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ORIGINAL ARTICLE

Detection of HPV infection in head and neck squamous cell carcinoma: a practical proposal

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Abstract Detecting human papillomavirus (HPV) infection in head and neck squamous cell carcinoma (HNSCC) is clinically relevant, but there is no agreement about the most appropriate methodology. We have studied 64 oropharyngeal carcinomas using p16 immunohistochemistry, HPV DNA in situ hybridisation (ISH) and HPV DNA polymerase chain reaction (PCR) followed by pyrosequencing. We have also evaluated a new assay, RNAscope, designed to detect HPV E6/E7 RNA transcripts. Using a threshold of 70 % labelled tumour cells, 21 cases (32.8 %) were p16 positive. Of these, 19 cases scored positive with at least one HPV detection assay. Sixteen cases were positive by HPV DNA-ISH, and 18 cases were positive using the E6/E7 RNAscope assay. By PCR and pyrosequencing, HPV16 was detected in 15 cases, while one case each harboured HPV33, 35 and 56. All p16-negative cases were negative using these assays. We conclude that p16 expression is a useful surrogate marker for HPV infection in HNSCC with a high negative predictive value and that p16-positive cases should be further evaluated for HPV infection, preferably by PCR followed by type determination. Using RNase digestion experiments, we show that the RNAscope assay is not suitable for the reliable discrimination between E6/E7 RNA transcripts and viral DNA.

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Introduction

An association of human papillomavirus (HPV) infection with head and neck squamous cell carcinomas (HNSCC) and specifically with oropharyngeal squamous cell carcinoma (OPSCC) has been reported already in the 1980s [1–3]. Recently, this observation has gained wider interest because patients diagnosed with HPV-associated oropharyngeal carcinoma differ from those with HPV-negative cancers. Importantly, patients with HPV-associated disease are younger, lack the classical risk factors of smoking and alcohol abuse, present with more advanced disease and are not uncommonly initially with cervical lymph node metastasis, yet display a markedly improved outcome when compared to HPV-negative cases [4–9]. Moreover, in contrast to alcohol and/or tobacco-associated cases, rising incidence figures of HPV-associated OPSCC, especially in younger patients, have been reported in American as well as in European populations [8, 10–13]. Therefore, detection of HPV infection has become clinically relevant.

While a variety of methods have proved useful in this respect, there is a remarkable degree of uncertainty as to which method(s) should be used in clinical practice. A particular problem is the definition of a gold standard for the detection of HPV infection against which all techniques can be tested [14]. One reason for this is the large spectrum of HPV types. Although the vast majority of HPV-associated HNSCC carries HPV16 [15], this implies that methods relying on the detection of viral DNA, such as polymerase chain reaction (PCR) or in situ hybridisation (ISH), may miss occasional HPV-associated cases, either because of mutations or deletions of primer binding sites

or because certain HPV types may not be included in probe cocktails used in in situ hybridisation. More recently, it has been suggested that detection of HPV E6 and E7 RNA-transcripts may serve as a gold standard [8, 16] since these viral gene products are essential for HPV-induced cell transformation through their interaction with the cellular p53 and pRb proteins [17–20]. While this is an attractive proposition, similar considerations apply as for the in situ detection of viral DNA. Moreover, it has been reported that the proportion of HPV DNA-positive cases is higher than that of cases with detectable E6/E7 transcripts [16, 21, 22]. It has been argued that the detection of HPV DNA in the absence of E6/E7 transcripts is functionally irrelevant and that such cases should be scored HPV negative [21, 23]. Nevertheless, this observation raises the interesting question as to what the role of HPV genomes in cancer cells in the absence of E6/E7 transcripts might be. When viral genomes are detected in DNA extracts from tumour tissues, one might argue that contamination by HPV-infected non-neoplastic cells adjacent to the tumour cannot be ruled out [8]. This, however, is not true for in situ hybridisation studies. Therefore, the recent introduction of a kit-based methodology aimed at the in situ detection of E6/E7 RNA transcripts has been met with great interest [24].

