HPV-related Oropharyngeal Squamous Cell Carcinoma: $p16^{INK4A}$ Immunohistochemistry or HPV Genotyping?

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Abstract. *Background: Infection with high-risk human papillomavirus (HPV) is linked to a sub-group of squamous cell oropharyngeal tumors (OPSCC). Our aim was to compare an HPV Polymerase Chain Reaction (PCR) assay and $p16^{INK4A}$ expression status by immunohistochemistry (IHC) as a surrogate marker. Materials and Methods: This was a retrospective study considering patients affected by squamous cell oropharyngeal tumors. All included samples were processed for IHC for $p16^{INK4A}$ and tested by PCR for detection of HPV DNA and HPV genotyping. Results: A total of 84 patients affected by squamous cell oropharyngeal tumors were included and tested. A significant positive correlation was found between HPV PCR and $p16^{INK4A}$ IHC but the agreement was poor (k coefficient of 0.25). In fact, the sensitivity of $p16^{INK4A}$ IHC positivity in detecting HPV PCR positivity was low (28.21%, 95% confidence interval=16.54% - 43.78%). Conclusion: Positivity of $p16^{INK4A}$ by IHC had a low sensitivity in detecting HPV DNA and our results suggest the need at least to test $p16^{INK4A}$ IHC- negative samples using HPV PCR to increase detection accuracy and provide valuable information for the clinical management of these patients.*

Head and neck squamous cell carcinomas (HNSCC) are the sixth most common type of cancer worldwide, accounting for 633,000 new cases annually (1-3). Common events in head and neck carcinogenesis are loss of heterozygosity (LOH), hypermethylation, deletion, and mutation of the *p16* gene locus (4). Therefore, loss of $p16^{INK4A}$ expression defines a sub-group of patients with oropharyngeal cancer (OPSCC) including those of the base of the tongue and tonsils, including lingual tonsil and Waldeyer's ring with worse clinical outcome (5). Historically, tobacco and alcohol consumption are the primary known risk factors for development of HNSCC; recently, it has become evident that infection with high-risk human papillomavirus (HPV) is etiologically linked to a sub-group of HNSCCs (6, 7). Viral DNA is detected in about 20% of HNSCCs, the strongest association being found in OPSCC, in which HPV prevalence has been estimated at between 25% and 50% (8, 9), where carcinomas of the tonsils are particularly associated with HPV infection, most frequently with HPV16 (10). Recent studies indicated that in HNSCC, patients with HPV-infected tumors have a more favorable prognosis compared to patients whose tumors are HPV-negative (11).

Most of the acquired data on HPV-driven carcinogenesis comes from the uterine cervix model in which E6 and E7 proteins play the principal role in the malignant transformation, interacting with retinoblastoma protein (pRb; 12, 13). The retinoblastoma protein is a nuclear phosphoprotein that regulates growth in the G_1 phase of the cell cycle. pRb exerts its growth-inhibitory effects by binding to and inhibiting critical regulatory proteins, including members of the E2F family of transcription factors; E2F activation is necessary for the G_1-S transition (14). E2F selectively associates with hypophosphorylated pRb, and phosphorylation of pRb appears to release E2F from an inhibitory complex, enabling it to promote the transcription necessary for cell progression into late G_1 and

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S phase (15, 16). In quiescent cells, pRb is hypophosphorylated and associates with E2F. When quiescent cells are exposed to mitogenic signals, cyclin-D associates with and activates the cyclin-dependent kinases 4 and 6 (CDK4 and CDK6, respectively), which phosphorylate pRb during the G_1 phase, causing release of E2F (17). Therefore, the free and active E2F promotes the transcription of genes that encode proteins essential for cell-cycle progression. The main inhibiting factor of this pathway is $p16^{INK4A}$, which associates with CDK4 and CDK6 and inhibits their kinase activity (17). Expression of $p16^{INK4A}$ causes accumulation of hypophosphorylated pRb and consequent G_1 cell arrest (18).

HPV infection leads to the association between viral protein E7 and pRb. This interaction results in release of active E2F and stimulation of cells to S-phase entry, even in the absence of active cyclin-D-CDK4/6 complexes (19). To counteract E2F activation, HPV-infected cells overexpress $p16^{INK4A}$.

In analogy with female genital (or cervical) carcinogenesis, HPV testing has been widely recommended in OPSCC, but there is no consensus on which test is considered the 'golden standard' (20) among the numerous detection methods available either as single tests or combinations (21).

However, although recent publication of Guidelines for Head and Neck Cancer and College of American Pathologists protocol for the Examination of Specimens From Patients With Carcinomas of the Pharynx suggest $p16^{INK4A}$ immunohistochemistry (IHC) as a screening method for HPV detection (22, 23), some questions remain regarding the accuracy of the test when used alone, without confirmatory molecular detection of HPV-DNA.

