Evaluation of the Anyplex II HPV28 Assay in the Detection of Human Papillomavirus in Archival Samples of Oropharyngeal Carcinomas

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• ***Context.***—**The improved survival and better response to treatments of human papillomavirus (HPV)–related oropharyngeal squamous cell carcinoma (OPSCC) highlight the need for effective tools in evaluating HPV status on formalin-fixed, paraffin-embedded (FFPE) cancer tissues. To date, there is no agreement regarding the most appropriate method for HPV testing on FFPE materials.**

Objective.—**We aimed to investigate the performance of the Anyplex II HPV28 (Anyplex) on FFPE OPSCC tissues and to compare it with 2 other methods for HPV-DNA detection and p16 overexpression.**

Design.—**One hundred sixty FFPE OPSCCs were evaluated, which had already been analyzed with the INNO-LiPA HPV assay, Xpert HPV assay, and p16 immunostaining.**

Results.—**All the samples but 1 provided valid results with the Anyplex, which showed the highest HPV detection rate and a good concordance with all the other methods ($\kappa = 0.75$, 95% CI, 0.65–0.85 versus INNO-LiPA; $\kappa = 0.80$, 95% CI, 0.70–0.89 versus Xpert; $\kappa = 0.76$, 95% CI, 0.65–0.86 versus p16). Moreover, the HPV-driven fraction, based on HPV-DNA and p16 double positivity, was higher with Anyplex (83 of 159, 52.2%) than with the other 2 assays, that is, 78 of 156 (50.0%) for INNO-LiPA and 80 of 160 (50.0%) for Xpert.**

Conclusions.—**Anyplex II HPV28 showed a higher HPV detection rate and HPV-associated fraction than the other methods used. This assay is suitable for HPV detection in archival OPSCC tissues.**

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Human papillomavirus (HPV) has been detected in a considerable fraction of oropharyngeal squamous cell carcinomas (OPSCCs) particularly in those arising at the tonsils,[1–3] and high-risk (HR)–HPVs have been recognized as the etiologic agents of these tumors.[4] HPV16 is responsible for 90% of HPV-positive OPSCCs.[5] Human papillomavirus represents a strong independent prognostic factor for OPSCCs, with HPV-positive OPSCCs showing better response to therapies than HPV-negative OPSCCs.[6] Based on these observations, clinical trials on the deintensification of treatments for patients with HPV-related OPSCC are currently in progress.[7] Moreover, based on HPV status, a less intensive follow-up protocol may be planned for these patients.[8] The role of HPV in the development of OPSCC has led to essential modifications both in the classification[9] and in the staging system of these carcinomas.[10] Many HPV-DNA testing options are available but, to date, there is no agreement regarding the most appropriate method for the detection of HPV-DNA on formalin-fixed, paraffin-embedded (FFPE) tissues in a clinical setting. The immunohistochemical evaluation of $p16^{INK4A}$ protein (p16 IHC) is considered by many authors as a reliable surrogate marker of transforming HPV infections.[11,12] It is easily applicable to FFPE specimens, which are available in routine clinical practice, and is currently accepted as a stand-alone test for the identification of HPV-associated OPSCCs according to American Joint Committee on Cancer guidelines. However, p16 may be upregulated also in response to virus-unrelated mechanisms, and may thus give false-positive results. In fact, HPV-negative but p16-positive tumors have been reported in numerous studies.[13,14] Although the presence of HPV-DNA alone is not sufficient to attribute a causal role to HPV in oropharyngeal carcinogenesis, the latest *World Health Organization (WHO) Classification of Head and Neck Tumours* underscores the importance of direct HPV testing. Detection of HPV-DNA, in combination with p16 and/or HPV mRNA evaluation, represents one of the most reliable diagnostic approaches for the identification of HPV-driven OPSCCs.[15] Staining for p16 alone, without confirmation of HPV status

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with an HPV-specific test, will negatively affect the clinical management of $p16^+/HPV^-$ patients.[16]

Both direct and indirect methods for the detection of HPV-DNA on FFPE tissues have been used.[17–21] Polymerase chain reaction (PCR)–based methods that use the SPF_{10} primer set, such as the INNO-LiPA HPV Genotyping assay, are the most effective because of the short size of the HPV target region, which limits the risk of false-negative/invalid results due to the unpredictable DNA fragmentation that occurs during formalin fixation. Indeed, HPV detection systems based on this primer set have been used in international surveys to assess HPV prevalence in archival cancer tissues.[1]