Several studies have suggested that immunohistochemical detection of overexpression of the cellular protein p16 may serve as a surrogate marker of functionally relevant HPV infection [4, 21, 25–29]. The viral E7 gene product interacts with the cellular pRb protein, thus disabling feedback inhibition and inducing overexpression of p16 [17, 25]. However, although many studies have investigated this approach, there is no consensus regarding the definition of p16 positivity [30]. Some studies have scored all cases positive showing at least 1 % labelled tumour cells, more recent studies have employed a threshold of >70 %, and still others have used cut-off values in between [13, 26, 30–34].

The purpose of this study was therefore to compare the suitability of HPV detection methods with particular emphasis on p16 immunohistochemistry in comparison to molecular detection methods such as in situ hybridisation for the detection of viral DNA or E6/E7 RNA transcripts and HPV DNA PCR.

Material and methods

Materials and controls

Paraffin blocks from 64 post-operative surgical specimens of primary OPSCC diagnosed between 1998 and 2010 were retrieved from the files of the Institute for Pathology, Unfallkrankenhaus Berlin according to availability of paraffin blocks. Hematoxylin–eosin-stained sections of all cases were evaluated for keratinising or non-keratinising histomorphology according to the WHO classification of tumours of the head and neck [35]. Tissue microarray (TMA) blocks were constructed for all OPSCC cases (BioCat, Heidelberg, Germany). From each case, three 2-mm-diameter cores, selected from three different representative tumour areas, were included. Additionally, two hyperplastic palatine tonsils were included for control purposes. All materials were submitted for diagnostic or therapeutic purposes and were used in accordance with national ethical principles. Furthermore, paraffin sections from three cell lines, HeLa (HPV18), CaSki (HPV16) and C33 (HPV negative), were available (Ventana, Roche Diagnostics, Mannheim, Germany).

Immunohistochemistry

TMA blocks were sectioned at a thickness of 3 μm. The CINtec®-p16 kit (Roche mtm laboratories AG, Germany) was used according to the manufacturer's instructions. In addition, an antibody specific against p16INK4a (Clone G175-405, BD Pharmingen, BD Biosciences, Franklin Lakes, NJ, USA) was employed. All reactions were carried out on a Ventana Benchmark XT slide stainer (Ventana, Roche Diagnostics, Mannheim, Germany) using diaminobenzidine as a chromogen. A case was considered p16 positive when at least 70 % of neoplastic cells showed strong nuclear and/or cytoplasmatic staining [13, 32, 36]. In addition, the H-score was applied as described recently [16]. In brief, the highest intensity of p16-stained tumour cells was measured on a scale of 0–3 and then multiplied with the percentage of positive tumour cells. The resulting score (0–300) was considered p16 positive when it exceeded 60.

DNA in situ hybridisation

DNA in situ hybridisation (DNA-ISH) for high-risk (hr) and low-risk (lr) HPV types was carried out automatically on a Ventana Benchmark XT slide stainer using INFORM® HPV Probes In Situ Hybridization assay (Ventana), consisting of an hr-HPV-probe cocktail (targeting types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66) and an lr-HPV probe cocktail (targeting types 6 and 11). Briefly, tissue sections were deparaffinised, heat-treated and subjected to protease digestion. The probe for hr types as well as lr types was incubated for 140 min, temporarily heating the tissue to 95 °C (12 min) and 52 °C (4 min). Counterstaining was performed using the red counterstain provided with the kit.

E6/E7 mRNA in situ hybridisation

For detection of E6/E7 RNA transcripts, a commercially available kit (HPV HR7, RNAscope 2.0, Advanced Cell Diagnostics, Hayward, CA, USA) was used according to the manufacturer's instructions. Briefly, tissue sections were

baked for 1 h at 60 °C, followed by deparaffinisation, incubation with pre-treatment reagent 1 for 10 min, pre-treatment reagent 2 for 15 min at 100 °C and proteinase digestion for 25 min at 40 °C. Tissue sections were then incubated with probe cocktail (targeting HPV types 16, 18, 31, 33, 35, 52 and 58) for 2 h at 40 °C. Detection of immobilised probes was done following the supplier's instructions. Diaminobenzidine was used as chromogen. Sections were counterstained with hematoxylin.

To evaluate the specificity for RNA transcripts, RNase digestion was performed with ribonuclease A (Sigma, USA) in a 33.3 μg/ml solution for 30 min at room temperature, followed by two washing steps in dH_2O before hybridisation with the target probe.

