for expression of p16 by IHC. p16 IHC was performed using a proprietary kit (Roche mtm laboratories AG, Basel, Switzerland). The positive control was determined as a squamous cell carcinoma of the tonsil with high p16 expression. Normal tonsil was used as a negative control. Staining was graded as: 0=negative; 1+=1% to 25% of cells positive; 2+=26% to 50%; 3+=51% to 75%; 4+=76% to 100%.[11]

High-risk (HR) HPV in situ hybridization

HR HPV ISH was performed using proprietary reagents (Inform HPVIII Family 16 Probe (B); Ventana Medical Systems Inc, Oro Valley, AZ, USA), which can detect high-risk HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66). Positive control was determined as a head and neck squamous cell carcinoma case that was HPV-positive, and negative control was determined as normal tonsil sections. The HR HPV ISH test was reported to be positive when there was any blue reaction product with the nuclei of malignant cells.[12]

Interpretation of HPV tests

Two head and neck pathologists independently evaluated the p16 IHC and HR HPV IHS tests. Consensus was established between pathologists in cases where results were inconsistent.

Statistical analysis

Statistical tests were performed using SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA). Descriptive statistics were used to define the characteristics of each group. The association of p16 expression with clinicopathological features was carried out by the chi-square test and Fisher's exact test, where appropriate. The statistical significance of all tests was set to a $p<0.05$.

Results

Our study included 23 patients, 21 of which were men (91%) and 2 were women (9%). All the patients were diagnosed with squamous cell cancer of the oropharynx. The mean age of the patients was 59.4 years, ranging from 41 to 74. Patient ages showed normal distribution (Kolmogorov-Smirnov test, Shapiro-Wilk test; p>0.05) Demographics of the patients, clinical findings and pathological characteristics of the tumor can be seen in **Table 1**. There was no statistically significant difference between HPV positivity and gender (Fischer's exact test, p=0.462), primary tumor site (chi-square test, p=0.519), smoking (chi-square test, p=0.283), alcohol use (chi-square test, p=0.665), T stage (chi-square test, p=0.093) and N stage (chi-square test, p=0.177).

Of all our patients with oropharyngeal SCC, 6 (26%) were HPV ISH (+). Seven of these 23 cases showed p16 expression. Of these 7 cases, six showed high p16 expression (++++) and one showed low p16 expression (++). All 6 cases that showed high p16 expression were HPV ISH (+) (**Fig. 1**). One case that showed low expression of p16 was HPV ISH (-) (**Fig. 2**). The recurrent biopsies of the HPV ISH and p16 (+) patients were also HPV ISH and p16 (+).

Six patients showed high p16 expression and were HPV ISH (+). In 4 of these patients, the tumor was localized in the tonsil and the tumor was localized in the base of tongue in two patients. All cases that were p16 (+) showed diffuse p16 expression and none of the cases showed focal p16 expression.

The mean ages of the HPV (+) and HPV (-) groups were 59.5 and 59.4, respectively, thus the ages of the two groups were similar. The data regarding smoking and alcohol use was available for 21 of the 23 cases. Of the 6 HPV (+) cases, data regarding smoking and alcohol use was available for 5 cases. Of these 5 patients that were HPV (+), 3 reported they did not smoke or use alcohol. Only one of these 5 HPV (+) cases had history of smoking

Table 1. Demographic, clinical and pathological characteristics of the patients.

