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In situ hybridization for high-risk HPV E6/E7 mRNA is a superior method for detecting transcriptionally active HPV in oropharyngeal cancer

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Running title: mRNA ISH detects active HR-HPV in oropharyngeal cancer

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ABSTRACT

Current human papillomavirus (HPV) detection methods in oropharyngeal squamous cell carcinoma (OPSCC) have varying sensitivity and specificity. We aimed to compare different HPV-detection methods against the test used in clinical practice i.e. p16 immunohistochemistry (IHC) and to evaluate whether another HPV-detection test additional to p16 IHC would be worthwhile in OPSCC specimen. The study cohort comprised 357 consecutive OPSCC patients during two time periods: 2000-2009 and 2012-2016. From tumor tissue slides, HPV mRNA via *in situ* hybridization (ISH), HPV DNA via ISH and HPV DNA via polymerase chain reaction (PCR) were detected. The results of these methods were compared with p16 IHC results. Additionally, clinicopathological factors were compared with the methods studied. The sensitivity of HPV mRNA ISH, HPV DNA ISH and HPV DNA PCR were 93.4 %, 86.3 % and 83.5 %, respectively. The corresponding specificity was 92.4 %, 95.3 %, and 89.1 %, respectively. The negative predictive value for p16 IHC was highest (89.0 %) when using mRNA ISH, and followed by DNA ISH (83.5 %). ISH for high-risk HPV E6/E7 mRNA was found to be a highly specific and sensitive method for detecting HPV in OPSCC. As p16 protein may be overexpressed due to HPV-independent mechanisms, all p16 IHC-positive OPSCCs should be considered for retesting using mRNA ISH in order to verify transcriptionally active HPV. This is especially critical when considering de-escalated treatment approaches for

patients with HPV-positive tumors and still maintaining favorable outcomes for this subgroup of patients.

Keywords

oropharynx; carcinoma; viral infection; RNA; DNA; polymerase chain reaction

1. INTRODUCTION

The incidence of oropharyngeal squamous cell carcinoma (OPSCC) is constantly increasing in developed countries and human papillomavirus (HPV) has been found to be the main reason for this phenomenon [1]. Nowadays up to 70 % of newly diagnosed OPSCCs are HPV positive in Northern Europe and in North-America [2, 3]. Nevertheless, tobacco smoking and heavy alcohol use are still the major risk factors for OPSCC and have a strong role in the pathogenesis of HPV-negative tumors [4, 5]. HPV-positive tumors have different clinicopathological features, biological background and the response to treatment is clearly more favorable compared with HPV-negative OPSCC [4-6]. Consequently, there are several ongoing trials to find new strategies and key factors for treatment de-escalation for HPV-positive OPSCC patients [7-9]**.**

However, for detecting biologically active HPV a wide range of available methods exists, with variable sensitivity and specificity levels [10, 11]. Especially for treatment de-escalation it would be crucial to know whether the patient really has a HPV-positive tumor. Currently, the HPV status can be detected by DNA or RNA *in situ* hybridization (ISH), by polymerase chain reaction (PCR) or by detecting HPV E6/E7 mRNA transcripts by quantitative reverse transcriptase-PCR (qRT-PCR). Immunohistochemistry (IHC) of a surrogate marker p16 protein ($p16^{INK4A}$) is an indirect way to indicate presence of HPV. As HPV-driven carcinomas are dependent on the permanent overexpression of the HPV E6 and E7 mRNAs, HPV E6/E7 mRNA qRT-PCR has been regarded as

a gold-standard method for testing HPV in tissue samples. [12, 13] However, the use of these techniques is not entirely straightforward [11, 14]. PCR techniques warrant DNA or RNA extraction from formalin-fixed paraffin-embedded (FFPE) samples and require more specific techniques to proceed for example fragmented or ruptured RNA after FFPE sample processing. On the other hand, use of frozen material forms a technically demanding process for routine and can add extra costs being more labour-intensive. [11] Additionally, detection of HPV DNA by PCR is highly sensitive but lacks specificity, as it does not distinguish between transcriptionally active and clinically irrelevant HPV infections and thus, the detected DNA may not originate from tumor tissue [11, 14]. Moreover, HPV DNA PCR and ISH have been reported to have inadequate sensitivity when viral load is low, although the HPV ISH specificity is based on direct visualization of virus in tumor cell nuclei [11, 14].