As a control for completeness of RNase digestion, in situ hybridisation was carried out using a commercially available kit for simultaneous detection of immunoglobulin kappa and lambda light chain transcripts (Zyto*Fast* human Ig-kappa/Ig-lambda CISH Kit, Zytovision, Bremerhaven, Germany). Briefly, the sections of hyperplastic tonsils were baked at 60 °C for 10 min before deparaffinisation. Enough pepsin solution to cover the section was applied for 20 min at 37 °C. Following protein digestion, RNase digestion was carried out. Next, the κ-λ-target probe was applied and incubated for 2 h at 55 °C followed by washing at 55 °C. Detection of bound probe was carried out according to the supplier's instructions using AEC and NBT/BCIP as chromogens for kappa and lambda light chain transcripts, respectively.

HPV typing

DNA was extracted from FFPE tissue sections of all p16-positive cases and of 15 p16-negative cases, using QIAamp DNA Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol.

The HPV L1 gene region amplification was performed using 25 ρmols of biotinylated GP5/GP6+ primers [37] (Eurofins MWG Operon, Ebersberg, Germany) and 2.5 U of HotStarTaq master mix (Qiagen, Germany), using 95 °C for 15 min for enzyme activation and 95 °C for 40 s for denaturation, a touchdown approach with an initial annealing temperature of 50 °C decreasing by 0.5 °C per PCR cycle down to 40 °C, followed by 1 min at 72 °C for extension [38]. Positive cases were submitted for HPV typing using pyrosequencing assay HPV sign PQ (Diatech Pharmacogenetics, Jesi, Italy) and a Pyromark Q24 instrument (Biotage, Uppsala, Sweden), according to the manufacturers' instructions.

Cases which were negative for HPV DNA PCR with the methodology described above were submitted for HPV DNA detection by nested PCR using PGMY [39] and GP05/06+ [37] primers. The amplicons were purified using GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences Corp., USA) and submitted for DNA sequencing using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction V3.1 (Applied Biosystems). The sequences were obtained from ABI3130 sequencer of Instituto Nacional de Câncer, Rio de Janeiro, Brazil. The HPV-type identification was performed using the software BLASTn and confirmed by phylogenetic analysis carried out with MEGA 5.0, neighbour-joining method, Kimura-2-parameters model [40].

Statistical analysis

A case was considered HPV-associated when positive in DNA-ISH, E6/E7 ISH and/or PCR. Based on this, the sensitivity, positive predictive value and negative predictive value of p16 immunohistochemistry were calculated. Pearson's chi-square and Fisher's exact test were used to test association between dichotomous variables. Differences were considered significant at $p<0.05$ in two-tailed tests. Data were analysed using Statistical Package for the Social Sciences 13.0 (SPSS).

Results

Overall, we examined 64 primary OPSCC. Fifty-three patients (82.8 %) were male, 11 female (17.2 %). Age distribution ranged from 33 to 89 years, with a median age of 66 years.

First, all cases were subjected to immunohistochemistry for the detection of p16 expression. A case was considered p16 positive when at least 70 % of neoplastic cells showed strong nuclear and/or cytoplasmatic staining (Fig. 1a). Using both p16-specific reagents, 21 cases (32.8 %) scored positive for p16 expression (Table 1). In a few cases, weak to moderate staining of smaller proportions of neoplastic cells was observed mainly in basal cells (Fig. 1d). This was particularly noticed with antibody G175-405, although application of the 70 % cut-off to score cases as positive or negative yielded identical results. Applying the H-score to staining with the CINtec®-p16 kit, the same 21 cases scored positive. By contrast, using the G175-405 reagent, eight additional cases scored p16 positive, and several were just below the threshold when applying this scoring system. All of these were HPV negative by all test methods employed.

Subsequently, all cases were tested for the presence of HPV DNA in the tumour cells using a commercially available assay using probe cocktails specific for low- and high-risk HPV types, respectively. All cases were negative for low-risk types, whereas 16 cases (25 %) were positive for high-risk HPV types showing a dot-like blue nuclear signal (Fig. 1b).

