

P16^{INK4A} immunostaining is a strong indicator for high-risk-HPV-associated oropharyngeal carcinomas and dysplasias, but is unreliable to predict low-risk-HPV-infection in head and neck papillomas and laryngeal dysplasias

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Human papillomavirus (HPV) is a risk factor for the development of benign and malignant mucosal head and neck lesions. P16^{INK4A} is often used as a surrogate marker for HPV-infection, although there is still controversy with respect its reliability. Our aim was to determine if p16^{INK4A} overexpression can accurately predict both high-risk and low-risk-HPV-presence in (pre)-malignant and benign head and neck lesions. P16^{INK4A} immunohistochemistry was performed on paraffin-embedded tissue sections of 162 oropharyngeal squamous cell carcinomas (OPSCC), 14 tonsillar and 23 laryngeal dysplasias, and 20 tonsillar and 27 laryngeal papillomas. PCR, enzyme-immunoassay and FISH analysis were used to assess HPV-presence and type. Of the 162 OPSCC and 14 tonsillar dysplasias, 51 (31%) and 10 (71%) were HPV16-positive, respectively. All tonsillar papillomas were HPV-negative and four laryngeal dysplasias and 26 laryngeal papillomas were positive for HPV6 or –11. P16^{INK4A} immunohistochemistry revealed a strong nuclear and cytoplasmic staining in 50 out of 51 HPV16-positive and 5 out of 111 HPV-negative OPSCC ($p < 0.0001$) and in all HPV16-positive tonsillar dysplasias, whereas highly variable staining patterns were detected in the papillomas and laryngeal dysplasias, irrespective of the HPV-status. In addition, the latter lesions generally showed a higher nuclear than cytoplasmic p16^{INK4A} immunostaining intensity. In conclusion, our data show that strong nuclear and cytoplasmic p16^{INK4A} overexpression is a reliable surrogate indicator for HPV16 in OPSCC and (adjacent) dysplasias. For HPV6 or –11-positive and HPV-negative benign and premalignant lesions of the tonsil and larynx, however, p16^{INK4A} immunostaining is highly variable and cannot be recommended to predict HPV-presence.

Key words: human papillomavirus, immunohistochemistry, FISH, PCR

Abbreviations: CDK: cyclin-dependent kinase; DAPI: 4,6-diamidino-2-phenyl indole; DFS: disease-free survival; FISH: fluorescence in situ hybridization; HNSCC: head and neck squamous cell carcinomas; HPV: human papillomavirus; HR: high-risk; IN: intraepithelial neoplasia; LR: low-risk; OPSCC: oropharyngeal squamous cell carcinomas; PCR: polymerase chain reaction; pRb: retinoblastoma protein; TSCC: tonsillar squamous cell carcinomas

Additional Supporting Information may be found in the online version of this article.

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Grant sponsor: Medical Research Foundation; **Grant number:** PF126; **Grant sponsor:** Maastricht University Medical Centre; **Grant sponsor:** Dutch Cancer Foundation, The Netherlands; **Grant number:** 2007–3935; **Grant sponsor:** TUBITAK (Turkey) –DFG (Germany) Scientific Exchange Program (to S.E.G.); **Grant number:** 2225-2006

DOI: 10.1002/ijc.28534

History: Received 2 May 2013; Accepted 12 Sep 2013; Online 11 Oct 2013

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What's new?

Human papillomavirus (HPV) is a risk factor for the development of benign and malignant mucosal head and neck lesions. P16^{INK4A} is often used as a surrogate marker for HPV infection, although there is still controversy with respect to its reliability. The aim of this study was to determine if p16^{INK4A} overexpression can accurately predict both high-risk and low-risk-HPV presence in (pre)malignant and benign head and neck lesions. This results show that p16^{INK4A} overexpression is a reliable surrogate indicator for high-risk-HPV16 in oropharyngeal squamous cell carcinomas (OPSCC) and tonsillar dysplasias, but not for low-risk-HPV6/11-positive benign and premalignant tonsillar and laryngeal lesions.