Detection of p16 protein overexpression by ICH has been used as a surrogate marker to indicate presence of an oncogenic HPV infection in OPSCC [15, 16]. Strong p16 overexpression has been shown in OPSCC to have high sensitivity and correlation between transcriptionally active HPV infection and outcome, independent of treatment strategy [12]. In addition, it is easily achievable and the recent 8th edition of TNM classification distinguishes OPSCC tumors as HPV positive and HPV negative by p16 IHC [17]. However, there is still a percentage of OPSCCs that are positive for p16 IHC but lack active HPV [9, 18], suggesting that molecular mechanisms other than HPV pathway oncogenesis can additionally lead to increased expression of p16 impairing the specificity [12]. This could be due to inactivation of pRb by gene deletions, point mutations, functional mutations, or other mechanisms of Rb pathway deregulation [9]. Moreover, several studies have shown reverse results reporting poor survival rates for p16-positive but HPV-negative HNSCC patients compared with double-positive (p16+/HPV+) patients [18]. Argumentation of p16 specificity and outcome correlation has been crucial when considering p16 IHC as a single test for HPV detection. During the last few years RNA ISH technique has been proven to be a feasible

method for detecting RNA transcripts i.e. for example the transcriptionally active HPV viral oncogenes E6/E7 mRNA in histological FFPE samples. HPV E6/E7 genes are highly type specific for different subtypes of HPV and can therefore be used to test for the most common and virulent viral types. Thus, a method detecting E6/E7 mRNA in a clinically relevant high-risk HPV type is thought to be appropriate tool for routine diagnostics when available in kit formats [14].

In the present study, our aim was to compare p16 IHC with three different detection methods for HPV DNA or HPV mRNA in a series of OPSCC samples. Additionally, the aim was to investigate whether it would be feasible to have another test beside p16 IHC to detect clinically relevant HPV in tissue samples.

2. MATERIALS AND METHODS

2.1. PATIENT MATERIALS

The present study is based on two different OPSCC patient cohorts, patient material I (PM I) and patient material II (PM II), which were collected during different time periods. The older PM I was collected retrospectively and the newer PM II prospectively, and they were processed individually as separate groups due to differences in HPV methodology and TNM classification.

2.1.1. PM I

The PM I is a cohort covering a 10-year period from 2000 to 2009 and comprises 202 consecutive OPSCC patients diagnosed and curatively treated at the HUS Helsinki University Hospital. Clinicopathological data were collected from hospital registries and recorded as described in our

previous study [19], and TNM classification and staging was done according to the 7th edition of AJCC Cancer Staging Manual [20].

2.1.2. PM II

The PM II consist of 155 consecutive biopsy-proven OPSCC patients diagnosed during a four-year time period between February 2012 and March 2016 at the HUS Helsinki University Hospital. Clinicopathological data were collected from hospital registries and the TNM classification and staging were done according to the 8th edition of AJCC Staging Manual [17]. The patient series is partly the same as in our two previous reports with more detailed description [6, 21].

2.2. Tissue microarray blocks

Tissue microarray blocks (TMA) were prepared from both materials and the methodology is described in our earlier studies [19, 22]. To produce TMA blocks of the H&E-stained tumor-tissue samples the tumor areas were annotated. Six representative core punctuates (one mm in diameter) of each tumor were detached and placed into a paraffin block with a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA).

2.3. *In situ* hybridization for high-risk HPV E6/E7 mRNA

For both PM I and II, ISH for high risk HPV E6/E7 mRNA was performed manually using the RNAscope® 2.5 HD Reagent kit (Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer´s protocol. Five-µm formalin-fixed and paraffin-embedded tissue microarray (TMA) sections were incubated for 1 hour at 58°C. After deparaffinization the sections were pretreated

with hydrogen peroxidase (RNAscope® Hydrogen Peroxide) for 10 minutes at RT. Target retrieval was performed (RNAscope® Target Retrieval Reagents) for 15 minutes at 100°C. The sections underwent protease treatment (RNAscope® Protease Plus) for 30 minutes at 40°C in hybridization oven followed by hybridization with HR HPV 18 cocktail probe (RNAscope®) for genotypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82 for 2 hours at 40°C in hybridization oven. Preamplifiers and amplifiers were hybridized consecutively, accompanied with chromogenic signal detection with DAB. Finally, the slides were counterstained with hematoxylin. An endogenous housekeeping gene HS-PPIB (RNAscope®) probe was used as a positive control and a bacterial gene DapB, diaminopimelate (RNAscope®) probe as a negative control. The staining was examined using a qualitative scoring system; punctate brown, granular nuclear and cytoplasmic dots were observed being present or not at the individual TMA spots. A finding of one to three brown dots or more per tumor cell was regarded as a positive staining result. The scoring was performed independently by two researchers (J.H. and R.RB.) and incoherent cases were re-scored.