In the present study, we aimed to compare two different commonly used HPV detection methods: an HPV Polymerase Chain Reaction (PCR) assay, and $p16^{INK4A}$ expression status by IHC as a surrogate marker.

Materials and Methods

Participants and study design. In this retrospective study we included all consecutive patients from January 2000 to December 2014 affected by primary, previously untreated OPSCC from the Institute of Anatomic Pathology of Udine University Hospital and from the Institute of Anatomic Pathology of University Politecnica delle Marche. Where available, in the majority of cases, we preferred diagnostic small biopsy, otherwise we used excision biopsy. We considered as exclusion criteria HNSCC localized on lips, buccal mucosa, hard palate, tongue margins and all cases with insufficient paraffin-embedded tumor tissue (FFPE) for performing HPV PCR and $p16^{INK4A}$ IHC, or cases where HPV PCR was not feasible. The included cases were revised by two independent pathologists (MO and LM) who confirmed the diagnosis. Clinicopathological information regarding the age of the patients, tumor staging according to the TNM classification (23), treatment performed and follow-up were gathered from clinical files.

Table I. *Population features*.

Characteristic	84 patients
Age at diagnosis, years	
Median	57
Range	40-83
Sex, no. (%)	
Male	64
Female	20
Site of tumor origin, no. (%)	
Base of tongue	34 (40.5%)
Pharynx	26 (30.9%)
Retromolar trigone	12 (14.3%)
Tonsils	10 (11.9%)
Cervical lymph nodes	2 (2.4%)

Table II. *Results of the different detection methods for human papillomavirus (HPV) infection in our series*.

$p16^{INK4A}$ immunostaining	
Positive	13 (15.5%)
Negative	71 (84.5%)
HPV DNA, no. (%)	
Negative	45 (53.5%)
Positive	39 (46.5%)
HPV-16	34 (85%)
HPV-39	3 (7.5%)
HPV-31	1 (2.5%)
HPV-11	1 (2.5%)
HPV-positive tumor site of origin, no. (%)	
Base of tongue	16 (41%)
Pharynx	11 (28.2%)
Tonsils	6 (15.4%)
Retromolar trigone	4 (10.3%)
Cervical lymph nodes	2 (5.1%)
$HPV^{+}p16^{INK4A+}$	11 (13.1%)
$HPV^{+}p16^{INK4A-}$	28 (33.3%)
$HPV^{-}p16^{INK4A-}$	43 (51.2%)
$HPV^{-}p16^{INK4A+}$	2 (2.4%)

Tissue microarray (TMA). Haematoxylin and eosin-stained sections were carefully examined to select representative regions from each selected donor block. TMAs were made using a microarrayer semi-automated instrument (Galileo TMA CK4500; Integrated Systems Engineering SRL, ISENET, Milan, Italy). A cylindrical, thin-walled needle (inner diameter of 1.5 mm) was used for coring tissues and for transferring cored samples into array cavities in the recipient block. We performed three core biopsies from each tumor sample in order to obtain good representativity of the tumor. From the recipient block, we obtained 4-μm-thick sections.