No	Age (Range)	Primary	T stage	N stage	HPV ISH	p16	Smoking (PPY)	Alcohol use
1	60–70	BOT	4	2c	-	-	60	+
2	60–70	BOT	2	0	+	++++	0	+
3	50–60	BOT	3	1	-	-	60	+
4	40–50	Tonsil	2	2b	-	-	15	-
5	60–70	BOT	2	2b	+	++++	25	+
6	60–70	BOT	3	1	-	++	50	+
7	>70	Tonsil	2	0	-	-	0	-
8	>70	Tonsil	2	1	+	++++	0	-
9	40–50	Tonsil	3	2c	-	-	25	+
10	40–50	BOT	3	0	-	-	ND	ND
11	>70	BOT	3	0	-	-	0	-
12	40–50	Tonsil	4a	2c	+	++++	ND	ND
13	50–60	Tonsil	3	0	-	-	80	+
14	60–70	Tonsil	1	2a	+	++++	0	-
15	60–70	Tonsil	2	1	-	-	40	SD
16	60–70	BOT	4a	2c	-	-	20	SD
17	50–60	Tonsil	3	2b	-	-	30	-
18	60–70	Tonsil	3	2b	-	-	40	+
19	50–60	Tonsil	2	2a	+	++++	0	-
20	50–60	BOT	2	0	-	-	20	+
21	60–70	BOT	3	2c	-	-	30	-
22	>70	BOT	2	2c	-	-	80	+
23	60–70	Tonsil	2	2b	-	-	80	+

BOT: base of tongue; **No:** patient number; **ND:** no data; **PPY:** pack per year; **SD:** social drinker

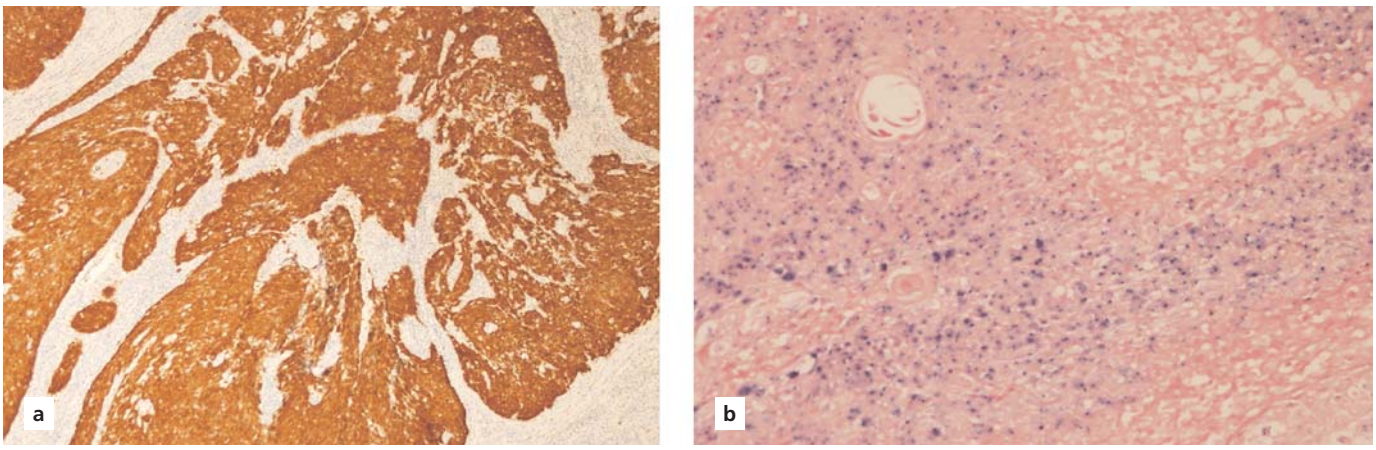

Fig. 1. Specimen of a case with oropharyngeal squamous cell cancer. (**a**) Tumor cells showing diffuse and strong nuclear and cytoplasmic p16 expression (×100 magnification). (**b**) The specimen was positive for high-risk HPV by in situ hybridization (×400 magnification). [Color figure can be viewed in the online issue, which is available at www.entupdates.org]

and this case smoked 25 PPY (pack per year). The nonsmokers were more common in the HPV (+) group.

Six cases reported that they did not smoke, and 4 of these cases were HPV (+). Fifteen cases reported that they smoked 43.6 PPY on average.

The age of the HPV16-positive group was similar to that of the HPV16-negative group (mean 59.5 vs. 59.4 years).

The control group comprised of 10 cases with normal oropharyngeal tissue without evidence of any malignancy. The mean age of the control group was 37.5 years, ranging from 25 to 55. Five cases (50%) in the control group were female and 5 (50%) cases were male.