2.4. p16-INK4a immunohistochemistry

For both PMI and PMII, p16-Ink4a IHC was performed and analyzed as described earlier [6, 19, 22]. Four-micrometer-thick tissue slides were cut, deparaffinized in xylene, and rehydrated in a graded series of alcohol. Slides were treated in a PreTreatment module (Lab Vision Corp., UK Ltd, UK) in Tris-HCl buffer (pH 8.5). Blocking of endogenous peroxidase was carried out with 0.3% Dako REAL Peroxidase-Blocking Solution. In both materials monoclonal mouse anti-human p16INK4a (9517 CINtec Histology Kit, MTM laboratories, Germany) was used as a primary antibody. Antibody incubation was carried out with the Dako REAL Antibody Diluent S2022. Epitope retrieval was carried out with Tris-HCl and Dako REAL DAB+ Chromogen was used for

staining visualization. The incubation time was 30 minutes for p16INK4a antibody and a positive control was used. Incubation of negative controls was performed in diluent lacking the primary antibody. Counterstaining was performed with hematoxylin. The tumor was regarded as p16 positive if over 70 % of cells were strongly immunopositive according to WHO recommendations [23]. In all cases p16 immunopositivity scoring was very clear and without discrepancies in interpretation.

2.5. *In situ* hybridisation for high-risk HPV DNA

With PM I, Ventana Inform HR-HPV *in situ* hybridization assay with a high-risk HPV probe and iVIEW Blue detection kit and the Benchmark XT series stainer (Tuscon, Arizona, USA) was used. Tissues sectioned at 5 μm thickness were used. Extended Ventana cell conditioning solution (CC2) was used and pretreatment was carried out with an incubation time of 32 minutes with ISH protease 3. In the assay, the following HR-HPV subtypes have been demonstrated to be detected: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. HPV status was regarded as positive if any spot had nuclear HPV-DNA positivity in the carcinoma sample [19].

2.6. HPV PCR

With PM II, PCR assay was used for detection of HPV DNA in tissue samples. DNA was extracted from 4-10 um thick sections cut from paraffin embedded formalin-fixed blocks and amplified with primer sets 1 and 2 from the Multiplex HPV Genotyping Kit® (DiaMex GmbH, Germany). Primer Set 1 contains all HPV primers: nine biotinylated forward and three reverse primers for amplifying the HPV types under investigation. Primer Set 2 (DNA quality control primers) contains primers for the amplification of a ß-globin gene fragment to verify the amount and the quality of human

genomic sample DNA. A negative control contained no genomic DNA to confirm the absence of a contamination in the amplification reactions. HPV genotyping was performed with a Multiplex HPV Genotyping Kit® (DiaMex GmbH, Germany), which detects 24 LR- and HR-HPV-genotypes as follows: LR-HPV6, 11, 42, 43, 44, and 70; and HR-HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. The labeled hybrids were analyzed with a Luminex LX-100 analyzer (Bio-Plex 200 System, Bio-Rad Laboratories, Hercules, USA). A median fluorescence intensity (MFI) of at least 100 beads was computed for each bead set in the sample. In the present study, only HR-HPV-positive tumors were classified as HPV-DNA PCR positive.

2.7. Statistical analysis

Data were analyzed with a statistical software program (IBM SPSS Statistics 25, IBM, Somers, IL, USA). Concordance and correlation with HPV status and the clinicopathological factors were analysed between mRNA ISH, p16 IHC, DNA ISH and DNA PCR (Supplementary Table 1) and (Supplementary Table 2). Sensitivity, specificity, positive and negative predictive values (PPV and NPV) for the studied methods were assessed and compared with reference test p16 IHC (Table 1). In addition, results of mRNA ISH were evaluated against those of DNA ISH and DNA PCR. Chi-squared and Fisher exact tests were used for categorical data cross tabulation (Supplementary Table 1 and 2). The independent samples t-test was used for comparing means of dichotomized variables (Supplementary Table 1 and 2). A two-sided *P*-value of less than 0.05 was considered statistically significant.