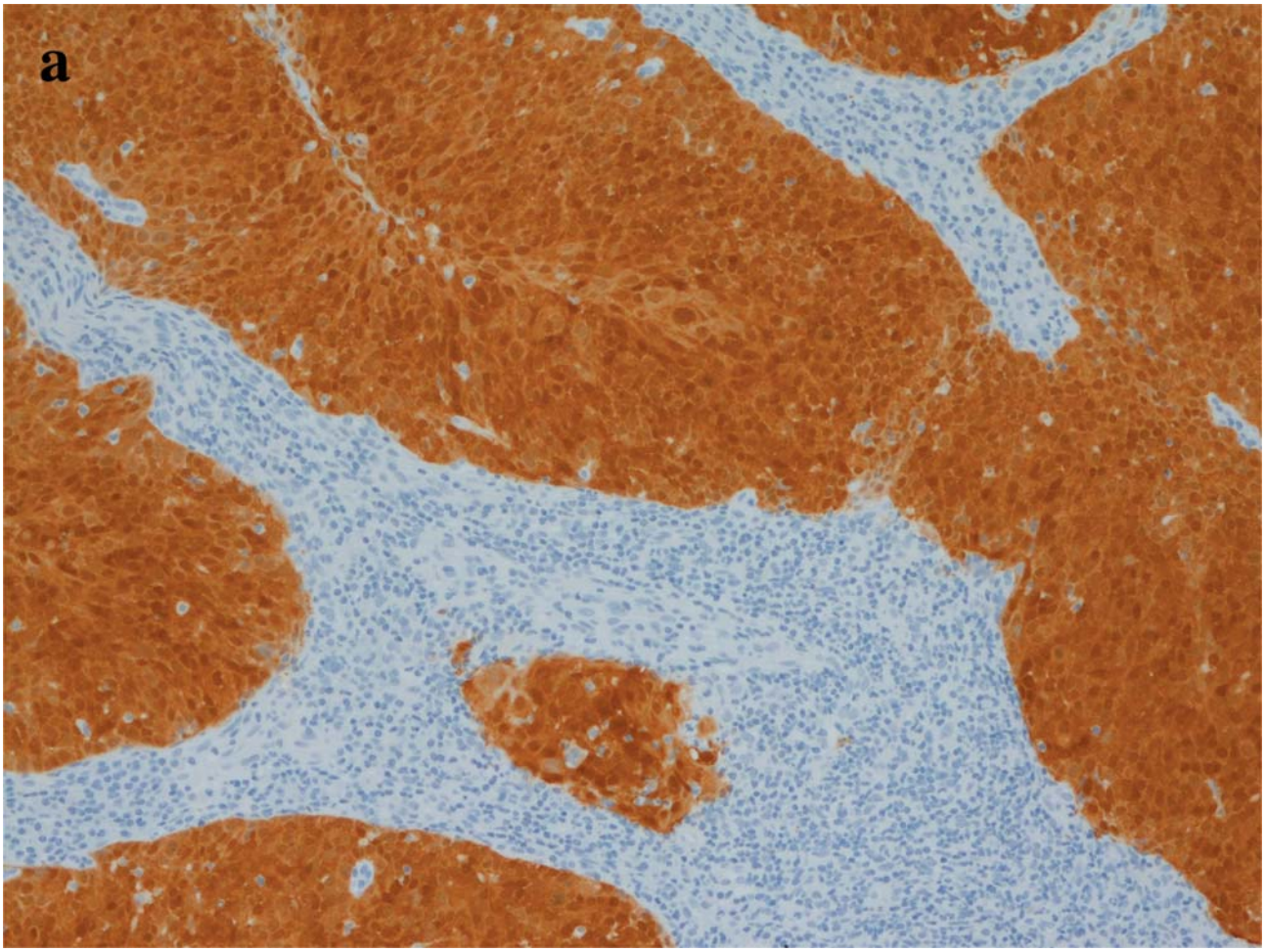

Figure 1. *Continued*

IHC for p16^{INK4A} and scoring of results. Tissue sections were cut at 4-μm-thick; for antigen retrieval and deparaffinization, slides were heated for 20 min at 98˚C in Target Retrieval Solution low pH (code K8005; DAKO, Glostrup, Denmark) with PT-link (DAKO). The slides were then incubated at room temperature in hydrogen peroxide for 10 min to block endogenous peroxidase activity. A fully automated immunohistochemistry kit, CINtec® Histology Kit (code 9517; CINtec® Ventana Medical Systems, Roche S.p.A., Segrate, Milan, Italy), was used for the qualitative detection of the p16^{INK4A} antigen according to the instruction manual. Cervical cancer sections known to be HPV-positive were used as a positive control and omission of primary antibody was used as a negative control. Among the 84 cases, 50 had sufficient tissue on the FFPE block to perform TMA, while the other 34 samples had an insufficient tumor area to be cored. For these samples, p16^{INK4A} staining was performed on the entire section. Tumors were classified as p16^{INK4A}-positive (strong, diffuse, nuclear and cytoplasmic staining in >70% of carcinoma cells), or as p16^{INK4A}-negative, following College of American Pathologists (CAP) guidelines (24).

IHC evaluation was carried out independently by two pathologist (MO and LM). Each pathologist was blinded to the results obtained by the other and to the results of the HPV test. Cases with discordant results were re-evaluated to determine a consensus.

DNA extraction. Genomic DNA was extracted from FFPE tumor samples using QIAamp® DNA FFPE Kit (code 56404; Qiagen, Milan, Italy) according to the manufacturer's protocol. The extracted DNA was suspended in 30 μl of sterile water.

PCR for detection of HPV DNA and HPV genotyping. Viral DNA was detected with the HPV Sign® Q24 Complete Real-Time PCR assay (code 979990; Diatech Pharmacogenetics, Jesi, Italy) according to the manufacturer's instructions using RotorGene Q Real-Time PCR platform (Qiagen, Milan, Italy). This assay allows detection of the most clinically relevant HPV genotypes. The presence and integrity of DNA in all samples was verified by β-actin gene amplification, which acts as an internal control. HPV-positive samples were genotyped by pyrosequencing assay according to the instructions of the HPV Sign® Q24 Complete Real-Time PCR kit. The obtained sequences were compared with the genotype-specific sequences available in the public database using IndentiFire software (Diatech Pharmacogenetics, Jesi, Italy).