Discussion

Squamous cell cancer of the oropharynx has increased significantly over recent years.[6,7] Tural et al. demonstrated a continuous increase in the proportion of HPV positive oropharyngeal squamous cancer from 33% between 1996 and 1999 to 70% between 2008 and 2011 in Turkey.[13] This increase makes detection of HPV status clinically important in our country, like the other countries. HPV status also has been shown to be important in oropharyngeal positive SCC (OPSCC) regarding prognosis and survival.[9,10]

There are a couple of methods to detect HPV, each with different sensitivity and specificity.[14,15] Strong association

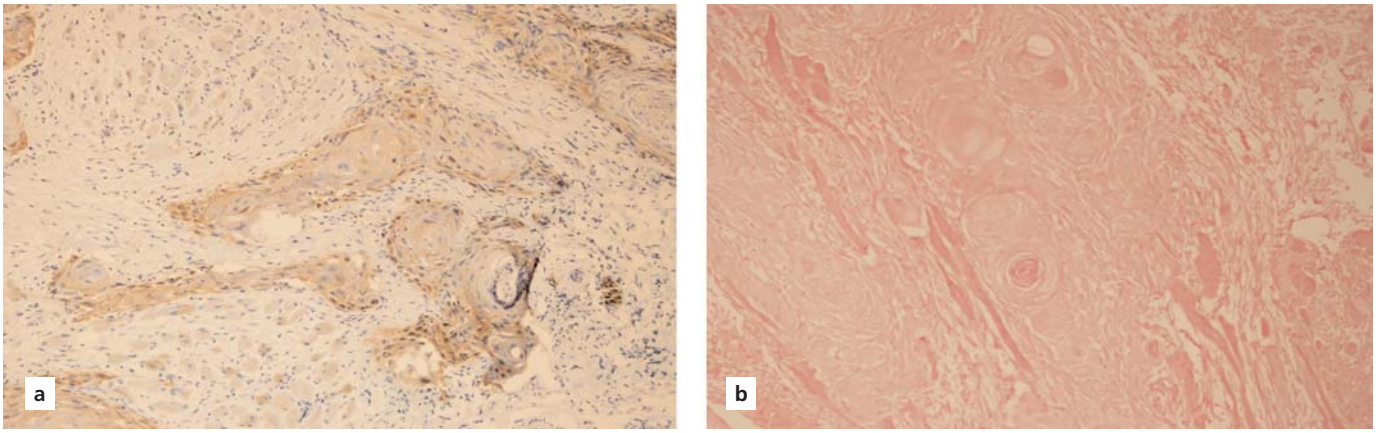

Fig. 2. Specimen of a case with oropharyngeal squamous cell cancer. (**a**) Tumor cells showing low p16 expression (×200 magnification). (**b**) The specimen was negative for high-risk HPV by in situ hybridization (×400 magnification). [Color figure can be viewed in the online issue, which is available at www.entupdates.org]

has been reported between detection of the integrated HPV and over-expression of p16 protein, thus it was suggested that p16 immunohistochemistry could be an alternative procedure for detection of HPV in clinical practice for patients with oropharyngeal carcinoma; as it was simple, inexpensive and highly sensitive.[16–18] It was reported that p16 IHC is a better alternative for detection of HPV mRNA.[19] OPSCC cases were suggested to be screened using p16 IHC, and if the case was p16 positive, then ISH can be used.[20] However, some studies suggest that no procedure like p16 IHC should be used as an alternative to the more reliable diagnostic procedures, based on HPV nucleic acid detection.[21]

The purpose of this study was therefore to compare different HPV detection methods, particularly p16 immunohistochemistry and in situ hybridization, and show the staining patterns.

The proportion of HPV (+) cases in our study was 26% (6/23). This is lower than the current literature, which demonstrates that HPV may account for 70–80% of oropharyngeal squamous cell carcinoma.[22,23] One reason suggested by several studies could be that oropharyngeal HPV infection may be acquired sexually.[24,25] Our community is conservative, therefore this could be attributed to rarity of high-risk sexual behavior. Besides, our study sample is small which could have an impact on the results.