Human papillomavirus (HPV)-infections may lead to the development of head and neck mucosal lesions. High-risk (HR)-HPV16 is involved in the carcinogenesis of oropharyngeal squamous cell carcinomas (OPSCC) and is an indicator of favorable prognosis, independent of the applied treatment modality. The reported incidence of HPV16 in OPSCC ranges from 25% to 93% in different studies, and appears to have increased during the last decade. Also the incidence of OPSCC and its proportion within the total head and neck squamous cell carcinoma (HNSCC) population are increasing.^{1,2} Low-risk (LR)-HPV6 and -11 especially play a role in the development of benign laryngeal lesions. HPV-positivity may help in these lesions to predict a higher risk for recurrence and a lower risk for malignant progression in relation to HPV-negative, smoking-induced laryngeal lesions.³

In general, two methods are used for the detection of HPV-DNA in clinical diagnosis and biomedical research, *i.e.*, polymerase chain reaction (PCR)-based methods and fluorescence in situ hybridization (FISH). PCR is a highly sensitive method, but may not be able to distinguish biologically and clinically relevant HPV-containing lesions from cases with an extracellular virus contamination. Although FISH is a less sensitive method, it is highly specific and has the advantage to visualise the virus-DNA in situ and allows the distinction between an episomal and integrated status. Because both techniques are relatively costly, technically demanding and requiring sophisticated laboratory facilities as well as experienced personnel,⁴ practical alternatives or complementary procedures for HPV-testing have been explored, including the immunohistochemical detection of p16^{INK4A}.⁵

HPV-positive carcinomas are characterized by overexpression of viral oncoproteins E6 and E7 leading to inactivation of p53 and the retinoblastoma protein (pRb), respectively, thereby inducing cell cycle deregulation and inhibition of apoptosis. As a result, cyclin-dependent kinase (CDK) inhibitors, including p16^{INK4A} and p21^{Cip1/WAF1}, and the MDM2 inhibitor p14^{ARF} are generally upregulated, which subsequently leads to downregulation of cyclin D1 and inhibition of its complex formation with CDK4.^{6–9} Of these HPV-associated changes in protein expression, p16^{INK4A} overexpression has been reported to be the most reliable surrogate marker for the presence of HR-HPV in OPSCC.^{6,10–12} In contrast, HPV-negative carcinomas, induced by smoking and alcohol consumption, are generally characterized by inactivation

of the p16^{INK4A} gene through loss of 9p21. Therefore, the choice of p16^{INK4A} immunostaining to distinguish HPV-positive and -negative malignancies is most obvious.^{6,7,13,14}

P16^{INK4A} immunostaining has long been identified as an objective biomarker for HR-HPV-positive (pre)malignancies of the uterine cervix, allowing the unambiguous identification of truly dysplastic cells in biopsies, thereby reducing interobserver disagreement and improving diagnostic specificity.^{6,15,16} In contrast, normal cervical epithelium and inflammatory or metaplastic lesions are usually p16^{INK4A} negative, while genital lesions containing LR-HPV6 or -11 often show only weak p16^{INK4A} immunostaining.^{14,17–19} Moreover, several studies reported a strong correlation between p16^{INK4A} immunostaining intensity and frequency of positive cells with increasing severity of the cervical lesion.^{18,20–22} The College of American Pathologists and the American Society for Colposcopy and Cervical Pathology has recently published their recommendations on using p16^{INK4A} immunostaining as a biomarker in lower anogenital squamous lesions, introducing the concept of “block-positivity,” defined as continuous strong nuclear or nuclear plus cytoplasmic p16^{INK4A} staining of the basal cell layer with upward extension. The authors state that this scoring method allows a categorization of precancerous disease when the differential diagnosis is between a premalignancy [-intraepithelial neoplasia (IN) 2 or -IN 3] and a mimic of a premalignancy known to be not related to neoplastic risk. Furthermore, it can be used when -IN 2 is in the differential diagnosis to distinguish between a premalignant disease and a low-grade lesion/a non-HPV-associated pathology. As such, the method is used as an adjudication tool for cases in which there is a professional disagreement in histologic specimen interpretation.²³

Despite the fact that p16^{INK4A} immunohistochemistry is a low-cost test that can easily be implemented in daily diagnostic practice, its reliability as a surrogate marker for the presence of HR-HPV has been subject of debate.^{18,24} HPV-negative but p16^{INK4A} positive cases, although small in number, have been described in many series. It has been suggested that this p16^{INK4A} positivity results from pRb inactivation by means of mechanisms independent of HPV-infection.^{4–9,25,26} Also the lack of standardization of p16^{INK4A} immunohistochemistry and its evaluation criteria may be a source of misinterpretations. The foregoing arguments have been a reason for hesitation to introduce the p16^{INK4A} test into the clinic.¹⁵

Table 1. HPV-status and clinicopathological features of all head and neck lesion studied