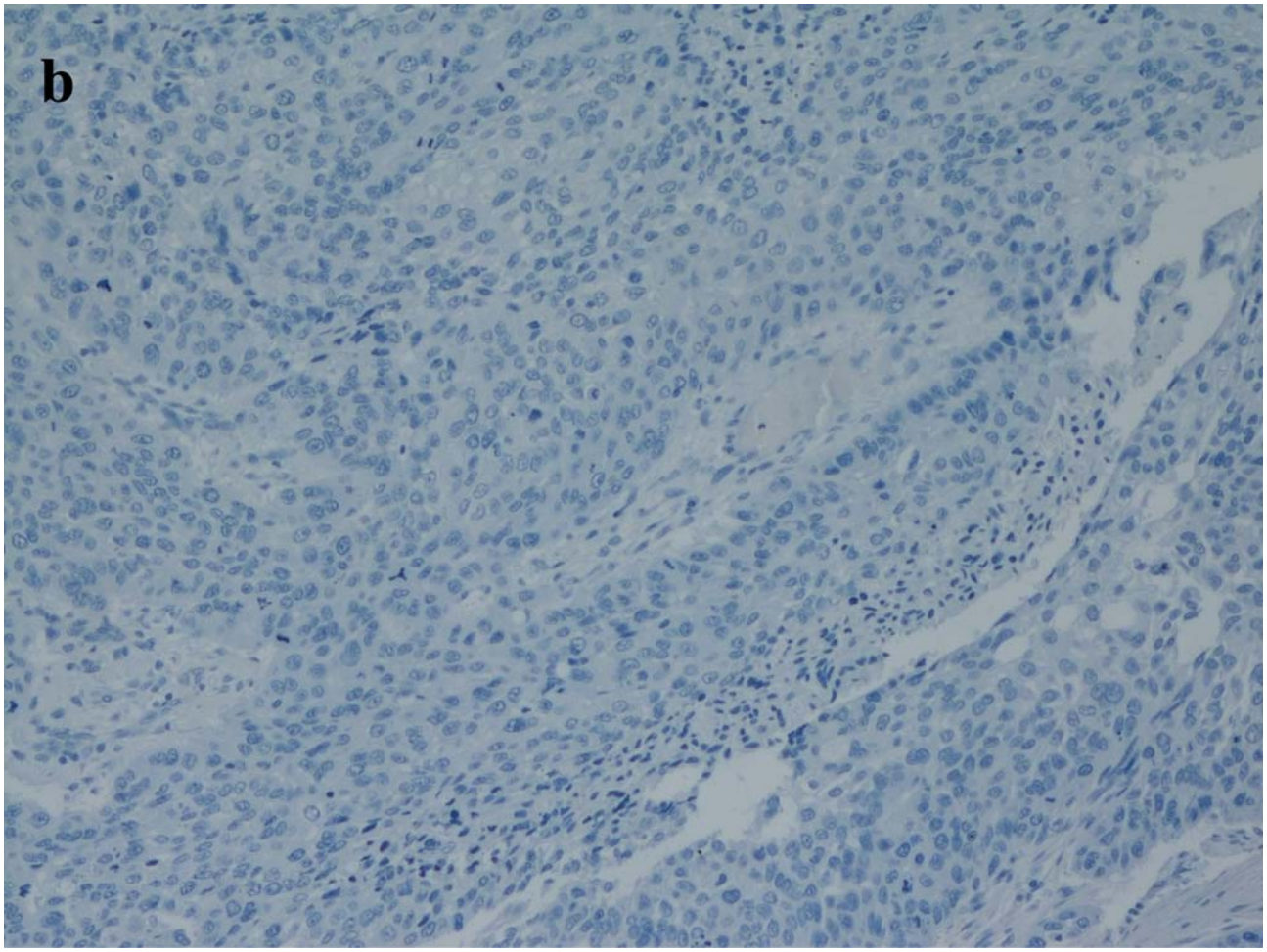

Figure 1. *Continued*

Statistical analysis. Statistical analysis was performed using R language and environment for statistical computing (version 3.1.2), from the R Foundation for Statistical Computing, Vienna, Austria (http://www.R-project.org/). The correlation between immunohistochemical $p16^{INK4A}$ staining and HPV DNA was evaluated by Chi-square test. Furthermore, the agreement between detection methods was assessed by Cohen's k coefficient. We considered Cohen's k coefficient values of less than 0.4 as indicating poor agreement, values between 0.4 and 0.75 as fair to good agreement and values over 0.75 as excellent agreement. In addition, we evaluated the accuracy $p16^{INK4A}$ staining positivity in predicting HPV DNA positivity.