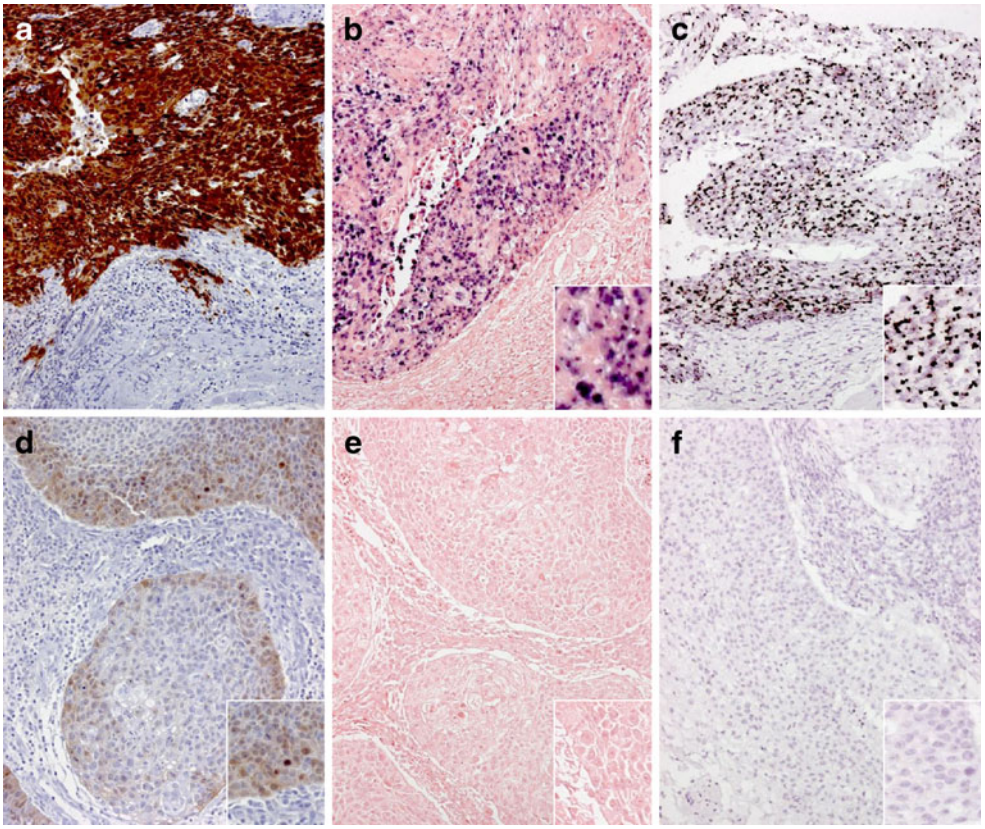

Fig. 1 Results of p16 immunohistochemistry (**a**, **d**), HPV DNA-ISH (**b**, **e**) and ISH using the RNAscope HPV E6/E7 in situ hybridisation assay (**c**, **f**). An HPV-positive OPSCC shows strong cytoplasmic and nuclear p16 expression (**a**), dot-like, blue, nuclear signals following DNA-ISH (**b**) and nuclear, dot-like as well as fine granular cytoplasmic brown signals using RNAscope E6/E7 ISH (**c**). An HPV-negative OPSCC showed weak p16 expression less than 75 % of tumour cells, predominantly in basal cells (**d**). This case was negative in both ISH assays (**e**, **f**)

Next, all cases were subjected to ISH using a commercially available assay designed to detect E6 and E7 RNA transcripts from various high-risk HPV types. Using this approach, 18 cases (28.1 %) scored positive (Fig. 1c).

Table 1 Summary of results

Case	p16	HR DNA-ISH	E6/E7 RNA-ISH	Typing[a]	Localisation	Type
1	+	+	+	HPV35	Base of tongue[b]	k
2	+	+	+	HPV33	Tonsil	nk
3	+	+	+	HPV16	Tonsil[b]	k
4	+	+	+	HPV16	Tonsil	k
5	+	+	+	HPV16	Tonsil	k
6	+	+	+	HPV16	Tonsil	k
7	+	+	+	HPV16	Tonsil	nk
8	+	+	+	HPV16	Tonsil	nk
9	+	+	+	HPV16	Tonsil	nk
10	+	+	+	HPV16	Tonsil	nk
11	+	+	+	HPV16	Base of tongue	k
12	+	+	+	HPV16	Base of tongue	k
13	+	+	+	HPV16	Base of tongue	nk
14	+	+	+	HPV16	Base of tongue	nk
15	+	+	+	HPV16	Base of tongue	nk
16	+	+	+	HPV16	Base of tongue	nk
17	+	-	+	HPV16	Tonsil[b]	nk
18	+	-	+	-	Tonsil	k
19	+	-	-	HPV56	Base of tongue	k
20	+	-	-	-	Base of tongue	k
21	+	-	-	-	Base of tongue	nk