2.8. Ethical considerations

Ethics approval and consent to participate: Research Ethics Board approval and an institutional study permission were granted at the Hospital District of Helsinki and Uusimaa, Finland (DNr: 51/13/03/02/2013). We declare that the study design complies with the Declaration of Helsinki.

3. RESULTS

We used two separate patient cohorts (PM I and II) for comparing different HPV assays (p16 IHC, mRNA ISH, DNA ISH and DNA PCR) and the results are described in Supplementary Table 1 and 2. The detailed discussion about the patient demographics have been published by Jouhi et al. [19] for PM I and by Carpen et al. [6] for PM II. As expected, we found strong concordance between all the methods when compared with clinical factors.

We evaluated the sensitivity, specificity, and positive and negative predictive values for mRNA ISH, DNA ISH and DNA PCR against p16 expression by IHC (Table 1). As the HPV-mRNA ISH analysis was done similarly for both cohorts, results were combined in order to reach larger numbers and thus statistical reliability. For HPV DNA, different methods were used in PM I and II, as described in the Materials and Methods section. All tests showed relatively high specificity, as expected (Table 1). mRNA ISH showed highest sensitivity with the reference test p16 IHC. In relation to the findings with p16 IHC, HPV mRNA ISH resulted in 15 (11 %) false negative and 10 (5 %) false positive samples of the 357 samples tested with mRNA. Two of the 15 discordant cases (p16+/mRNA ISH-) and four of the other 10 discordant cases (p16-/mRNA ISH+) were HPV positive by a DNA-based method (Table 3).

When mRNA ISH status was used to classify for clinical factors, the results were concordant with other HPV detecting methods, as expected (Supplementary Table 1 and 2). Non-smokers and ex-

smokers were mostly HPV positive by mRNA ISH whereas current smokers were mRNA ISH negative in both cohorts (P<0.001). Similarly, in both groups lower alcohol consumption correlated to mRNA ISH positivity (P<0.001). Tumor grade of mRNA ISH -positive group was significantly higher compared with mRNA ISH-negative group in both materials (P<0.001). Tumor spread to local lymph nodes was significantly more common in HPV mRNA-positive tumors compared with mRNA-negative ones both in PM I and PM II. Clinical stage III-IV represented most of the cases in HPV mRNA ISH-positive group (P<0.007). In PM II, significantly (P<0.001) higher number of patients with HPV mRNA-positive tumor had stage I-II disease compared with patients with a mRNA-negative tumor. Male gender was dominant among HPV mRNA ISH-positive patients in PM II (P<0.026).

4. DISCUSSION

Numerous studies have demonstrated patients with HPV-positive OPSCC to have clearly improved survival outcomes compared with patients having HPV-negative cancer [4, 8, 9]. Several on-going trials aim to de-escalate treatment for HPV-positive patients who usually have better prognosis [7-9]. This highlights the need to distinguish the actual HPV-positive cancers from the negative ones. Our aim was to compare p16 IHC to mRNA ISH and other commonly used methods to detect HPV in OPSCC patients. Additionally, the aim was to survey whether another test besides p16 IHC would be advantageous to detect clinically relevant HPV infection and to be used at a clinical pathology laboratory setting. Our results indicate the mRNA-ISH method to have excellent specificity without compromising sensitivity in detecting truly HPV-induced oropharyngeal cancers. Our results confirm findings in earlier reports [24].