Results

In our registers, we found 94 patients affected by primary previously untreated OPSCC (81 from Udine and 13 from Ancona); 10 cases were excluded because HPV PCR was not feasible. The reform the total number of cases analyzed was 84.

The age of the patients at the time of diagnosis ranged from 40 to 83 years, with a mean age of 57 years (Table I). The primary tumor location was mainly the base of the tongue (40.5%), and pharynx (30.9%) (Table I).

$p16^{INK4A}$ is normally weakly- expressed in restricted basal-parabasal layers of oral squamous epithelium, whereas it is generally overexpressed in OPSCC. The protein expression in positive OPSCCs was diffusely distributed in the nuclei and cytoplasm of almost the entire cancer cell population. In the evaluation of our cases, tumors were classified as 13 cases (15.5%) with $p16^{INK4A}$-positive staining (strong, diffuse, nuclear and cytoplasmic staining in >70% of carcinoma cells) (Figure 1a), and 71 cases (84.5%) with $p16^{INK4A}$-negative staining (Figure 1b), following CAP guidelines (24). In our cohort, six cases exhibited focally weak-to-moderate $p16^{INK4A}$ staining in fewer than 70% of carcinoma cells (Figure 1c), categorizing these cases as negative.

HPV-DNA was found in 39 out of 84 cases of OPSCC (64 male and 20 female patients) and HPV 16 was the most frequent genotype detected (Table II), as described in the literature for Italy (25).