ISH in situ hybridisation, *k* keratinising squamous cell carcinoma, *nk* non-keratinising squamous cell carcinoma

[a]DNA for KRAS gene was amplifiable in all cases by DNA PCR

[b]Initial presentation as lymph node metastasis (CUP syndrome)

Notably, all cases which scored p16 negative by applying the 70 % threshold were also negative using both in situ hybridisation assays (Fig. 1d–f)

All 21 p16-positive cases were next analysed using single-round PCR followed by pyrosequencing of PCR products (Table 1). In this analysis, 18 cases scored positive (28.1 %). Fifteen cases harboured HPV16, one was positive for HPV33, one was positive for HPV56, and a further case contained HPV35 genomes. Three cases scored negative in this assay, as well as in further examination using nested PCR (Table 1). For control purposes, 15 p16-negative cases were also investigated by PCR, yielding negative results in all cases.

A strong correlation was observed between p16-IHC and DNA-ISH ($p<0.0005$, Fisher's test) and p16-IHC and E6/E7-ISH ($p<0.0005$, Fisher's test) as well as p16-IHC and PCR ($p<0.0005$, Fisher's test) (Table 2). As expected, all HPV-associated cases identified by molecular methods (DNA-ISH, E6/E7-ISH or PCR) were also positive for p16 (Table 1).

However, 5/21 (23.8 %) p16-positive cases were negative using hr-HPV DNA-ISH, 3/21 (14.3 %) were negative as assessed using hr-HPV E6/E7 RNA-ISH, and 3/21 (14.3 %) were negative in the PCR assays. Of note, one p16-positive case (case 18, Table 1) was negative as assessed by hr-HPV DNA-ISH as well as by both PCR assays, yet yielded an unequivocal positive result using hr-HPV E6/E7 RNA-ISH. One other p16-positive case (case 19, Table 1) was HPV negative by both in situ hybridisation assays but scored positive by PCR (HPV56; not included in E6/E7 RNA-ISH target probe). These two cases were judged to be HPV positive. There remained two p16-positive cases (cases 20 and 21, Table 1) that were negative using all molecular HPV detection techniques. These cases were scored HPV negative. Thus, in summary, HPV infection was detected in 19 of 64 OPSCC (29.7 %). Three cases (all HPV+) initially presented as carcinoma of unknown primary site (CUP) with lymph node metastasis (Table 1).

Table 2 Correlation between p16 immunohistochemistry results and E6/E7 RNA-ISH, DNA-ISH results and PCR methodology results

	p16+ (%) 21/64 (33)	p16− (%) 43/64 (67)
E6/E7 RNA-ISH		
+	18/21 (85.7)	0/43
-	3/21 (14.3)	43/43 (100)
DNA-ISH		
+	16/21 (76.2)	0/43
-	5/21 (23.8)	43/43 (100)
PCR		
+	18/21 (85.7)	0/43
-	3/21 (14.3)	43/43 (100)
All methods		
+	19/21 (90.5)	0/43
-	2/21 (9.5)	43/43 (100)

Keratinising histomorphology (Table 1) correlated with the absence of HPV infection as detected by all three HPV detection methods ($p<0.0005$). However, 9 of 19 HPV-positive cases and 10 of 21 p16-positive cases showed keratinisation. Moreover, one of the p16+/HPV− cases showed keratinising histology, while three of the p16-/HPV− cases showed non-keratinising histomorphology.

When all of the molecular methods are considered together in comparison to p16-IHC, p16-IHC showed a sensitivity of 100 %, a specificity of 95 %, a positive predictive value of 90 % and a negative predictive value of 100 %.