	OPSCC (n = 162)	Tonsillar dysplasia (n = 14)	Tonsillar papilloma (n = 20)	Laryngeal dysplasia (n = 23)	Laryngeal papilloma (n = 27)
Number of different patients	162	12	20	19	14
Gender					
Male	117 (72%)	9 (75%)	12 (60%)	18 (95%)	12 (86%)
Female	45 (28%)	3 (25%)	8 (40%)	1 (5%)	2 (14%)
Mean age at first diagnosis (range)	60 (39–87)	60 (36–79)	42 (5–66)	63 (43–82)	44 (1–72)
TNM status					
T1	23 (14%)				
T2	54 (33%)				
T3	46 (29%)				
T4	39 (24%)				
N0	51 (31%)				
N1	29 (18%)				
N2	64 (39%)				
N3	15 (9%)				
Missing	3 (3%)				
M+	4 (3%)				
Histology grade (all samples)					
Mild		0 (0%)		6 (26%)	
Moderate		3 (21%)		8 (35%)	
Severe		11 (79%)		9 (39%)	
Number of HPV-positive samples	51 (31%)	10 (71%)	0 (0%)	4 (17%)	26 (96%)
HPV-PCR (all samples)					
HPV 6				4 ¹	17
HPV 11				2	9
HPV 16	51	10			
HPV-FISH (all samples)					
Integrated	51	9			1 ²
Episomal		1		4	26

¹Two out of these four HPV6-positive samples were also positive for HPV11.

²This sample showed both episomal and integrated HPV6.

Although numerous studies analyzed the expression of p16^{INK4A} in HR- and LR-HPV-associated cervical premalignancies, these data are scarce for premalignant and benign head and neck lesions, and therefore the aim of the present study was to determine the usefulness of this biomarker in neoplasms of the oropharynx and larynx. For this purpose we analysed a series of 162 OPSCC as well as a range of tonsillar and laryngeal dysplasias and papillomas, part of which was expected to harbor HR- and LR-HPV-types.

Material and Methods

Tumor material and patient data

Biopsy and resection material was obtained from 227 patients resulting in 246 samples, taken at different moments in time. Since our main goal was to evaluate the

potential of p16^{INK4A} immunostaining patterns as a surrogate marker for HPV in daily routine diagnostic practice, *i.e.*, in every single biopsy sample a pathologist receives from the clinic, we present the analyses in the total number of samples in the main text (Table 1 and Figs. 1a and 1b). For comparison we have included the analyses per patient in Supporting Information Table 1 and Supporting Information Figs. 1a and 1b.

Formaldehyde-fixed and paraffin-embedded tissues were selected from the archives of the Departments of Pathology of the University of Cologne, Germany and the Maastricht University Medical Centre, The Netherlands. Patients were diagnosed between 1986 and 2007.

Table 1 summarizes the clinicopathological features of the cases used in this study.