The Anyplex II HPV28 (Anyplex), by Seegene (Seoul, South Korea), is based on a multiplex real-time PCR, which offers the advantage of a complete genotyping for 28 HPVs, including both HR and low-risk (LR) types. To date, this method has only been used on cervical samples.[22–24] To the best of our knowledge, only 1 study has evaluated the Anyplex on FFPE samples but it included very few OPSCC cases.[25]

In this context, we evaluated the performance of the Anyplex in detecting and genotyping HPV-DNA on a large series of FFPE OPSCCs. To this aim, we compared the Anyplex results with those obtained with 2 other HPV-DNA detection systems (the INNO-LiPA by Fujirebio [Pomezia, Italy] and the Xpert HPV by Cepheid [Sunnyvale, California]), as well as with p16 immunostaining. We evaluated the agreement between HPV testing methods both in terms of HPV status and genotyping.

MATERIALS AND METHODS

Study Samples

A retrospective series of 160 FFPE primary OPSCC cases, diagnosed between 2010 and 2017 (2010: n = 14; 2011: n = 26; 2012: n = 26; 2013: n = 21; 2014: n = 19; 2015: n = 21; 2016: n = 19; 2017: n = 14), were retrieved from the archives of the Pathology Department of the Regina Elena National Cancer Institute of Rome, Italy. All the cases that had already been characterized for the presence of HPV-DNA with the INNO-LiPA and Xpert systems, and for the expression of p16 with IHC, were selected. The study was approved by the Ethics Committee of the Regina Elena National Cancer Institute (CE/975/17).

Nucleic Acid Extraction Methods

Depending on the size of the lesion, 1 to 3 × 5-μm FFPE sections were obtained. The utmost care was taken during sectioning to avoid contamination.[26] A final section was stained by hematoxylin-eosin and reviewed by a certified pathologist to confirm the diagnosis. Sections were deparaffinized with 1 mL of xylene at 56°C for 5 minutes. The xylene was removed by washing with 1 mL of absolute ethanol. Tissue lysis was obtained by overnight incubation at 56°C in 180 μL of ATL buffer with 20 μL of Proteinase K (Qiagen, Milan, Italy), followed by 1-hour incubation at 90°C. The DNeasy Blood and Tissue Kit (Qiagen) or QIAamp DNA FFPE Tissue Kit (Qiagen) were used for total nucleic acid extraction, following the manufacturer's instructions. Elution was performed with 50 or 100 μL of elution buffer, based on the size of the FFPE tissues.

HPV Testing

INNO-LiPA HPV Genotyping Extra and Extra II Assays.—Briefly, for INNO-LiPA HPV Genotyping Extra or Extra II (Fujirebio), 10 μL of total nucleic acid extract obtained from FFPE tissues were used for amplification. All the hybridization steps were carried out in a Profiblot T48 instrument (Tecan, Mannedorf, Switzerland).

INNO-LiPA HPV Genotyping Extra and Extra II are based on the SPF_{10} primer set for the amplification of a 65-bp fragment within the L1 region of the viral genome and are able to detect individually, among others, the 12 genotypes classified as HR (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), the HPV68 classified as probably HR, and the HPV66 classified as possibly HR by the International Agency for Research on Cancer.[4]

Xpert HPV Assay.—The assay was performed by using crude lysates, obtained from deparaffinized FFPE tissues as previously described; 100 μL of the crude lysate was added to 900 μL of nuclease-free water and tested as previously described.[21]

The Xpert HPV (Cepheid) is a qualitative test that detects the same 14 HR-HPV or possibly/probably HR-HPV types detected by INNO-LiPA. This test is based on a real-time PCR that targets a sequence of 80 to 150 bp, depending on the genotype, in the E6/E7 region. The test result is given with a concurrent partial genotyping. The Xpert assay detects HPV16 as a single genotype, while for the other genotypes it provides a pooled result (HPV 18 and 45; HPV 31, 33, 35, 52, and 58; HPV 51 and 59; HPV 39, 56, 66, and 68).

