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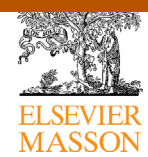

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Original article

# Study of the concordance between p16 immunohistochemistry and HPV-PCR genotyping for the viral diagnosis of oropharyngeal squamous cell carcinoma

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ABSTRACT

*Objective:* The diagnosis of HPV-related oropharyngeal cancer in clinical practice is based on p16 immunohistochemistry and PCR detection of viral DNA (HPV-PCR). The primary objective of this study was to evaluate the concordance between these 2 diagnostic tests. The secondary objective was to study the clinical characteristics of these patients.
*Materials and methods:* This single-centre prospective study was conducted between February 2010 and July 2012. Immunohistochemical analysis of p16 and HPV-PCR were performed on tumour biopsies. Concordance was evaluated according to Cohen's kappa coefficient and was interpreted according to the Landis and Koch scale. The patients' clinical data were analysed as a function of the diagnostic test results.
*Results:* Seventy-one patients were included in this study. The prevalence of HPV was 43.7% according to p16 and 31% according to HPV-PCR. The concordance study revealed a kappa coefficient of 0.615. A tumour of the tonsil or base of the tongue was detected in 100% of p16+/HPV-PCR+ cases. Smoking and alcohol abuse were significantly less frequent among HPV+ patients regardless of the method of detection. These patients were older and presented tumours with a lower grade of histological differentiation.
*Conclusion:* p16 immunohistochemistry or HPV-PCR used alone appear to be insufficient. These results confirm the high prevalence of HPV-related oropharyngeal squamous cell carcinoma (OSCC) and the previously reported specific clinical and histological features, apart from age. It appears essential for future clinical trials to be stratified according to smoking and tumour HPV status, defined by means of reliable virological tests targeting E6/E7 mRNA and no longer a simple positive response to the p16 marker, as is frequently the case at the present time. New tests suitable for use in routine practice therefore need to be developed.



## 1. Introduction

The association between upper aerodigestive tract squamous cell carcinomas and human papilloma virus (HPV) infection has been demonstrated since 1983 [1]. Subsequent epidemiological studies have demonstrated a growing predominance of HPV infection in oropharyngeal squamous cell carcinomas (OSCC), particularly involving the tonsils. In the USA, the incidence of HPV-positive OSCC increased by 225% between 1984 and 2004 while that of HPV-negative OSCC decreased by 50% over the same period [2]. In 2011, the prevalence of HPV-positive OSCC in France was 46.5% [3].

HPV-positive OSCC have a better prognosis than HPV-negative OSCC. It has been demonstrated that HPV infection is an independent prognostic factor, even in smoking patients, with a 58% reduction of the risk of death [4]. This difference can be mainly explained by different oncogenetic mechanisms. A meta-analysis published in 2013 demonstrated a significant difference in terms of overall survival and recurrence-free survival in favour of patients with HPV-positive OSCC treated by radiotherapy with or without chemotherapy and/or surgery [5]. Although changes to

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conventional treatment protocols may be indicated in this particular patient category in the future, the modalities of these changes have not yet been defined. At the present time, it is not recommended to modify treatment decisions as a function of HPV status.

Before considering a possible modification of treatment protocols, a reliable virological diagnosis that can be performed in routine practice is absolutely necessary in order to accurately identify these patients [6]. Various HPV diagnostic methods are currently available. The gold standard accepted by most authors is detection of viral oncoprotein E6 and E7 mRNA by quantitative PCR, which confirms the presence of transcriptionally active virus [7,8]. However, this test is still expensive and difficult to perform routinely. Detection of p16 overexpression is the most widely used test because it is the least expensive and the easiest to perform [6]. However, this test has a sensitivity of 94% but an insufficient specificity of 82% [9]. Detection of HPV DNA by in situ hybridization or PCR are more direct methods allowing characterization of the integrated or episomal status of the virus and identification of the serotypes involved by genotyping, but routine use of these tests remains limited.

The primary objective of this study was to analyse the concordance between the two diagnostic tests performed routinely in our institution (detection of protein p16 overexpression by immunohistochemistry and detection of HPV DNA by PCR with genotyping) on a population of patients referred for OSCC. The secondary objective was to more clearly define the clinical characteristics of this population of patients with HPV-positive OSCC.