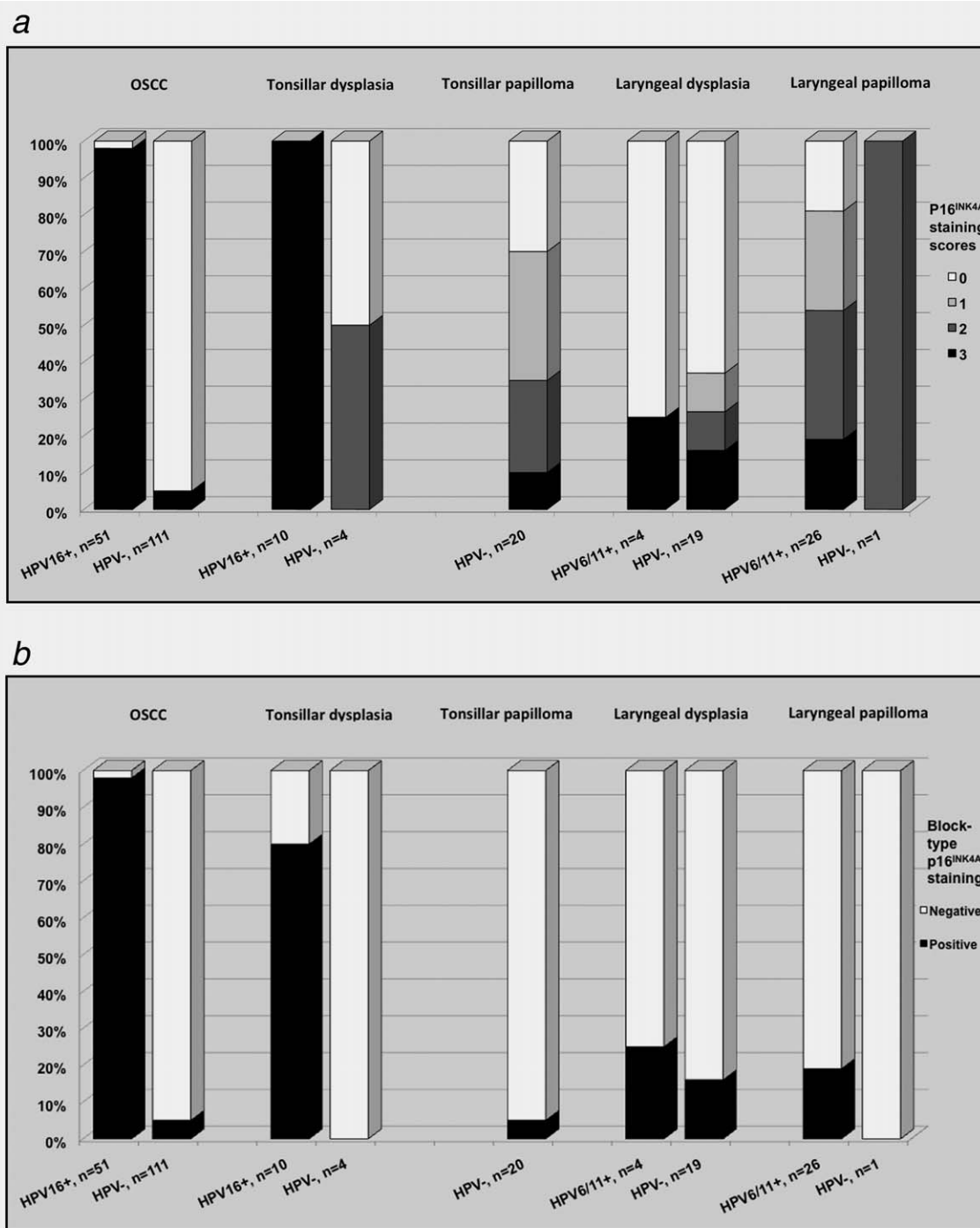


Figure 1. (a) P16^{INK4A} immunostaining scores in all (pre)malignant and benign head and neck lesions. Scoring criteria: Score 0 negative; Score 1: 5–10% of the cells show a nuclear reactivity with or without cytoplasmic positivity; Score 2: focal staining pattern with 10–25% of the cells showing nuclear reactivity with or without cytoplasmic positivity; Score 3: >25% cells show a strong nuclear staining reaction with or without cytoplasmic positivity.¹⁴ (b) Immunostaining results for p16^{INK4A} in all (pre)malignant and benign head and neck lesions based on the block-type scoring approach, defined as p16^{INK4A} only being interpreted as positive if continuous (>70%) strong nuclear with or without cytoplasmic staining is present (in all head and neck lesions) and is observed in the basal cell layer with extension upwards (in the benign and premalignant lesions).²³

Patient age at first diagnosis ranged from 39–87 years (mean 60) in the OPSCC group with no significant difference between the HPV-positive and the HPV-negative cases. In

the papilloma group, the age ranged from 1 to 72 years (mean 43) and in the dysplasia group from 36 to 82 years (mean 62). The specimens included 162 OPSCC (from 162

patients) and 84 benign and premalignant samples (from 65 patients), *i.e.*, 14 tonsillar dysplasias from 12 patients, 13 of which were adjacent to an invasive carcinoma, 20 tonsillar papillomas (from 20 patients), 23 laryngeal dysplasias (from 19 patients) and 27 laryngeal papillomas (from 14 patients).

A correlation of the HPV16 status with the disease-free survival (DFS) data with a maximum follow-up time up to 180 months and a mean of 36 months revealed that the after 5 years was 55% for patients with an HPV-negative carcinoma and 74% for patients with an HPV-positive carcinoma (Hazard ratio = 0.4; 95% Confidence Interval = 0.2–0.8), indicating that we used a representative OPSCC patient group.

The OPSCC and dysplasias were histologically classified according to the criteria of the World Health Organization (C.J.P.-K. and H.P.D.).²⁷ Eleven out of 14 tonsillar dysplasias were graded as severe and three as moderate. From the laryngeal dysplasias six were graded as mild, eight as moderate and nine cases as severe (see Table 1).