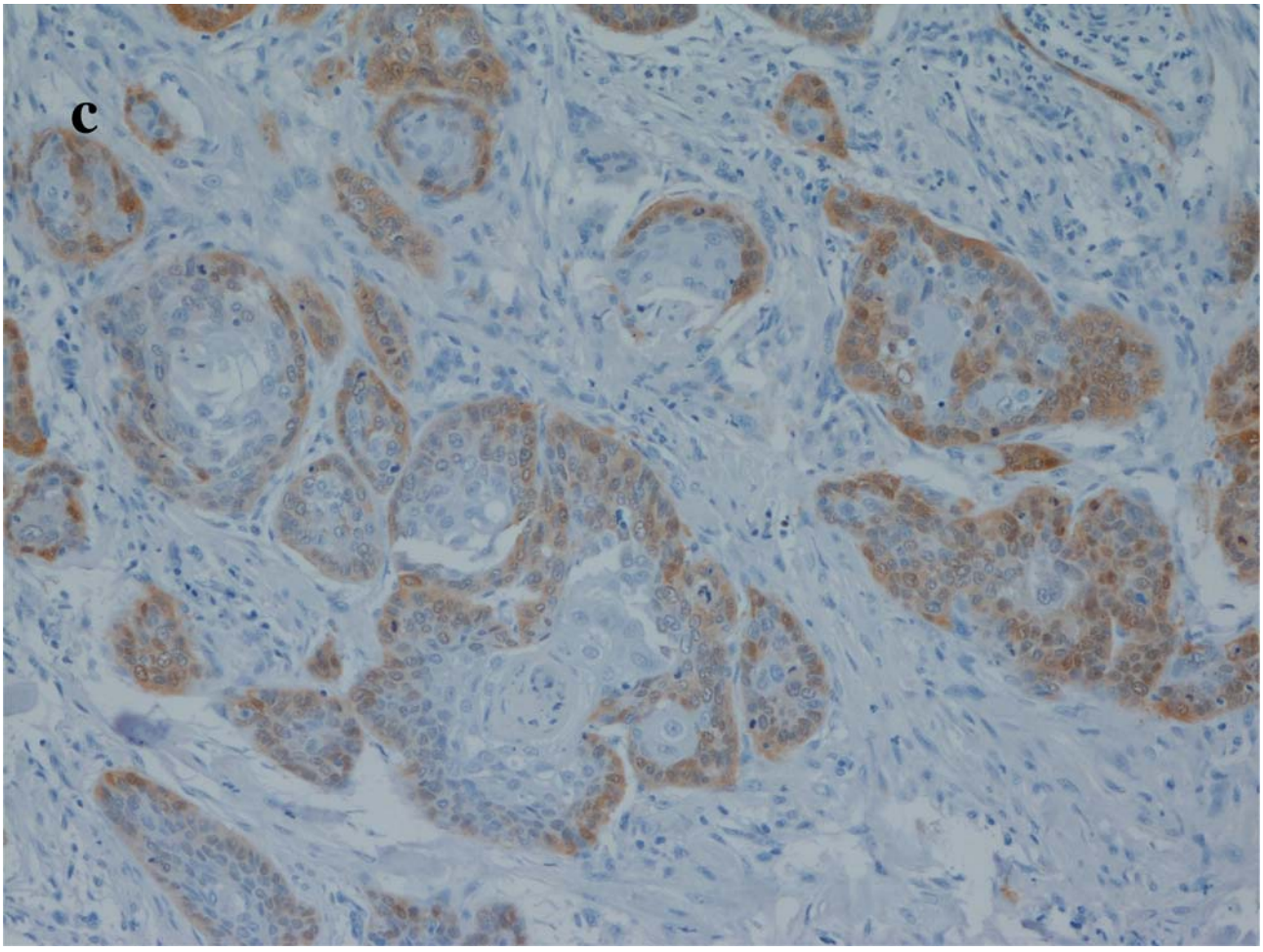

Figure 1. *Representative examples of immunohistochemical staining of tissue sections for $p16^{INK4A}$: a: strong and diffuse $p16^{INK4A}$ staining in tumor cells (×10), b: negative $p16^{INK4A}$ staining in tumor cells (×10), c: focally weak to moderate $p16^{INK4A}$ staining in fewer than 70% of carcinoma cells, categorizing such cases as negative (×10).*

The majority of tumors (51.2%) were both HPV- and $p16^{INK4A}$-negative. Out of the HPV-positive tumors, 11/39 (13.1%) were $p16^{INK4A}$-positive, while only 2/45 (2.4%) of the HPV-negative tumors were $p16^{INK4A}$-positive (Table II). What is interesting to note is the high number of tumors (28/39, 33.3%) that were HPV-positive but $p16^{INK4A}$-negative (Table II). The tumor site for which HPV genotype detection was higher was the base of the tongue, with 16 cases (41%) (Table II). HPV-positive $p16^{INK4A}$-negative cases that were analyzed for expression of $p16^{INK4A}$ by TMA were also analyzed over the entire section in order to be sure that the negativity in $p16^{INK4A}$ was not due to the coring of a small area not representative of the whole tumor. The data obtained confirmed that in all cases, the neoplasm was homogeneously negative for $p16^{INK4A}$.

We found a significant correlation between $p16^{INK4A}$ staining and HPV DNA ($p<0.05$). However, Cohen's k coefficient of 0.25 shows a poor agreement between the two tests. In addition, sensitivity of $p16^{INK4A}$ staining in predicting HPV DNA was 28.21% (95% confidence interval=16.54-43.78%), the specificity was 95.56% (95% confidence interval=85.17-98.77%), the positive likelihood ratio was 6.35 (95% confidence interval=1.59-25.27), the negative likelihood ratio was 0.75 (95% confidence interval=0.61-0.92), and accuracy was 64.29% (95% confidence interval=53.62-73.7%).

Discussion

In the preesst study, we found a significant positive correlation between HPV PCR and $p16^{INK4A}$ IHC but the agreement was poor. In fact, the sensitivity of the $p16^{INK4A}$ IHC against the HPV PCR was low (28.21%, 95% confidence interval=16.54-43.78%).

The *p16* gene (CDKN2a/INK4a) functions as a negative regulator of the cell cycle through its inhibition of CDK4/6 and subsequent blockage of the cyclin-dependent phosphorylation of the Rb. In oral epithelial pre-cancer and cancer, the *p16* gene is frequently inactivated via the following events: LOH, hypermethylation, deletion, mutation (4); this inactivation and loss of $p16^{INK4A}$ expression defines a sub-group of patients with OPSCC with worse clinical outcome (5). $p16^{INK4A}$ protein overexpression has been proposed as surrogate marker of HPV infection initially in cervical cancer (26), and subsequently the same concept was applied for squamous cell carcinomas from sites other than the uterine cervix (27), and especially in head and neck cancers (28).

However, the overall low specificity of $p16^{INK4A}$ IHC for the diagnosis of high-risk HPV infection in recent series of patients (29) is certainly very different to what was observed for the cervix, in which virtually all SCCs are associated with high-risk HPV and $p16^{INK4A}$ overexpression. In the HNSCC, more heterogeneous causative factors, such as as lifestyle (tobacco and smoke) and molecular profiles, contribute to the pathogenesis of the disease (30); therefore $p16^{INK4A}$ overexpression may derive from different mechanisms other than HPV infection (31).

In our study, only 11 cases (13.1%) with HPV positivity on PCR overexpressed $p16^{INK4A}$, undermining the usefulness of $p16^{INK4A}$ as a diagnostic surrogate for HPV infection in the oropharynx, which was the site previously was seen to have the highest concordance between IHC and PCR results (32).

In head and neck tumors, up to now, the only consistent clinical predictors for disease specific survival of patients with SCCs is TNM stage (33, 34). In spite of the histological homogeneity (most head and neck cancer cases are of SCC), there is a wide biological heterogeneity, and this contributes to a lack of consistency in treatment planning and in tumoral treatment response. The better characterization at a molecular level may better define homogeneous groups of patients for prognosis, as well as for responsiveness to treatments (35). The growing evidence of the prognostic significance of HPV status in OSCC (36, 37), indicating that HPV is associated with a better prognosis and an increased radiosensitivity, suggests that it can be used as a molecular marker in OPSCC.