Different methods to test the presence of HPV from FFPE samples are available and all of them have their advantages and challenges [11, 13]. IHC staining as a diagnostic method is robust, cost-

effective and easy to be arranged for routine use, which together with high sensitivity supports the use of p16 IHC as a test for HPV detection in tissue samples [18]. Numerous studies have shown p16 overexpression to be a sensitive marker of biologically relevant HPV infection in OPSCC tissue samples [18]. Both our patient materials showed high correlation between the HPV-mRNA ISH and p16 IHC assays. Altogether, ten cases were HPV-mRNA ISH positive and p16 negative (Table 2). The reason for this could be transcription of viral mRNA that gives a natural target amplification step that remarkably improves viral detection in tissue samples and can lead to improved sensitivity of mRNA ISH method [25, 26]. Another reason may be a technically undetectable threshold for p16 immunostaining and thus a lower sensitivity for HPV [27]. In addition, a negative staining result for p16 can be due to HPV-mRNA expression without any protein translation and no effect on p16-protein expression. More importantly, 15 cases were p16 positive but HPV-mRNA ISH negative (Table 2). This is consistent with earlier studies where p16 has been reported to have suboptimal specificity for HPV infection [12]. According to previous studies, we could speculate that in these cases, pRb inactivation leading to p16 positivity may be induced by other mechanisms such as point deletions and gene mutations without the presence of HPV [9, 27]. Clinically this patient group is most relevant as without confirmed HPV positivity the p16-positive OPSCC patients may be treated by de-escalated therapy, leading to poorer outcomes [8, 9]. Many previous studies have reported that 8-20 % of the p16-positive OPSCCs do not contain HPV DNA and this percentage may even be increased in other HNSCC subsites [11, 27]. Thus, it could be suggested that HPV status should be confirmed in all p16-positive cases by another method. Further, results from earlier studies have introduced similar recommendations [25, 26].

In many clinical pathology laboratories, HPV-DNA ISH has been established as a routine assay to detect HPV infection in OPSCC samples besides p16-IHC testing. DNA ISH is easy to perform on FFPE samples and has good specificity [12]. HPV-DNA positivity is detected either as integrate (punctuate nuclear) or episomal (diffuse nuclear) staining [10]. However, detected expression

cannot differentiate biologically active HPV from inactive DNA [10]. In our material, when compared to p16, DNA ISH had lower sensitivity than mRNA ISH. This may be due to low HPV-DNA amount in tumor tissue that remains nonrecognizable to DNA ISH [10, 12]. Possible detection of HPV DNA as reported before might reflect silent HPV-infection without any viral replication [28]. Instead, with mRNA ISH only E6/E7 transcripts are detected [29].

In clinical practice, several PCR assays are used and their diversity in HPV diagnostics is rather wide [11]. PCR is well recognised as a high-sensitivity test but still has limited specificity, several studies reporting p16 positive cases even to remain HPV-PCR negative [12, 24, 28]. On the contrary, when compared with p16, our results showed HPV-DNA PCR to have lower sensitivity and better specificity, consistent with the idea of p16 upregulation being HPV independent. Falsely, negative DNA-PCR results may result from inadequate formalin fixation, in case of low viral DNA concentration or poor DNA integrity [30]. In addition, competition for reagents can cause some drawbacks in PCR due to many type of HPV infections with low copy number leading to false negative results [31]. Therefore, either the presence of HPV or its´ genotypes can remain undetected with PCR [31]. In our study, when compared to p16 specificity HPV PCR was the lowest of all the methods studied. When using PCR-based methods, it is not always possible to determine whether the DNA is from the tumor cells or surrounding normal tissue and additionally, contamination is possible [32].

Our experience of using HPV-mRNA ISH for detection of HPV-E6/E7 transcripts in OPSCC samples is extremely promising. Concordant with earlier studies, we found mRNA ISH to be highly sensitive and specific for HPV detection in OPSCC when compared to p16. [24, 25]. mRNA ISH is straightforward to be performed manually but is additionally available in a clinical automated platform [14]. Automaticity shall enhance standardization and allow large sample volumes to be tested within a reasonable time in a clinical laboratory setting. Therefore, fast automatic and standardized laboratory analyses will obviously save time, labor costs, and presumably lead to more