The molecular methodologies showed a strong correlation between them. Considering the E6/E7 RNA-ISH assay, 16/18 (88.9 %) positive cases were also positive by hr-HPV DNA-ISH, and 17/18 (94.4 %) positive cases yielded a positive result by PCR ($p<0.0005$, Fisher's test).

According to the manufacturer's specification, ISH using the RNAscope assay was designed to detect E6 and E7 HPV RNA transcripts only. To test this, we subjected all cases to ISH using the RNAscope assay subsequent to RNase digestion. RNase digestion conditions were validated using ISH for the detection of κ and λ light chain RNA transcripts on sections of human tonsils, confirming complete abolition of the signal following RNase digestion (not shown). Furthermore, paraffin-embedded samples of CaSki and HeLa cervical cancer cells were examined (Fig. 2a–f). Following RNase digestion, CaSki cells showed only a minimal reduction of signal intensity (Fig. 2a, b), while the signal in HeLa cells was largely abolished, with only a few nuclear dot-like signals remaining (Fig. 2d, e). The signal remaining after RNase digestion was very similar to that obtained by HPV DNA in situ hybridisation in both cell lines (Fig. 2c, f). Notably, the fine granular cytoplasmic staining was lost after RNase digestion.

All of the cases of oropharyngeal squamous cell carcinoma initially positive using the HPV E6/E7 RNA-ISH assay maintained positivity following RNase digestion, however showing a noticeable reduction of signal, especially of the fine granular staining pattern (Fig. 2g–i).

Discussion

It is becoming increasingly clear that HPV-associated HNSCC cases differ from HPV-negative cases in terms of aetiology, epidemiology and outcome [4–8]. The reliable detection of HPV infection in tumour samples has therefore become clinically relevant. In spite of a large number of studies, there is still controversy as to which method or