Anyplex II HPV28 Assay.—The Anyplex (Seegene) is a qualitative multiplex real-time PCR-based method for the simultaneous genotyping of 28 HPV types with targets a fragment of 100 to 200 bp in length in the L1 region. For each sample, 2 PCRs were performed by using 5 μL of nucleic acid extract and 15 μL of reaction mixture, containing either primer set A or B, for the detection of the 14 HR-HPV or possibly/probably HR-HPV types and of the 14 possibly HR/LR types (HPV 26, 53, 69, 70, 73, 82; and HPV 6, 11, 40, 42, 43, 44, 54, 61), respectively. A housekeeping human gene, that is, β-globin, is detected as an internal control to monitor extraction efficiency, cell adequacy, and PCR inhibition. For HPV-positive samples, a low (+), intermediate (++), or high (+++) positivity is also indicated. Samples were considered HPV positive when the signal was + or greater, according to manufacturer's instructions. All runs of PCR included negative and positive controls and were performed with CFX96 Real-time PCR System (BioRad, Hercules, California).

$p16^{INK4A}$ Immunohistochemistry

The CINtec Histology Kit (Roche Diagnostics, Milan, Italy) was used to assess p16 expression following the manufacturer's instructions. The samples were considered positive when more than 75% of tumor cells showed a strong nuclear expression according to the current TNM system/WHO classification.[27]

Statistical Analysis

The raw agreement and Cohen κ coefficient were estimated in order to assess the agreement for HPV status (HPV negative versus positive regardless of the specific genotype[s] identified) of the Anyplex versus INNO-LiPA, and the Anyplex versus Xpert. The Cohen κ coefficient was also used to evaluate the agreement between the Anyplex results and p16 expression. The agreement was also calculated for HPV type-specific positivity.

The McNemar test was used to evaluate the statistical significance of differences in positivity rate among assays. Differences were considered significant when $P < .05$. The SPSS statistical package (version 17.0, SPSS Inc, Chicago, Illinois) was used for all analyses.

RESULTS

Study Samples

Of the 160 OPSCC cases retrieved for the study, 121 had been diagnosed in men and 39 in women (median age, 61 years; interquartile range, 54–68). Table 1 summarizes the case characteristics. Almost half of the cases were tonsillar carcinomas (72, 45.0%), while 68 (42.5%) arose from the base of the tongue and 20 (12.5%) from other oropharyngeal subsites.

Table 1. Characteristics of the 160 Oropharyngeal Squamous Cell Carcinomas Included in the Study

Age, median (IQR), y	61 (54–68)
Sex distribution, n (%)	
Men	121 (75.6)
Women	39 (24.4)
Cancer subsite, n (%)	
Tonsils	72 (45.0)
Base of the tongue	68 (42.5)
Other[a]	20 (12.5)
Year of diagnosis, n (%)	
2010	14 (8.7)
2011	26 (16.3)
2012	26 (16.3)
2013	21 (13.1)
2014	19 (11.9)
2015	21 (13.1)
2016	19 (11.9)
2017	14 (8.7)
Total	**160** (100)

Abbreviation: IQR, interquartile range.

[a] Soft palate, uvula, epiglottic vallecula, amygdaloglossus sulcus, posterior wall.

HPV Results With the Anyplex, INNO-LiPA, and Xpert Assays

Tables 2 and 3 show the results obtained with the 3 HPV-DNA detection methods used, in terms of HPV status and genotyping for the HPV-positive cases, respectively. The Anyplex gave only 1 invalid result (1 of 160, 0.6%). Specifically, the Anyplex showed 13 invalid results when extraction was carried out with the DNeasy Blood and Tissue Kit (13 of 160, 8.1%); however, extraction was successful for all but 1 sample when nucleic acids were re-extracted with the QIAamp DNA FFPE Tissue extraction kit. The INNO-LiPA gave an invalid result (no band observed for the housekeeping gene) in 4 of the 160 cases (2.5%). All of the samples were valid with the Xpert. Of the 160 analyzed samples, the HPV-positive cases totaled 98 (61.3%), 79 (49.4%), and 81 (50.6%) with the Anyplex, the INNO-LiPA, and the Xpert, respectively. Considering only the valid results, a marginal statistical difference in positivity rate among assays was observed (Anyplex versus INNO-LiPA: $\chi^2 = 3.86$, $P = .05$; Anyplex versus Xpert: $\chi^2 = 3.91$, $P = .05$).