reliable and accurate diagnostics. By allowing direct single-molecule visualization of viral transcripts in tissue sections, HPV-mRNA ISH confirms the presence of transcriptionally active HPV virus [24, 26]. In addition, direct visualization in tissue reduces the risk of false positive results [11]. Double z-probe design has a relatively short target region, which allows hybridization of partially degraded RNA [29]. Our study revealed 15 cases with p16 IHC positivity but HPV-mRNA ISH negativity. Patients with a discordant (p16 IHC+/HPV-) tumor have been related to significantly worse prognosis compared with patients who has a confirmed HPV-positive (p16 IHC+/HPV+) tumor [9, 33]. Thus, the use of p16 IHC as a stand-alone test for HPV detection can mislead diagnostics and treatment approaches, particularly when considering treatment de-escalation [9]. p16 IHC is only a surrogate marker for HPV detection and even though the recent guideline of College of American Pathologists (CAP) recommend the use of p16 IHC to detect HPV, they suggest that an additional HPV-specific test is considered to be performed on the discretion of the pathologist´s, in agreement with the clinician or in the context of a clinical trial [34]. DNA ISH and DNA PCR have been used with p16 IHC as a two-step combined algorithm for testing HPV but they have resulted in relatively limited sensitivity and specificity, respectively [11, 14, 34]. In our study DNA ISH and DNA PCR advocated lower sensitivity when compared with p16 IHC than mRNA ISH. This reinforces the idea of the mRNA ISH method being one of the two methods in the combination HPV testing as also earlier studies has proposed [25, 26]. At the same time when the combination test may increase diagnostic accuracy, eventually a single test would be preferable in HPV testing since combined algorithms are often laborious and usually increase costs and workload [11, 32]. The costs related to the mRNA ISH methodology can decrease if more evidence of its strenghts can be confirmed in the future and if it could even work as a single test in clinical routine as recently speculated [18]. The CAP has also speculated that mRNA ISH may be the test of choice in future if it comes widely available in clinical practice [34]. A PCR-based method is presented to be the most expensive HPV-detection method, followed by mRNA ISH,

DNA ISH and p16 IHC [18]. However, the most accurate method/combination of all methods that lead to precise diagnosis and treatment modalities, and further resulting in an as favorable as possible outcome presumably should decrease overall health-care costs.

In our material there were two cases in which HPV-mRNA ISH was negative but all the other methods (p16 IHC and DNA ISH/PCR) showed positivity, being in concordance with earlier studies. This demonstrates DNA ISH and DNA PCR to have lower specificity, when compared with mRNA ISH, also suggesting that DNA-based methods may find false positive cases [24, 35]. Additionally, low or absent expression of E6/E7 mRNA leading to low sensitivity of mRNA ISH could be the reason for false-negative cases as suggested before [11, 28]. Pre-analytical factors such as insufficient fixation and sample processing may produce suboptimal preservation of target mRNA molecules. This may lead to reduced sensitivity of mRNA ISH and may offer an explanation to our false-negative results [11].

Limitations of the present study include the lack of parallel HPV DNA results obtained by an identical method in both patient materials. Patient materials were collected in different time periods and different methods were used to detect HPV. Due to a relatively small amount of sample material in individual spots in the TMA blocks, we were not able to apply both techniques for both materials. Additionally, our tissue material lacked matched fresh frozen samples to detect viral HPV E6/E7 mRNA transcripts by qRT-PCR, which would have allowed us to compare it to mRNA ISH. The large number of patients is a strength of this study. Additionally, our result of mRNA ISH on OPSCC samples supports the extension of the method to other HPV-associated carcinomas.

5. CONCLUSION

Our aim was to compare different existing HPV detection methods using p16 IHC as a reference test, and to evaluate the need for a parallel test with p16 IHC in detecting HPV infection in OPSCC

patient samples. Our results show that ISH for high risk HPV E6/E7 mRNA is highly specific and sensitive for detecting active HPV in OPSCC. As there is some risk in the use of p16 only as the biomarker for HPV status we suggest the use of HPV E6/E7 mRNA ISH as a verifying test for all p16-positive tumors in routine HPV diagnostics. The use of as highly sensitive and specific method as possible is critical especially when considering de-escalation treatment approaches for patients with a HPV-positive OPSCC.

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Figure captions

Fig. 1 (A) HPV mRNA ISH with punctate-positive staining. (B) HPV mRNA ISH -negative staining. (C) HPV DNA ISH -positive staining. (D) HPV DNA ISH -negative staining. (E) Endogenous PPIB, Cyclophilin B positive control. (F) Bacterial gene DaP negative control. Magnification x120.