P16^{INK4A} immunohistochemistry

Immunostaining of p16^{INK4A} was performed on 4-μm-thick formaldehyde fixed, paraffin-embedded tissue sections, which were deparaffinized using xylol. As a primary p16^{INK4A} antibody clone E6H4 (Roche, Almere, The Netherlands) was used and detected using Powervision (DAKO A/S, Glostrup, Denmark) and peroxidase-DAB visualisation, as previously described.⁷

Three independent observers (J.J.M., S.E.G., E.-J.S.) performed evaluations of the immunostained samples and consensus was acquired about the scores.

P16^{INK4A} immunostaining patterns were first scored on a semiquantitative scale, using the following evaluation criteria: Score 0 indicates that the majority of the cells are negative and that only up to 5% of the cells show nuclear immunoreactivity with or without cytoplasmic staining; Score 1 indicates that 5–10% of the cells show a nuclear reactivity with or without cytoplasmic positivity; Score 2 indicates a focal staining pattern with 10–25% of the cells showing nuclear reactivity with or without cytoplasmic positivity; Score 3 indicates that >25% cells show a strong nuclear staining reaction with or without cytoplasmic positivity.¹⁴

The proportion of positive cells is the primary parameter, with the intensity as a secondary scoring parameter. All cases scored as 2 and 3 showed a strong immunostaining intensity, while few cases scored as 1 stained less intensely positive.

In addition, we scored the p16^{INK4A} staining patterns according to the “block-type” immunopositivity approach, defined as p16^{INK4A} only being block positive if continuous (>70%) strong nuclear with or without cytoplasmic staining is present (in all head and neck lesions) and staining is observed in the basal cell layer with extension upwards (in the benign and premalignant lesions).²³ Controls included sections of uterine cervical IN grade 2 and 3 (positive controls) and sections from the same cases on which the p16^{INK4A} antibody was substituted by a monoclonal mouse

IgG2a antibody to *Aspergillus Niger* or by buffer without a primary antibody (negative controls).

DNA isolation and HPV typing by PCR

For DNA isolation, tissues were processed with the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Total cellular DNA was eluted with 250 μl of the AE-buffer (Qiagen) and 5 μl were used in each of the PCR analyses.

To test the quantity and quality of the DNA samples and to demonstrate that the samples were free from inhibitory substances, PCR was performed for the β-Globin gene, resulting in a 268 bp PCO4/GH20 PCR product.²⁸ As negative controls water and as positive controls human placental DNA were included in each PCR run.

HPV sequences were detected by highly sensitive group-specific nested PCR protocols with degenerate primers A5/A10 and A6/A8 for HPV as previously described.²⁹ PCR products (5 μl) were separated on 2% agarose gels and visualized by ethidium bromide staining. For HPV typing internal biotinylated A6/A8-PCR products (270 bp) were hybridized with 37 type-specific digoxigenin-labeled oligonucleotide probes in an enzyme-immunoassay as described earlier.^{30,31}

Detection of HPV6-, 11-, 16- and 18 by FISH

FISH was performed on 4-μm thick formaldehyde fixed and paraffin-embedded tissue sections tissue as described previously.^{32–34} Briefly, sections were deparaffinized, pre-treated with 85% formic acid/0.3% H₂O₂, 1 M NaSCN and 4 mg/ml pepsin, post-fixed in 1% formaldehyde in PBS, dehydrated in an ethanol series and hybridized with digoxigenin-labeled HPV6-, 11-, 16- and 18-specific probes (PanPath, Budel, The Netherlands), respectively, depending on PCR results, according to the manufacturer's instructions. After hybridization the preparations were washed stringently in 50% formamide, 2× SSC at 42°C (two times 5 min). The probes were detected by subsequent application of mouse anti-digoxigenin (Sigma, St. Louis, MO), peroxidase-conjugated rabbit anti-mouse and peroxidase-conjugated swine anti-rabbit (both DAKO), and visualized by a peroxidase based amplification reaction using rhodamine-labeled tyramide.^{35,36} Preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenyl indole (DAPI; Sigma: 0.2 g/ml). Microscope images were recorded with the Metasystems Image Pro System (black and white CCD camera; Sandhausen, Germany) mounted on top of a Leica DM-RE fluorescence microscope equipped with DAPI and rhodamine filters.

Evaluation of nuclear hybridization signals was performed by two investigators (J.J.M., E.-J.M.S.) according to the criteria described by Mooren *et al.*,³⁷ *i.e.*, nuclear punctate signals were considered to indicate integrated HPV-DNA and diffuse signals to indicate episomal HPV-DNA. Controls included hybridizations on HPV6-, -11-, -16- and 18-positive

formaldehyde-fixed, paraffin-embedded tissues of known human uterine cervical and head and neck (pre)malignancies. Negative controls consisted of HPV-negative tissue sections as determined by PCR and hybridizations omitting the viral probe.