Despite clinical advances, there exist no commercially available, validated, and universally accepted tests for the determination of tumor HPV status, nor any guidelines. With our study, we underline the need for a consensus on the methods to be used for HPV detection in OPSCC (20, 38) and we re-inforce the idea, proposed by other authors (29), that the gold standard for the identification of HPV infection in OPSCC should be the molecular method, at least for those with negative $p16^{INK4A}$ immunostaining.

The main limit of this study was its retrospective design and the inclusion in the study of tumor tissue embedded in paraffin 10 years before the analysis; in fact in 10 samples, HPV PCR detection was not feasible. However, the wide number of cases analyzed was the main strength of our study. Furthermore, we need prospective studies in this field and a consensus is mandatory for the cut-off used to define positivity of $p16^{INK4A}$ by IHC because very little direct evidence exists for using it as a screening method for HPV detection, and only some authors have suggested algorithms for this purpose (22, 31, 39).

References

1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer *127(12)*: 2893-917, 2010.

2 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61(2): 69-90, 2011.

3 Siegel R, Naishadham D and Jemal A: Cancer statistics 2013. CA Cancer J Clin *63(1)*: 11-30, 2013.

4 Reed AL, Califano J, Cairns P, Westra WH, Jones RM, Koch W, Ahrendt S, Eby Y, Sewell D, Nawroz H, Bartek J and Sidransky D: High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res *56(16)*: 3630-3633, 1996.

5 Rischin D, Young R, Fisher R, Le Q, Peters L, Choi J, O'Sullivan B, Giralt J and McArthur G: Prognostic significance of HPV and p16 status in patients with oropharyngeal cancer treated on a large international phase III trial [abstract]. J Clin Oncol 27(15s): Ab 6004, 2009.

6 Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV and Sidransky D: Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst *92(9)*: 709-720, 2000.

7 Argiris A, Karamouzis MV, Raben D and Ferris RL: Head and neck cancer. Lancet *371(9625)*: 1695-1709, 2008.

8 Mehanna H, Beech T, Nicholson T, El-Hariry I, McConkey C, Paleri V and Roberts S: Prevalence of human papillomavirus in oropharyngeal and nonoropharyngeal head and neck cancer-systematic review and meta-analysis of trends by time and region. Head Neck *35(5)*: 747-755, 2013.

9 Rietbergen MM, Leemans CR, Bloemena E, Heideman DA, Braakhuis BJ, Hesselink AT, Witte BI, Baatenburg de Jong RJ, Meijer CJ, Snijders PJ and Brakenhoff RH: Increasing prevalence rates of HPV attibutable oropharyngeal squamous cell carcinomas in the Netherlands as assessed by validated test algorithm. Int J Cancer *132(7)*: 1565-1571, 2013.

10 Fakhry C and Gillison ML: Clinical implications of human papillomavirus in head and neck cancers. J Clin Oncol *24(17)*: 2606-2611, 2006.

11 Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, Forastiere A and Gillison ML: Improved survival of patients with human papillomaviruspositive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst *100(4)*: 261-269, 2008.

12 Munger K, Phelps WC, Bubb V, Howley PM and Schlegel R: The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol *63(10)*: 4417-4421, 1989.