Fig. 2 Patient ID 39 in PM I: HPV mRNA ISH positive (A) but HPV DNA-ISH negative (B). Patient ID 39 in PM I was p16 IHC positive. Patient ID 96 in PM II: HPV mRNA ISH negative (C) but p16 IHC positive (D). Patient ID 96 in PM II was HPV PCR negative. Patient ID 140 in PM I: HPV mRNA ISH negative (E) but HPV DNA-ISH positive (F). Patient ID 140 in PM I was p16 IHC negative. Magnifications were x290 and x170.

Table 1. Sensitivity, specificity, positive predictive value and negative predictive value when p16 IHC used as a reference test.

	Sensitivity	Specificity	PPV	NPV
[a]mRNA ISH (TOTAL)	93.4	92.4	95.5	89.0
DNA ISH (PM I)	86.3	95.3	96.2	83.5
DNA PCR (PM II)	83.5	89.1	94.8	69.5

Abbreviations: NPV = negative predictive value; PM = patient material; PPV = positive predictive value. [a]Detection of HPV mRNA for PM I and PM II was combined.

Table 2. Comparison of different HPV detection methods.

Variable	N	p16 + N (%)	p16 – N (%)
[a]mRNA ISH + (TOTAL)	221	211 (95)	10 (5)
[a]mRNA ISH – (TOTAL)	136	15 (11)	121 (89)
DNA ISH + (PM I)	105	101 (96)	4 (4)
DNA ISH – (PM I)	97	16 (16)	81 (84)
DNA PCR + (PM II)	96	91 (95)	5 (5)
DNA PCR – (PM II)	59	18 (31)	41 (70)

Abbreviations: N = number of patients; PM = patient material.
[a]Detection of HPV mRNA for PM I and PM II was combined.

Table 3. Discordant cases (p16/mRNA ISH) and their HPV detection by DNA-based methods.

Variable	Total (N)	DNA ISH + N	DNA ISH – N	DNA PCR + N	DNA PCR – N
p16 -/mRNA ISH + (PM I)	5	2	3		
p16 -/mRNA ISH + (PM II)	5			2	3
p16 +/mRNA ISH - (PM I)	9	1	8		
p16 +/mRNA ISH – (PM II)	6			1	5

Abbreviations: N = number of patients; PM = patient material; ISH = in situ hybridization; PCR = polymerase chain reaction. HPV DNA for PM I was detected by ISH and for PM II by PCR.

Highlights

ISH for high-risk HPV E6/E7 mRNA is a superior method for detecting HPV in OPSCC

All patients with p16-positive tumors should be considered to be retested by mRNA ISH

Precise HPV testing is essential when considering de-escalated treatment in OPSCC