## 2. Materials and methods

This single-centre prospective study was conducted between February 2010 and July 2012 in the Otorhinolaryngology and Head and Neck Surgery departments. All patients referred with histologically confirmed OSCC were included. Biopsies were performed during upper aerodigestive tract panendoscopy under general anaesthesia. Two tumour biopsies were systematically performed. The first biopsy was fixed in 10% formalin and was sent to the Pathology laboratory for histological and immunohistochemical examination. The second biopsy was placed in RNAlater® (Ambion) and was sent to the Virology laboratory, where it was stored at 4 °C.

The following parameters were recorded for each patient: age, gender, history of smoking, which was considered to be positive for ongoing smoking or smoking cessation for less than 5 years greater than or equal to 10 pack-years and a history of alcohol abuse, considered to be positive for an alcohol consumption greater than 30 g/day in men and 20 g/day in women. The anatomical tumour site, cTNM stage and grade of histological differentiation were also recorded. p16 and HPV-PCR status was determined from the tests performed.

### 2.1. Determination of p16 status

Fixed tissues were paraffin-embedded then stained with standard haematoxylin-eosin stain for morphological diagnosis. Immunohistochemistry screening for protein p16 was systematically performed using the p16CINK4 murine monoclonal antibody (CINtec Technology Histology Kit clone E6H4) on a Dako Autostainer Plus® automat (Dakocytomation) with antibody incubated for 12 hours, then revealed by the Envision® revelation system (Dakocytomation). A negative control for p16 omitting the primary antibody was performed for each test. Slides were examined by the pathologist to evaluate labelling. Labelled samples were evaluated according to a binary classification system left to the pathologist's discretion, comprising "positive" and "negative" characteristics.

### 2.2. Determination of HPV-PCR status

Biopsies were first treated in 20 mg/mL SDS-proteinase K buffer at 56 °C for 2 hours until complete digestion of the biopsy. Nucleic acid extraction was performed on a MagNA Pure Compact® automat (Roche Diagnostics) using the kit Pure MagNA Total LC Nucleic Acids Insulation Kit® (Laboratory Roche Diagnoses). After extraction, DNA was quantified by UV spectrophotometry (NanoDrop™, Thermo Scientific). Viral DNA detection and PCR HPV genotyping were performed with the INNO-LIPA HPV Genotyping Extra System® kit (Innogenetics) and the results were interpreted with LiRAS software. This technique allows the detection of 28 genotypes: 18 high-risk HPV (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82), 7 low-risk HPV (6, 11, 40, 43, 44, 54, 70) and 3 indeterminate-risk genotypes (69, 71, 74).

### 2.3. Statistical analysis

Cohen's kappa correlation coefficient was calculated between the 2 tests studied with interpretation on a Landis and Koch scale: between 0 and 0.20: very low concordance; between 0.21 and 0.40: low concordance; between 0.41 and 0.60: moderate concordance; between 0.61 and 0.80: satisfactory concordance; between 0.81 and 1: excellent concordance. Differences between groups were evaluated by $Chi^2$ test or Fisher's exact test for categorical variables and Student's *t*-test for continuous variables. All statistical tests were two-tailed and a *P*-value < 0.05 was considered statistically significant. Statistical analysis was performed with SPSS Statistics 19.0 software (IBM Inc.).

## 3. Results

Seventy-one patients were included in the study. Patient characteristics are described in Table 1. Protein p16 overexpression was detected in 31 patients (43.7%). This p16+ group presented a significantly lower incidence of smoking and alcohol abuse ($P < 0.001$). A significant predominance of tumours of the tonsils and base of the tongue was observed in this group ($P = 0.008$). HPV-PCR identified a high-risk HPV in 22 patients (31%): HPV-16 in 20 cases, corresponding to a prevalence of 28.2%, and HPV-33 in 2 cases. A low-risk HPV-6 was detected in 1 case. Smoking and alcohol abuse were significantly less frequent in the HPV-PCR+ group ($P = 0.006$ and $P = 0.002$, respectively). A tumour of the tonsil or base of the tongue was detected in 100% of HPV-PCR+ cases ($P = 0.001$). All clinical characteristics of the various groups according to their p16 or HPV-PCR status are described in Table 2. These 2 tests were concordant in 20 patients (81.7%) and discordant in 13 patients (19.3%). The estimated kappa correlation coefficient was 0.615, indicating a satisfactory concordance according to the Landis and Koch scale.