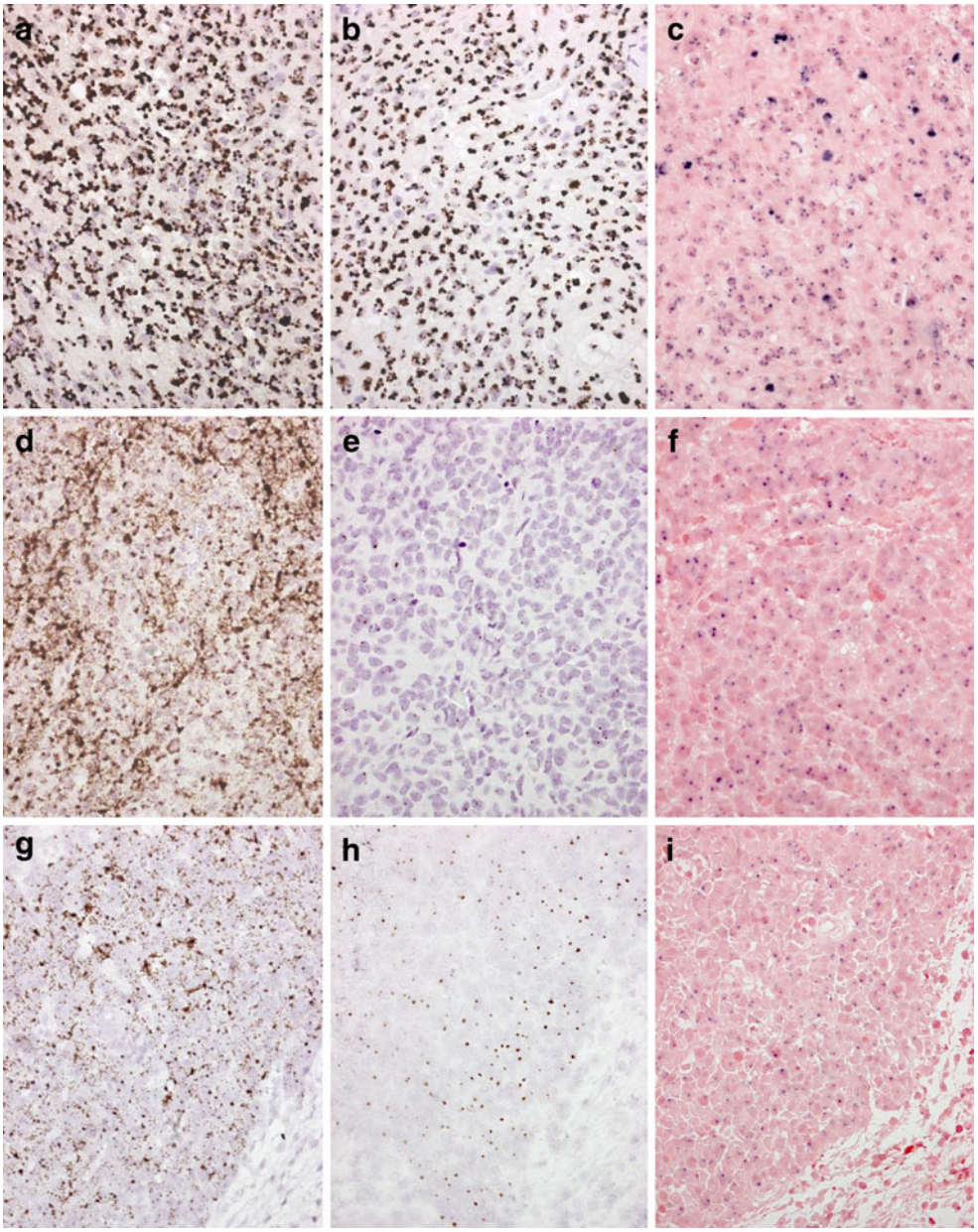

Fig. 2 Validation of the RNAscope HPV E6/E7 RNA-ISH assay by ISH without (**a**, **d**, **g**; brown staining) and following RNase digestion (**b**, **e**, **h**; brown staining) in comparison to HPV DNA-ISH (**c**, **f**, **j**; blue staining). CaSki cells (HPV16 positive) showed a strong coarse predominantly nuclear signal subsequent to ISH with the RNAscope HPV E6/E7 assay (**a**). This signal was only slightly reduced following RNase digestion (**b**). The remaining signal was slightly stronger but essentially similar to that obtained following HPV DNA-ISH (**c**). HeLa cells (HPV18 positive) showed a predominantly cytoplasmic signal using the RNAscope HPV E6/E7 ISH assay (**d**). Following RNase digestion, this signal was largely abolished, but a dot-like nuclear signal remained (**e**) which was again very similar to that obtained by HPV DNA-ISH (**f**). An HPV16-positive OPSCC showed nuclear and cytoplasmic signals using the RNAscope HPV E6/E7 assay (**g**). Following RNase digestion, a dot-like nuclear signal remained (**h**) similar to that seen with HPV DNA-ISH (**i**)

combination of methods is most suited to this purpose. This, however, is of paramount importance since HPV status of HNSCC has to be taken into consideration, e.g. in view of de-escalating therapy for HPV-positive patients as well as for the design of clinical studies.

Several authors have suggested that immunohistochemical detection of p16 overexpression may provide a useful surrogate marker for transformation-relevant HPV infection in HNSCC [4, 21, 25, 26, 28, 29]. We have therefore subjected all cases to p16 immunohistochemistry using two different commercially available reagents. Cases were scored as p16 positive if a minimum of 70 % of tumour cells showed cytoplasmic and/or nuclear labelling as described previously [13, 32, 36]. Using this threshold, 21 of 64 cases (32.8 %) scored p16 positive with both reagents employed, although results obtained with the CINtec®-p16 kit were generally more clear-cut, while immunohistochemistry using the p16-specific monoclonal antibody, G175-405, produced more heterogeneous staining.

Next, all 21 p16-positive cases were subjected to three different molecular methods for the detection of HPV nucleic acids. Furthermore, all p16-negative cases were submitted to HPV DNA-ISH and HPV E6/E7 RNA-ISH. Additionally, 15 p16-negative cases were analysed by HPV DNA PCR. The first conclusion from this analysis is that all p16-negative cases were also negative for HPV nucleic acids in all three assays, indicating that p16-IHC has a high negative predictive value and may be a suitable tool for excluding cases from further analysis. In our hands, application of the threshold of 70 % labelled tumour cells identified the

same cases as p16 positive as application of the more complex H-score [16] when using the CINtec®-p16 kit. By contrast, when using immunohistochemistry and the G175-405 antibody, additional eight cases were scored p16 positive when applying the H-score, all of which were HPV negative as assessed by all three molecular detection systems. Thus, in our hands, usage of the H-score did not prove superior to the application of the simple threshold of 70 % labelled tumour cells.