Statistical analysis

HPV-status and p16^{INK4A} immunostaining were correlated using cross-tabulations and the two-tailed Fisher exact test and/or Chi-square test. We regarded a p -value ≤ 0.05 as level of significance. DFS in the OPSCC group was calculated from the date of diagnosis until the date of recurrence (local, regional or distant, whichever occurred first). Patients without recurrence were censored at the date of the last follow-up or the date of death. The statistical significance of differences between survival times was determined by the log rank test in univariate analysis. All statistical analyses were performed by PASW Statistics version 18.0.

Results

HPV-status of the head and neck lesions examined in this study

The HPV-status of the OPSCC, the premalignant and the benign head and neck lesions is presented in Table 1. Only when the presence of HPV was detected by both PCR and FISH the sample was judged to be HPV-positive.

Of the 162 OPSCC 51 (31%) were found to be positive for HPV16. All 51 cases showed integrated HPV16, as concluded from the FISH-analyses. No other HPV-subtypes were detected in these lesions.

Forty of the 84 benign and premalignant lesions (48%) were HPV-positive: 10 (71%) of the 14 tonsillar dysplasias harbored HPV16; 4 (16%) of the 23 laryngeal dysplasias harbored HPV6, of which two also contained HPV11; of the 27 laryngeal papillomas 17 (63%) were positive for HPV6, and nine (33%) for HPV11. The frequency of HPV-infections in these lesions is in accordance with previous studies.^{38–41} Remarkably, all 20 samples of tonsillar papillomas were HPV-negative.

In all HPV16-positive tonsillar dysplasias the virus was integrated (Fig. 2b), whereas in one sample additionally an episomal FISH pattern was detected in the cell nuclei surrounding tonsillar crypts (data not shown). In all, except one sample, of the HPV6- or 11-positive laryngeal lesions the virus was episomal (Fig. 2h).

In the Supporting Information Table 1, the HPV-positivity per patient is presented, for comparison, showing only a slightly difference in the frequency of HPV-positivity in the tonsillar dysplasias (83% per patient vs. 71% per sample) and the laryngeal papillomas (93% vs. 96%).

Strong correlation between p16^{INK4A} overexpression and HR-HPV16 in OPSCC and tonsillar dysplasias

The p16^{INK4A} scores in the HPV-positive and -negative lesions, using the two different scoring methods, are shown

per subgroup in Figs. 1a and 1b. The analyses per patient are presented in Supporting Information Figures 1a and 1b.

The presence of HR-HPV16 in the OPSCC was significantly associated ($p < 0.0001$) with a strong p16^{INK4A} immunostaining reaction (staining Score 3) and with a block positive p16^{INK4A} immunostaining: 50 of 51 HPV16-positive OPSCC showed strong nuclear and cytoplasmic p16^{INK4A} immunopositivity in more than 70% of the tumor cells, whereas only five of the 111 HPV16-negative tumors were p16^{INK4A} positive.

Also all 10 HPV16-positive tonsillar dysplasia samples showed strong (Score 3) p16^{INK4A} expression (Fig. 2a). In eight of these an equally strong staining intensity in the nuclei and cytoplasm could be observed in more than 70% of the dysplastic cells and these were scored as block positive. The remaining two HPV16-positive samples showed a strong (Score 3) p16^{INK4A} immunostaining reaction in 40–50% of the lesion, being block negative.

Of the 4 HPV-negative tonsillar dysplasia samples, two showed an intermediate p16^{INK4A} staining pattern (Score 2) and two samples from one patient were negative (Score 0), implying that these were all block negative.

Highly variable p16^{INK4A} immunostaining patterns in the tonsillar papillomas and laryngeal lesions

In the 20 HPV-negative tonsillar papillomas, the p16^{INK4A} immunostaining patterns were highly variable and ranged from a negative score to a strong staining intensity in 30–80% of the cells (Figs. 2d–2f and Fig. 1a). Only one sample was p16^{INK4A} block positive (Fig. 1b). In 12 (86%) out of the 14 p16^{INK4A} positive tonsillar papillomas, the immunostaining intensity of the nucleus was stronger than that of the cytoplasm.