Table 2. Human Papillomavirus (HPV) Results for the 160 Oropharyngeal Squamous Cell Carcinomas Tested With the Anyplex, INNO-LiPA, and Xpert Assays

	HPV Test, n (%)		
	Anyplex[a]	**INNO-LiPA[b]**	**Xpert[c]**
Negative	61 (38.1)	77 (48.1)	79 (49.4)
Positive	98 (61.3)	79 (49.4)	81 (50.6)
Invalid	1 (0.6)	4 (2.5)	0 (0.0)
Total	**160** (100)	**160** (100)	**160** (100)

[a] Seegene, Seoul, South Korea.
[b] Fujirebio, Pomezia, Italy.
[c] Cepheid, Sunnyvale, California.

Table 3. Human Papillomavirus (HPV) Type-Specific Results for the Oropharyngeal Squamous Cell Carcinomas That Tested HPV-Positive With the Anyplex, INNO-LiPA, and Xpert Assays

	HPV Genotypes in HPV-Positive Cases, n (%)		
	Anyplex[a]	**INNO-LiPA[b]**	**Xpert[c]**
HPV6	1 (1.0)	0 (0.0)	Not included
HPV16	86 (87.8)	65 (82.3)	69 (85.2)
HPV18	2 (2.0)	2 (2.5)	2 (2.5)[d]
HPV26	1 (1.0)	1 (1.3)	Not included
HPV33	4 (4.1)	3 (3.8)	
HPV35	6 (6.1)	6 (7.5)	10 (12.3)[e]
HPV58	1 (1.0)	1 (1.3)	
HPV51	1 (1.0)	1 (1.3)	0 (0.0)
Total	**102**[f]	**79**	**81**

[a] Seegene, Seoul, South Korea.
[b] Fujirebio, Pomezia, Italy.
[c] Cepheid, Sunnyvale, California.
[d] HPV 18, 45.
[e] HPV 31, 35, 33, 52, 58.
[f] The number of type-specific positive cases exceeds that of HPV-positive cases because of 4 multiple infections: 6-16, 16-35, 16-51, 33-35; the percentages for single genotype were calculated over the 98 HPV-positive cases.

Regarding HPV genotyping, only HR-HPV or possibly HR-HPV genotypes were detected with the 3 assays, except for 1 case in which HPV6 was detected by Anyplex in coinfection with HPV16 (Table 3). Coinfections were detected with Anyplex in 3 other cases, which harbored HPV 16-35, 16-51, 33-35, respectively. Conversely, only single infections were revealed with the INNO-LiPA. It is not possible to establish the presence of multiple infections for the Xpert assay because of pooled results of more than 1 genotype, as previously mentioned.

All the genotypes detected with the Anyplex were among those also detectable with the INNO-LiPA, whereas the Xpert is not able to detect HPV6 and HPV26, which are not included in the Xpert HPV genotype panel. For all 3 assays the genotype mainly represented was HPV16, which was detected in 86 of 98 (87.8%), 65 of 79 (82.3%), and 69 of 81 (85.2%) of the samples positive with the Anyplex, the INNO-LiPA, and the Xpert, respectively. HPV16 was followed by HPV 35, 33, and 18. In 1 case the Anyplex detected HPV26, which was also revealed with the INNO-LiPA. Considering the 78 cases with a positive HPV result both with Anyplex and INNO-LiPA, type-specific data were perfectly concordant in 74 cases with an agreement of 94.9%, while in the remaining 4 cases the results were compatible, that is, the genotyping results only partially overlapped. In fact, in these 4 cases, as already mentioned, the Anyplex identified multiple infections by 1 INNO-LiPA concordant genotype (HPV16 both for the HPV6-16– and HPV16-51–positive cases; HPV35 both for the HPV16-35– and 33-35–positive cases) but also an additional genotype, which was not detected by the INNO-LiPA. Type-specific agreement could not be determined for Xpert, which cannot provide a result for individual genotypes except for HPV16. However, in 12 cases, the Xpert gave a result that was compatible with that observed with Anyplex and INNO-LiPA, that is, they tested positive with the probe that recognizes the genotype identified by the other 2 methods.

Table 4. Concordance of the Human Papillomavirus Results for the Anyplex Versus INNO-LiPA, Xpert, and p16 Expression						
	INNO-LiPA,[b] n = 155[c]		Xpert,[d] n = 158[c,e]		p16 IHC, n = 159[c]	
Anyplex[a]	Negative	Positive	Negative	Positive	Negative	Positive
Negative	58 (37.4)	1 (0.7)	61 (38.6)	0 (0.0)	57 (35.9)	4 (2.5)
Positive	18 (11.6)	78 (50.3)	16 (10.1)	81 (51.3)	15 (9.4)	83 (52.2)
Cohen κ (95% CI)	0.75 (0.65–0.85)		0.80 (0.70–0.89)		0.76 (0.65–0.86)	

Abbreviation: IHC, immunohistochemistry.