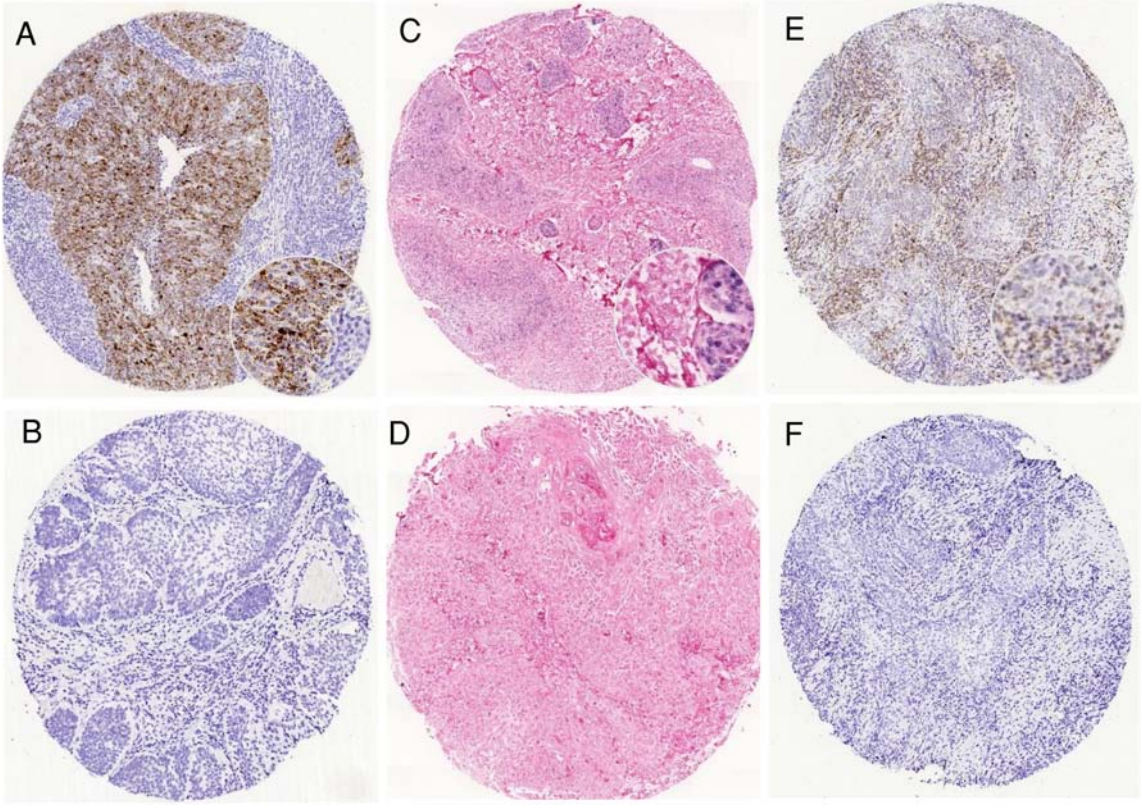

Figure 1

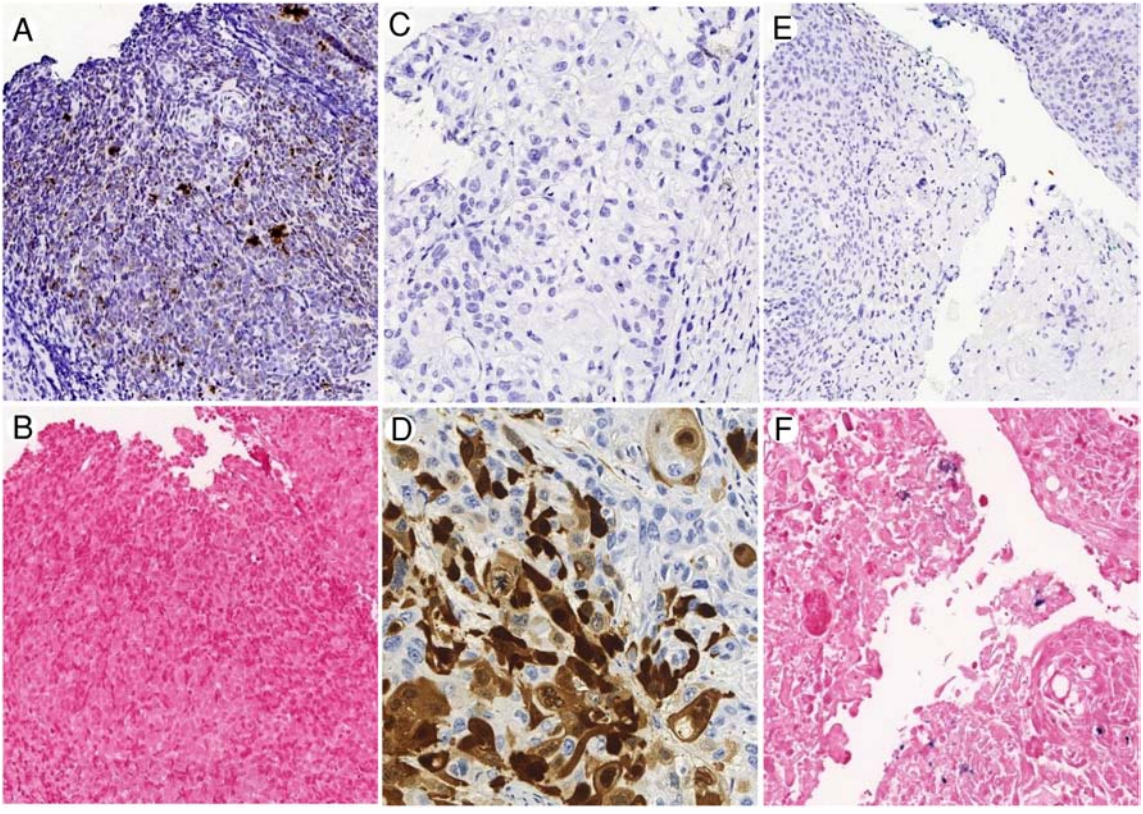

Figure 2