Of the 23 laryngeal dysplasias, four samples (two from one patient) showed a strong, four a low to intermediate and 15 (two from one patient) no p16^{INK4A} expression (Fig. 1a). Three of the four HPV6-positive lesions, of which two samples from the same patient harbored HPV11-DNA, were p16^{INK4A} negative. The remaining HPV6-positive dysplasia sample showed a strong p16^{INK4A} immunostaining (Score 3) and was also block positive (Figs. 2c and 2g and Figs. 1a and 1b). There was no association between p16^{INK4A} score and the degree of dysplasia. Although all five mild dysplasias were also p16^{INK4A} negative, the moderate and severe dysplasias showed negative to strong p16^{INK4A} staining patterns.

p16^{INK4A} staining patterns in the HPV6 and -11-positive laryngeal papillomas varied from negative to strongly positive. Five out of the 26 HPV-positive laryngeal papillomas were p16^{INK4A} block positive, three of these being from the same patient. Similar to most tonsillar papillomas, all of the p16^{INK4A} positive laryngeal lesions showed a stronger nuclear than cytoplasmic staining intensity.

In the Supporting Information Figures 1a and 1b, the p16^{INK4A}-positivity per patient is presented, showing only minor differences in the frequencies of p16^{INK4A}-

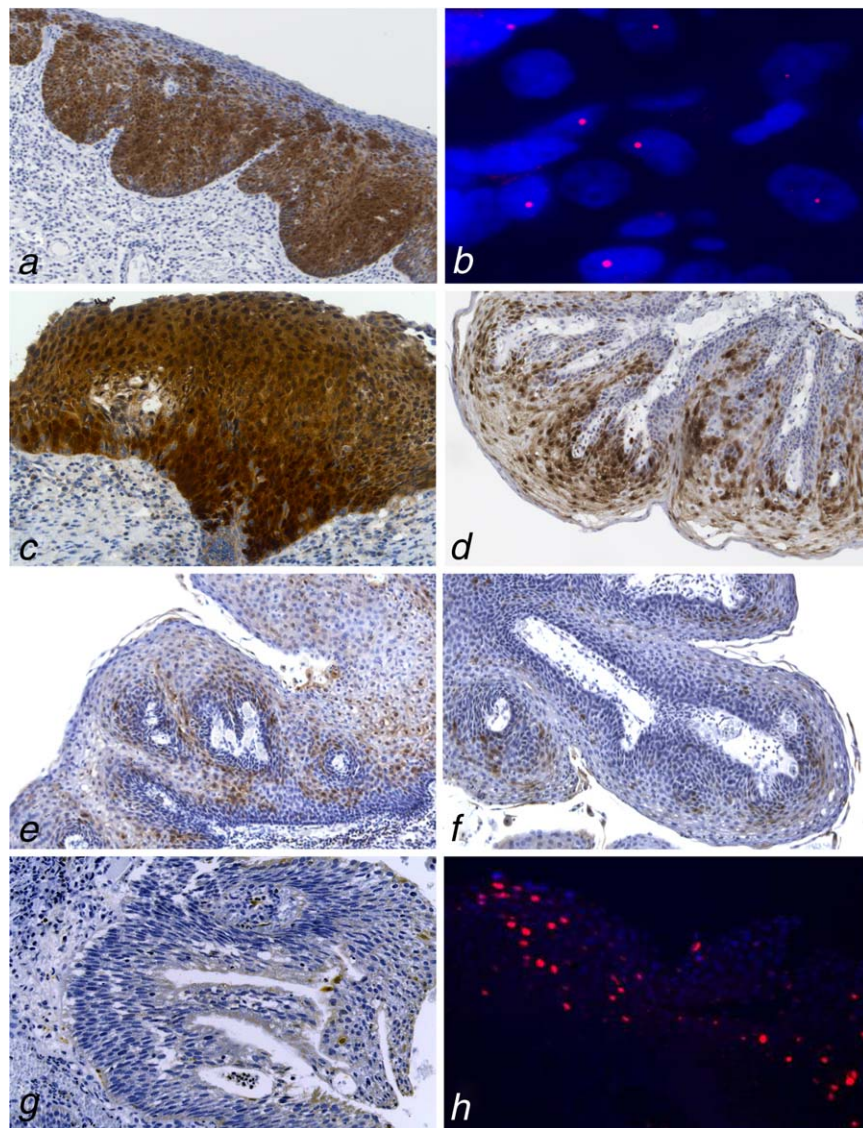


Figure 2. Representative examples of p16^{INK4A} immunohistochemistry (*a, c–g*) and HPV-specific FISH analysis (*b, h*) in head and neck lesions. (*a*) Strong nuclear and cytoplasmic p16^{INK4A} immunostaining (Score 3) and also block-positive p16^{INK4A} staining pattern in a tonsillar dysplasia; (*b*) Punctate signal (red) per nucleus (blue DAPI) indicating integrated HPV16 in a tonsillar dysplasia; (*c*) Strong nuclear and cytoplasmic p16^{INK4A} immunostaining (Score 3) and also block-positive p16^{INK4A} staining pattern in a laryngeal dysplasia sample; (*d*) Tonsillar papilloma sample showing strong nuclear and weaker cytoplasmic p16^{INK4A} immunostaining (Score 3), but scored as being block-negative. (*e, f*) Tonsillar papilloma samples showing a focal staining pattern with 10–25% of the cells showing nuclear and cytoplasmic positivity (Score 2; *e*), and showing 5–10% of the cells with a nuclear and cytoplasmic positivity (Score 1; *f*). (*g*) Laryngeal dysplasia sample showing 0–5% of the cells showing nuclear and cytoplasmic p16^{INK4A} immunopositivity (Score 0); (*h*) Diffusely (red) stained nuclei (blue DAPI) indicating episomal presence of HPV6 in a laryngeal papilloma. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

immunostaining scores, compared to those presented in Figures 1*a* and 1*b* (analyses per sample).

Discussion

Overexpression of p16^{INK4A} has been put forward as a specific surrogate biomarker for the presence of HR-HPV, in particular in (pre)malignancies of the uterine cervix, as well as those in the head and neck region.^{6,7,13,14} However, there still exists controversy on the reliability of p16^{INK4A} expression as indicator for the presence of HPV, particularly in the