History of tobacco use was another factor related to with HPV status. Our cases with HPV infection tended not to have a history of smoking. Six cases were HPV ISH (+) and 5 of these cases had data regarding smoking and alcohol history. Of these 5 HPV (+) cases, 4 were non-smokers. Only one of these 5 HPV (+) cases had history of smoking and these cases smoked 25 PPY. Compared to the average tobacco consumption of the smoking cases (43.6 PPY), this number is well below the average PPY. Besides, in total, 6 cases had no history of smoking and 4 of these cases (67%) were HPV (+). All these findings are consistent with the literature, as HPV (+) squamous cell carcinoma is less strongly associated with alcohol and tobacco use compared with HPV-negative SCC.[5,8]

The age of the HPV16-positive group was similar to that of the HPV16-negative group (mean 59.5 vs. 59.4 years). However, it is stated in the literature that HPV (+) tumors are more likely to be seen in younger population.[4,12,22] This difference might result from the small number of our study population.

In the literature, oropharyngeal cancers that are HPV positive were reported to be more likely to present with an early T stage, but relatively advanced involvement of the lymph nodes.[4,12,22] In our study, the HPV (+) patients were more likely to be present with early T stage but relatively advanced stage, consistent with the literature, although this was not statistically significant.

Our study showed that almost all of the specimens (6/7) that were p16 (+) on IHC were HPV ISH (+). The amount of HPV (+) / p16 (-) specimens was 0%, like in similar studies.[17] Only one specimen (1/7), which was p16 (+), was HPV ISH (-) (14%). This specimen was weak p16 (+). The amount of HPV (-) / P16 (+) tumors differ in the literature, changing from 5% to 20.45%.[6,14,17] The result of our study (14%) is in this range. However, we would like to point out that the p16 (+) specimen that was HPV ISH (+) was noted to show low p16 expression on IHC. There are also studies that classify >70% staining as high p16 (+). According to this classification, again, 6 of the 7 p16 (+) specimens were classified as high p16 (+), thus our results did not change according to different classifications among different studies.[21] Therefore, it can be concluded that high p16 expression is a reliable marker of HPV positivity. Additional tests are needed only if the specimen shows low p16 expression, as this would be much more cost-effective. IHC is a simple test, with low cost and high sensitivity.[16,18] p16 IHC can be combined with PCR or ISH to improve its specificity as a test.

A cancerous tissue might exhibit distinct molecular features, known as tumor heterogeneity, resulting in resistance to treatment.[26] However, in our study, all our cases that were p16 positive showed diffuse p16 expression, thus did not show tumor heterogeneity. This is particularly important. This would suggest that even a biopsy specimen showing diffuse p16 expression shows p16 positivity of the whole tumoral tissue.

Our study has a few limitations, one of which is the retrospective design of the study. All clinical and pathological data were collected from patient charts, which may decrease the reliability of the data. In addition, the number of our study population is small and the number of female cases is much lower than the male cases (2 female and 21 male patients). Lastly, it is reported that paraffin-embedded specimens are poorer in detection of HPV compared to fresh specimens.[16]

Conclusions

High p16 expression is a reliable marker of HPV positivity. IHC is a simple test, with low cost and high sensitivity. Combining p16 IHC with HPV ISH will further improve

its specificity as a test. The specimens did not show tumor heterogeneity, suggesting that even a biopsy specimen showing diffuse p16 expression shows p16 positivity of the whole tumoral tissue. Studies with larger number of patients would be beneficial to show the value of p16 expression and IHC for detection of HPV.

Conflict of Interest: No conflicts declared.

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Please cite this article as: Önerci Çelebi Ö, Şener E, Aydın MÇ, Güler Tezel G. Detection of human papilloma virus in normal and tumoral oropharyngeal tissue using HPV DNA in situ hybridization and p16 expression and its clinicopathologic importance. ENT Updates 2018;8(1):13–18.