The characteristics of p16+/HPV-PCR+ patients were compared to those of the rest of the population. The mean age of p16+/HPV-PCR+ patients was higher than that of the rest of the population (62.8 vs. 55.5 years; $P = 0.008$). In contrast, smoking (55% vs. 88.2%; $P = 0.002$) and alcohol abuse (20% vs. 68.6%; $P < 0.001$) were less frequent in this group. All patients (100%) of the p16+/HPV-PCR+ group presented a tumour of the tonsil or base of the tongue versus 64.7% of the rest of the population ($P = 0.002$). This difference in terms of tumour site was even more significant when the p16+/HPV-PCR+ group was compared to the p16-/HPV-PCR− group ($P < 0.001$).

Patient characteristics according to their combined p16 and HPV-PCR status are presented in Table 3. Two of the patients with discordant p16 immunohistochemistry and HPV-PCR results were p16-/HPV-PCR+ and 11 were p16+/HPV-PCR−. The characteristics of p16+/HPV-PCR− patients were compared to those of

**Table 1**
Clinical characteristics of all patients included.

| | Total $n = 71$ |
|---|---|
| Mean age (years) | 57.5 |
| Gender | |
| Male | 54 (76.1%) |
| Female | 17 (23.9%) |
| Chronic smoking | 56 (78.9%) |
| Chronic alcohol abuse | 39 (54.9%) |
| Site | |
| Tonsil | 27 (38%) |
| Base of tongue | 23 (32.4%) |
| Vallecula | 7 (9.9%) |
| Posterior pharyngeal wall | 7 (9.9%) |
| Soft palate | 4 (5.6%) |
| Glossotonsillar sulcus | 3 (4.2%) |
| Tumour grade | |
| G1 well-differentiated | 23 (32.4%) |
| G2 moderately-differentiated | 35 (49.3%) |
| G3 poorly-differentiated | 13 (18.3%) |
| cT stage | |
| T1 | 6 (8.5%) |
| T2 | 29 (40.8%) |
| T3 | 15 (21.1%) |
| T4a | 18 (25.4%) |
| T4b | 3 (4.2%) |
| cN stage | |
| N0 | 10 (14.1%) |
| N1 | 6 (8.5%) |
| N2a | 4 (5.6%) |
| N2b | 24 (33.8%) |
| N2c | 27 (38%) |
| N3 | 0 (0%) |
| cM stage | |
| M0 | 68 (95.8%) |
| M1 | 3 (4.2%) |

p16+/HPV-PCR+ patients. A statistically significant difference was observed between the 2 groups in terms of tumour site, with 27.3% of sites other than the tonsil or base of the tongue in the discordant group ($P = 0.037$). Two p16-/HPV-PCR+ patients were smokers with a smoking history of 50 and 70 pack-years and an alcohol consumption of 80 and 40 g/day, respectively.

## 4. Discussion

The results of this study show that the two diagnostic tests performed routinely in our institution were not sufficiently concordant, and that the use of only one of these tests would be inadequate to reliably confirm the viral origin of the carcinoma. With these limitations in mind, our results confirm the high prevalence of HPV-positive OSCC and the clinical and histological characteristics specific to this subpopulation of patients.

p16 overexpression detected by immunohistochemistry reflects altered functioning of pRb induced by viral oncoprotein E7. This altered functioning may also be secondary to a mutation of the pRb gene independent of HPV infection. In 2012, Thomas also suggested the possibility of p16 overexpression unrelated to pRb [10]. This test therefore presents a risk of false-positive results, which can explain the 11 discordant patients observed in the p16+/PCR− group. Another hypothesis to explain these results would be the failure of PCR to detect viral DNA, which could be explained by non-recognition of the L1 sequence of the viral genome targeted by complementary primers used for PCR. HPV would no longer be detected in tumours in which genomic progression has resulted in loss of identifiable viral DNA sequences [11]. In a retrospective study of 108 patients, Schache et al. also reported these discordances with 7% of p16+/HPV-PCR− patients [9].

PCR genotyping is a very sensitive technique, as it is able to detect very small quantities of viral DNA, but it has a limited specificity to identify the aetiopathogenesis of these tumours. PCR can demonstrate simple HPV infection even in the absence of integration of the HPV genome with expression of E6 and E7. The high sensitivity of PCR could explain its ability to detect viral DNA contamination of the sample during sample processing [9,10]. Such contamination could explain the results observed in 2 patients of the p16-/HPV-PCR+ group. The hypothesis of false-positive results for these 2 patients is all the more likely in that they were both heavy smokers and drinkers.