13 McDougall JK: Immortalization and transformation of human cells by human papillomavirus. Curr Top Microbiol Immunol *186*: 101-119, 1994.
14 Duronio RJ, O'Farrell PH, Xie JE, Brook A and Dyson N: The transcription factor E2F is required for S phase during *Drosophila* embryogenesis, Genes Dev *9(12)*: 1445-1455, 1995.
15 La Thangue NB: DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem Sci *19(3)*: 108-114, 1994.
16 Weinberg RA: The retinoblastoma protein and cell cycle control. Cell *81(3)*: 323-330, 1995.
17 Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev *13(12)*: 1501-1512, 1999.
18 Lukas J, Parry D, Aagaard L, Mann D J, Bartkova J, Strauss M, Peters G and Bartek J: Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature *375(6531)*: 503-506, 1995.
19 Tommasino M and Crawford L: Human papillomavirus E6 and E7: proteins which deregulate the cell cycle. Bioessays *17(6)*: 509-518, 1995.
20 Braakhuis BJ, Brakenhoff RH, Meijer CJ, Snijders PJ and Leemans CR: Human papilloma virus in head and neck cancer: The need for a standardized assay to assess the full clinical importance. Eur J Cancer *45(17)*: 2935-9, 2009.
21 Venuti A and Paolini F: HPV detection methods in head and neck cancer. Head Neck Pathol *6(Suppl 1)*: S63-74, 2012.
22 National Comprehensive Cancer Network (NCCN) Guidelines™ Version 2.2013-Updates Head and Neck Cancers. [http: //www.nccn.org/professionals/physician_gls/f_guidelines.asp]. 05/29/13
23 AJCC Cancer Staging Manual. American Joint Committee on Cancer. Seventh Edition. Edge SB, Byrd, DR, Compton CC, Fritz AG, Greene FL, Trotti A (eds.). New York: Springer, 2010.
24 College of American Pathologists (CAP) Checklist and protocols Based on AJCC/UICC TNM, 7th edition-Protocol web posting date: October 2013 [http: //www.cap.org/apps/cap.portal?_nfpb=true&cntvwrPtlt_actionOverride=/portlets/contentViewer/show&_windowLabel=cntvwrPtlt&cntvwrPtlt{actionForm.contentReference}=committees/cancer/cancer_protocols/protocols_index.html&_pageLabel=cntvwr]. Updated December 2, 2014.
25 Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El GF, Brahim-Tallaa L, Guha N, Freeman C, Galichet L and Cogliano V: A review of human carcinogens-Part B: biological agents. Lancet Oncol *10(4)*: 321-322, 2009.
26 Amortegui AJ, Meyer MP, Elborne VL and Amin RM: p53, retinoblastoma gene product, and cyclin protein expression in human papillomavirus virus DNA-positive cervical intraepithelial neoplasia and invasive cancer. Mod Pathol *8(9)*: 907-912, 1995.
27 O'Neill CJ and McCluggage WG: p16 expression in the female genital tract and its value in diagnosis. Adv Anat Pathol *13(1)*: 8-15, 2006.
28 Fischer CA, Kampmann M, Zlobec I, Green E, Tornillo L, Lugli A, Wolfensberger M and Terracciano LM: p16 expression in oropharyngeal cancer: its impact on staging and prognosis compared with the conventional clinical staging parameters. Ann Oncol *21(10)*: 1961-1966, 2010.
29 Bussu F, Sali M, Gallus R, Vellone VG, Zannoni GF, Autorino R, Dinapoli N, Santangelo R, Martucci R, Graziani C, Miccichè F, Almadori G, Galli J, Delogu G, Sanguinetti M, Rindi G, Valentini V and Paludetti G: HPV infection in squamous cell carcinomas arising from different mucosal sites of the head and neck region. Is p16 immunohistochemistry a reliable surrogate marker? Br J Cancer *108(5)*: 1157-62, 2013.
30 Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortes ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA and Grandis JR: The mutational landscape of head and neck squamous cell carcinoma. Science *333(6046)*: 1157-1160, 2011.
31 El-Naggar AK and Westra WH: P16 expression as a surrogate marker for HPV-related oropharyngeal carcinoma: a guide for interpretative relevance and consistency. Head Neck *34(4)*: 459-461, 2011.
32 Doxtader EE and Katzenstein AL: The relationship between p16 expression and high-risk human papillomavirus infection in squamous cell carcinomas from sites other than-uterine cervix: a study of 137 cases. Hum Pathol *43(3)*: 327-332, 2012.
33 Head and neck surgery and oncology. Shah, Jatin P; Patel, Snehal G. Head and neck surgery. 3rd ed. Edinburgh ; New York: Mosby, 2003.
34 Spector JG, Sessions DG, Haughey BH, Chao KS, Simpson J, El Mofty S and Perez CA: Delayed regional metastases, distant metastases, and second primary malignancies in squamous cell carcinomas of the larynx and hypopharynx. Laryngoscope *111(6)*: 1079-1087, 2001.
35 Almadori G, Bussu F, Cadoni G, Galli J, Paludetti G and Maurizi M: Molecular markers in laryngeal squamous cell carcinoma: towards an integrated clinicobiological approach. Eur J Cancer *41(5)*: 683-693, 2005.
36 Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axelrod R, Silverman CC, Redmond KP and Gillison ML: Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med *363(1)*: 24-35, 2010.
37 Gillison ML: HPV and prognosis for patients with oropharynx cancer. Eur J Cancer *45(Suppl 1)*: 383-385, 2009.
38 Schlecht NF, Brandwein-Gensler M, Nuovo GJ, Li M, Dunne A, Kawachi N, Smith RV, Burk RD and Prystowsky MB: A comparison of clinically utilized human papillomavirus detection methods in head and neck cancer. Mod Pathol *24(10)*: 1295-1305, 2011.
39 Jordan RC, Lingen MW, Perez-Ordonez B, He X, Pickard R, Koluder M, Jiang B, Wakely P, Xiao W and Gillison ML: Validation of methods for oropharyngeal cancer HPV status determination in US cooperative group trials. Am J Surg Pathol *36(7)*: 945-54, 2012.

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