[a] Seegene, Seoul, South Korea.
[b] Fujirebio, Pomezia, Italy.
[c] Only samples with a valid test result with both tests being compared were included.
[d] Cepheid, Sunnyvale, California.
[e] The sample positive for HPV26 was excluded from the analysis, since it was not detectable by the Xpert.

Concordance of the Anyplex With the INNO-LiPA, the Xpert, and p16 Expression

The analysis of the concordance for HPV-DNA positivity between the Anyplex and the other 2 assays is shown in Table 4. We observed a good concordance between the Anyplex and the INNO-LiPA with a Cohen $\kappa = 0.75$ (95% CI, 0.65–0.85), and this was even higher comparing the Anyplex with the Xpert ($\kappa = 0.80$; 95% CI, 0.70–0.89). Also when we compared the HPV-DNA status determined with the Anyplex and the p16 immunostaining, we found a good concordance ($\kappa = 0.76$; 95% CI, 0.65–0.86).

Using the double positivity for HPV-DNA and p16 to identify the cases that are etiologically associated to HPV infection, the HPV-driven fraction was 83 of 159 (52.2%) for the Anyplex, 78 of 156 (50.0%) for the INNO-LiPA, and 80 of 160 (50.0%) for the Xpert.

Table 5. Cases Discordant for Human Papillomavirus Status in the Comparison Among the Anyplex, INNO-LiPA, Xpert, and p16 Results[a]				
Sample ID	Anyplex[b]	INNO-LiPA[c]	Xpert[d]	p16
24	Pos	Neg	Neg	Neg
29	Neg	Pos	Neg	Neg
30	Pos	Neg	Pos	Pos
31	Pos	Neg	Pos	Pos
32	Pos	Neg	Pos	Neg
89	Pos	Neg	Neg	Neg
93	Pos	Neg	Neg	Neg
102	Pos	Neg	Neg	Neg
129	Pos	Neg	Neg	Neg
146	Pos	Neg	Neg	Neg
147	Pos	Neg	Neg	Neg
149	Pos	Neg	Neg	Neg
150	Pos	Neg	Neg	Neg
152	Pos	Neg	Neg	Pos
153	Pos	Neg	Neg	Neg
154	Pos	Neg	Neg	Pos
155	Pos	Neg	Neg	Neg
157	Pos	Neg	Neg	Neg
159	Pos	Neg	Neg	Neg

Abbreviations: Neg, negative; Pos, positive.

[a] Only samples with a valid test result with all the tests and with genotype(s) detectable by all the assays were included.
[b] Seegene, Seoul, South Korea.
[c] Fujirebio, Pomezia, Italy.
[d] Cepheid, Sunnyvale, California.

When comparing HPV status among methods, taking into account only the 14 genotypes included in all the 3 HPV-DNA detection methods, and considering only the samples with a valid result for all the assays, in 19 cases the Anyplex showed a discordant result with at least 1 other method (Table 5). In particular, the Anyplex showed HPV positivity in 13 cases, all harboring HPV16, which were negative with the other 2 HPV-DNA tests and also with p16 immunohistochemistry. Regarding the remaining cases, in 3 of them (sample ID: 29, 30, and 31), the Anyplex results were concordant with all the other methods except for the INNO-LiPA. In the other 3 cases (sample ID: 32, 152, and 154), the Anyplex was concordant only with 1 of the other 3 assays, in particular with p16 in 2 of 3 cases.

DISCUSSION

In this study, we analyzed the performance of the Anyplex on a large series of FFPE samples from patients with OPSCC, and compared the results with those obtained with the INNO-LiPA, the Xpert HPV, and with the immunohistochemical staining for p16, which were available for all the cases.

Because of the recent understanding of the prognostic and therapeutic implication of HPV etiology in OPSCC, it has become very important to optimize HPV diagnostic workflow in these samples. Often, in patients with OPSCC, an FFPE biopsy sample is the only available material that can be analyzed for HPV detection. Most commercially available assays for HPV-DNA detection have been designed and validated for cytologic cervicovaginal specimens to be used in cervical cancer screening. However, formalin fixation may cause protein cross-linking and nucleic acid degradation, and HPV-DNA methods must be technically validated on this kind of specimen. To the best of our knowledge, only 1 study has investigated the performance of the Anyplex on FFPE tissues.[25] However, this previous investigation analyzed very few head and neck cancer specimens, with most of the samples being from cervical cancer, and the authors did not assess p16 overexpression.