In our study, the statistical concordance between these 2 tests was considered to be satisfactory according to the Landis and Koch

**Table 2**
Clinical characteristics of the patients as a function of p16 or HPV-PCR status.

| | p16+ $n = 31$ (43.7%) | p16− $n = 40$ (56.3%) | *P* | HPV-PCR+ $n = 22$ (31%) | HPV-PCR− $n = 49$ (69%) | *P* |
|---|---|---|---|---|---|---|
| Age (years) | 58.71 | 56.6 | *NS* | 63.05 | 55.04 | *0.007* |
| Smoking | 18 (58.1%) | 38 (95%) | *<0.001* | 13 (59.1%) | 43 (87.8%) | *0.006* |
| Alcohol | 7 (22.6%) | 32 (80%) | *<0.001* | 6 (27.3%) | 33 (67.3%) | *0.002* |
| Tumour site | | | | | | |
| Anterior/lateral oropharynx[a] | 28 (90.3%) | 25 (62.5%) | *0.008* | 22 (100%) | 31 (63.3%) | *0.001* |
| Other sites | 3 (9.7%) | 15 (37.5%) | | 0 (0%) | 18 (36.7%) | |
| Tumour grade | | | | | | |
| G1/G2 | 21 (67.7%) | 37 (92.5%) | *0.007* | 15 (68.2%) | 43 (87.8%) | *0.049* |
| G3 | 10 (32.3%) | 3 (7.5%) | | 7 (31.8%) | 6 (12.2%) | |
| cTNM stage | | | | | | |
| T | | | | | | |
| T1/T2 | 18 (58.1%) | 17 (42.5%) | *NS* | 11 (50%) | 24 (49%) | *NS* |
| T3/T4 | 13 (41.9%) | 23 (57.5%) | | 11 (50%) | 25 (51%) | |
| N | | | | | | |
| N0 | 2 (6.5%) | 8 (20%) | *NS* | 1 (4.5%) | 9 (18.4%) | *NS* |
| N+ | 29 (93.5%) | 32 (80%) | | 21 (95.5%) | 40 (81.6%) | |
| M | | | | | | |
| M0 | 30 (96.8%) | 38 (95%) | *NS* | 22 (100%) | 46 (93.9%) | *NS* |
| M1 | 1 (3.2%) | 2 (5%) | | 0 (0%) | 3 (6.1%) | |

HPV: human papilloma virus; *NS*: non significant.
[a] Anterior and lateral oropharynx comprises palatine tonsil, base of the tongue and glossotonsillar sulcus.

**Table 3**
Patient characteristics as a function of combined p16 and HPV-PCR status.

| | p16+ HPV-PCR+ $n = 20$ (28.2%) | p16- HPV-PCR− $n = 38$ (53.5%) | $P$[b] | p16+ HPV-PCR− $n = 11$ (15.5%) | $P$[c] | p16- HPV-PCR+ $n = 2$ (2.8%) |
|---|---|---|---|---|---|---|
| Age (years) | 62.8 | 56.13 | *0.15* | 51.27 | *0.17* | 59 |
| Smoking | 11 (55%) | 36 (94.7%) | *0.001* | 7 (63.6%) | *NS* | 2 (100%) |
| Alcohol | 4 (20%) | 30 (78.9%) | *<0.001* | 3 (27.3%) | *NS* | 2 (100%) |
| Tumour site | | | | | | |
| Anterior/lateral oropharynx[a] | 20 (100%) | 23 (60.5%) | *0.001* | 8 (72.7%) | *0.037* | 0 (0%) |
| Other sites | 0 (0%) | 15 (39.5%) | | 3 (27.3%) | | 2 (100%) |
| Tumour grade | | | | | | |
| G1/G2 | 13 (65%) | 8 (72.7%) | *0.23* | 2 (100%) | *NS* | 35 (92.1%) |
| G3 | 7 (35%) | 3 (27.3%) | | 0 (0%) | | 3 (7.9%) |
| cTNM stage | | | | | | |
| T | | | | | | |
| T1/T2 | 10 (50%) | 8 (72.7%) | *NS* | 1 (50%) | *NS* | 16 (42.1%) |
| T3/T4 | 10 (50%) | 3 (27.3%) | | 1 (50%) | | 22 (57.9%) |
| N | | | | | | |
| N0 | 1 (5%) | 1 (9.1%) | *NS* | 0 (0%) | *NS* | 8 (21.1%) |
| N+ | 19 (95%) | 10 (90.9%) | | 2 (100%) | | 30 (78.9%) |
| M | | | | | | |
| M0 | 20 (100%) | 10 (90.9%) | *NS* | 2 (100%) | *NS* | 36 (94.7%) |
| M1 | 0 (0%) | 1 (9.1%) | | 0 (0%) | | 2 (5.3%) |