By comparing the molecular HPV detection methods, HPV DNA-ISH proved to be slightly less sensitive than the other two assays, although the results obtained with all three showed a good correlation. In total, we detected HPV nucleic acids in 19 of 21 p16-positive OPSCC cases, resulting in a total detection rate of HPV in 29.7 % of cases. The most commonly detected HPV type was HPV16, but three other types (HPV33, HPV35 and HPV56) were also detected in one case each. This indicates that strategies testing HNSCC cases only for HPV16 are not sufficient and may miss a proportion of HPV-associated cases. The type distribution is well in line with previous studies [15]. The rate of HPV detection in our study was below the prevalence reported in American studies [6, 7, 10, 27], but it is in agreement with previous studies of OPSCC from European populations [3, 13, 15]. Differences regarding the prevalence of HPV infection in OPSCC series may therefore be accounted for by geographical factors [41]. It has also been suggested that the incidence of HPV-associated cancers has been increasing in recent years [8, 10–12, 42, 43].

Three cases (all HPV+) initially presented as CUP with lymph node metastasis underlining the high incidence of CUP syndrome among HPV-associated OPSCC [9, 36, 44]. The significance of two p16-positive/HPV-negative cases in our series remains uncertain. Some groups have regarded such cases as HPV-associated in case of non-keratinising histology [7, 45]. In our series, non-keratinising histology did not significantly correlate with HPV positivity as detected by any of the HPV detection methods. Thus, while keratinising histology appears suitable in predicting the absence of HPV infection, non-keratinising histology is not a suitable surrogate marker for the presence of HPV infection in OPSCC. Also, it is possible that HPV types not covered by our assays may account for the p16-positive/HPV-negative cases. The single p16+/HPV DNA PCR-/E6-E7 RNA-ISH+ case, even though a rare event in this study, illustrates that HPV DNA PCR using consensus primers may not detect all HPV-positive cases. Possibly, additional use of different primer pairs may improve the sensitivity of HPV DNA PCR [46, 47]. Alternatively, p16 expression may have been up-regulated by other mechanisms, e.g. gene mutation or epigenetic regulation.

Finally, according to the manufacturer's specification, ISH using the RNAscope assay was designed to detect E6 and E7 HPV RNA transcripts only. Two facts led us to assume that this might not be the case. Firstly, the protocol provided by the supplier included a 100 °C heat treatment step, which we suspected to be sufficient to denature viral DNA. Secondly, we observed two types of signal in positive cases, a fine granular cytoplasmatic staining as well as strong staining consisting of variably sized clusters localised mainly in tumour cell nuclei. We show that the signals obtained using the RNAscope assay with HPV E6/E7-specific probes are not completely abolished following RNase digestion. Specifically, the coarse nuclear signal remains in a pattern similar to the signals obtained using HPV DNA in situ hybridisation. We therefore conclude that the RNAscope technique, while a sensitive tool for the detection of HPV infection, is not suitable in discriminating reliably between viral RNA transcripts and viral DNA.

In summary, we show that p16 immunohistochemistry is a useful surrogate marker for HPV infection in HNSCC with a high negative predictive value. p16-positive cases should be further investigated for the presence of HPV nucleic acids. In situ hybridisation provides a sensitive tool for the localisation of HPV genomes in tumour cells. However, using commercially available kits, a precise determination of HPV types is not possible, and therefore, we prefer HPV DNA PCR followed by sequencing of PCR products. In a recent study by Rietbergen et al., this algorithm reached an accuracy of 98 % when compared to an alleged gold standard of viral mRNA expression detected by quantitative real-time PCR on snap frozen tissue [13]. In the rare event of p16-positive and HPV DNA PCR negative results, additional investigation by HPV ISH may help in preventing misclassification. Finally, the RNAscope assay with HPV E6/E7-specific probes is a sensitive assay for the in situ detection of HPV nucleic acids but does not reliably discriminate between E6/E7 transcripts and viral DNA.

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Conflict of interest The authors indicate no potential conflicts of interest.

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