We found good agreement between the Anyplex and both the other 2 methods for HPV-DNA detection as well as p16 immunostaining. In particular, compared with the INNO-LiPA, which is based on the most widely used primer set for HPV-DNA identification in FFPE tissues,[1,28] the Anyplex showed a good agreement and gave a lower rate of invalid results. Moreover, in 2 cases the Anyplex showed HPV positivity, in agreement with Xpert and p16 results, and only the INNO-LiPA had negative findings. These cases are likely to be HPV related but, based on INNO-LiPA results,

these patients would have been deprived, in the near future, of the opportunity of less intense treatments, whereas they have been correctly identified as HPV-positive by Anyplex.

Both Anyplex and INNO-LiPA revealed an HPV26 infection, which is not detectable with Xpert, leading arguably to a false-negative result with this last assay. In a worldwide survey that analyzed more than 1000 OPSCCs, HPV26 was found in 2.6% of these tumors.[1]

Regarding p16, although this biomarker is currently considered as the reference method to establish the HPV etiology in OPSCC,[10] recently it has been suggested that the double positivity for HPV-DNA and p16 can provide better diagnostic accuracy and prognostic value than 1 test alone and can allow a more accurate patient classification.[15] In our study, based on the double positivity for HPV-DNA and p16, 2 additional cases would have been classified as HPV driven with the combination Anyplex/p16 (sample ID 152 and 154), whereas they would have been missed with the INNO-LiPA and Xpert. Accordingly, the HPV-driven fraction was higher with the Anyplex and p16 than by combining p16 with the other 2 HPV detection methods. This finding may support the use of this assay to establish the real fraction of HPV-associated tumors.

The HPV detection rate was slightly higher for the Anyplex than for all the other methods. In particular, we identified 13 cases that were HPV positive only with this method. It is worth noting that these samples were all positive for genotype 16 when using Anyplex. These results may be due to the higher analytical sensitivity of the Anyplex than that of the other assays, although false-positive results cannot be excluded. Moreover, we observed, only with the Anyplex, 4 cases in which 2 genotypes were present. This could be due to the ability of this assay to detect different genotypes even when the relative viral load is unbalanced in favor of 1 predominant type. Interestingly, in all these cases, the Anyplex result was given at low positivity (+) for the genotype not revealed by the INNO-LiPA and high positivity (+++) for the genotype detected also by the INNO-LiPA, suggesting that the former was present at low viral load.

It must be noted that in OPSCC, it is not possible to evaluate the clinical accuracy indicators of an HPV diagnostic method because of the lack of a reference assay and of an unequivocal clinical or diagnostic endpoint. The E6/E7 HPV mRNA detection has been considered the reference standard for the identification of the HPV-driven cases.[29,30] However, its application on FFPE tissues remains challenging. In absence of a gold standard assay, it is not possible to establish which are the really HPV-driven cases and the clinical significance of the presence of HPV viral sequences in cancer samples. Only data on response to therapies and investigations on the clinical outcome of these OPSCCs, which are currently not available for our series, may clarify this point.

We have to acknowledge a few limitations in the use of the Anyplex and, in general, of HPV-DNA detection methods. HPV-DNA does not identify transcriptionally active HPV within the tumor and this limitation may lead to misdiagnosis because of the high analytical sensitivity (ie, very few viral copies detected) and inability to distinguish active and transforming infections. In OPSCC diagnostic workup, a high specificity is required to avoid false-positive cases that may improperly be considered candidates for deintensified treatment protocols. Therefore, HPV-DNA detection methods should not be used as a stand-alone diagnostic test to identify HPV-driven OPSCC, and should be combined with other markers, such as p16 and/or HPV mRNA. HPV mRNA assays, indeed, can detect the infections with an active transcription of viral oncogenes, which are likely to have an etiologic role in tumor development. Unfortunately, we did not analyze our series for HPV mRNA, even though we analyzed all cases for p16 overexpression, which is considered a surrogate marker for HPV activity.

In conclusion, in our study, the Anyplex II HPV28 was suitable for HPV detection in FFPE tissues and showed a higher detection rate than the other methods taken into consideration, being able also to detect coinfections. Longitudinal studies on clinical outcome are needed and could clarify which OPSCCs were truly HPV related.

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