HPV: human papilloma virus. The p16-/HPV-PCR+ group was not compared to the other groups because of its small sample size; *NS*: non significant.
[a] Anterior and lateral oropharynx comprises palatine tonsil, base of the tongue and glossotonsillar sulcus.
[b] Comparison p16+/HPV-PCR+ vs. p16-/HPV-PCR−.
[c] Comparison p16+/HPV-PCR+ vs. p16+/HPV-PCR−.

scale (kappa correlation coefficient of 0.615). However, this concordance appears to be insufficient in view of the prognostic and potential therapeutic implications, with 18.3% of discordant results between p16 and HPV-PCR. In 2010, Ang also reported discordances between these two tests, with a correlation coefficient slightly higher than 0.8 [4]. The definitive diagnosis of HPV-related OSCC therefore clearly cannot be based on only one of these 2 tests. The gold standard accepted by most authors is detection of viral oncoprotein E6 and E7 mRNA. Smeets et al. were the first to evaluate a diagnostic algorithm using detection of p16 overexpression and PCR genotyping by comparing these methods to the gold standard. They reported 100% specificity and sensitivity with the following algorithm: p16 immunohistochemistry for all tumours then HPV-PCR for p16 positive tumours [7]. Schache et al. reported a sensitivity of 97% and a specificity of 94% for the p16/HPV-PCR combination compared to PCR detection of E6 mRNA [9].

Based on the positivity of these 2 tests, the prevalence of HPV-related OSCC in our study would be 28.2%. In 2011, in a French multicentre series of 523 patients, Lacau St Guily et al. reported a prevalence of 46.5% based exclusively on PCR [3]. In the meta-analysis published by Kreimer et al., based on 27 studies (969 patients) all over the world, the prevalence of HPV-positive OSCC according to PCR was 35.6% [12]. In contrast, in a prospective series of 68 cases of OSCC derived from Latin America, a region in which smoking remains the leading aetiological factor, Ribeiro et al. reported a prevalence of 4.4% by PCR [13]. The prevalence of HPV-positive OSCC is therefore largely influenced by the diagnostic methods used and the study population.

In our study, the clinical characteristics of p16+/HPV-PCR+ patient were remarkable from several points of view. Firstly, the tumour was systematically situated in the palatine tonsil or lingual tonsils (base of the tongue). This predominance has already been reported in many studies, regardless of the diagnostic test used [2,14,15]. Some authors have proposed the hypothesis that tonsillar crypts may promote retention of viral particles [16]. Secondly, these patients presented a lower rate of smoking (55% versus 94.7%). It has been clearly demonstrated that smoking constitutes an oncogenic factor independent of HPV infection [4,17]. In 2010, Ang showed that the response to treatment of HPV-positive OSCC decreased with increasing levels of smoking, thereby creating intermediate risk profiles, although they were unable to define a critical threshold [4]. Smoking induces specific genetic changes, which modify the tumour response to treatment. Our study comprised a limited description of smoking, preventing any subgroup analysis according to the level of smoking. Thirdly, these patients were older, which is in contradiction with most of the results published in the literature [2,4,9]. This discordant result can be explained by the specific geographic origin of our population. These results emphasize that the viral origin of OSCC must be systematically considered, regardless of the patient's age and smoking status. Finally, these patients' tumours presented significant histological differences in terms of grade of differentiation, with less well-differentiated tumours. The higher frequency of poorly-differentiated tumours could be related to the specific nature of the tonsillar crypt epithelium particularly targeted by HPV compared to the epithelium of the rest of upper aerodigestive tract [16].

## 5. Conclusion

p16 immunohistochemistry or PCR detection of HPV viral DNA combined with genotyping give discordant results. These techniques are insufficient when used alone to reliably confirm the viral origin of an OSCC. The combined use of these two tests would be more reliable, but cannot eliminate all ambiguity. It appears essential for future clinical trials to be stratified according to smoking status and tumour HPV status, which must be defined by reliable virological tests targeting E6/E7 mRNA and no longer simply based on positivity of the p16 marker, as is all too frequently the case. New tests need to be developed before considering a possible modification of conventional treatment regimens for patients with HPV-positive OSCC.

## Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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