latter group. In our study, we analysed the relationship between HR- and LR-HPV-status and p16^{INK4A} immunostaining patterns in 246 samples of benign, premalignant and malignant head and neck lesions. A strong (Score 3) and block positive nuclear and cytoplasmic p16^{INK4A} staining pattern was predominantly found to correlate with HPV16-containing OPSCC and tonsillar dysplasias. In contrast, the tonsillar and laryngeal papillomas, as well as the laryngeal dysplasias showed a highly variable p16^{INK4A} staining pattern, independent of the HPV-status. By using the block-type

scoring approach, however, most of these lesions were interpreted as being p16^{INK4A} block negative.

It is now well established that HPV-associated HNSCC represent a separate entity, which is clinically and molecularly distinct from its tobacco- and/or alcohol-induced counterpart.^{2,7,37} The upregulation of p16^{INK4A} results from inactivation of pRb by the HPV-E7 oncoprotein, or alternatively through E7-mediated epigenetic induction of KDM6B, subsequently leading to activation of the p16^{INK4A} gene.^{6,16}

Although several studies have reported a high interobserver conformity in the evaluation of p16^{INK4A} immunostaining patterns,^{4,14,18,19,42} others have indicated limitations in this procedure. The immunohistochemical p16^{INK4A} staining procedure is subject to variations in the protocol and to difficulties in the interpretation of the staining patterns as a result of different scoring criteria.¹⁷ There is an emerging consensus that only samples showing strong nuclear and cytoplasmic p16^{INK4A} immunostaining, observed in more than 25% of tumor cells, should be considered HPV-positive.^{5,13,14,18,20–22} Recent studies even suggest to use 70% immunopositivity of the carcinoma tissue as a cut-off point.^{4,11,12} In our study, we indeed observed the strong and block positive p16^{INK4A} immunostaining in all except one HPV16-positive OPSCC. Despite the fact that a small fraction of the HPV-negative OPSCC did show p16^{INK4A} positivity, the correlation of this surrogate marker with HPV16-presence is highly significant.

Also in all HPV16-positive tonsillar dysplasias of the present study a strong, and in most cases block positive, p16^{INK4A} immunostaining pattern could be detected, which is in accordance with the results in cervical dysplasias.^{14,19} Similar to OPSCC, also in these lesions we identified p16^{INK4A} positive staining in two HPV-negative samples, albeit with an intermediate score and being block negative.

The use of p16^{INK4A} immunostaining as surrogate marker for HPV-presence in laryngeal dysplasias and head and neck papillomas, however, is unreliable. In the HPV6/11-positive laryngeal dysplasia samples the majority of cases was p16^{INK4A} negative, while 1/3 of the HPV-negative samples did show p16^{INK4A} positivity. LR-HPV-integration seldomly occurs and the only laryngeal papilloma sample harboring nuclear punctate HPV6-FISH signals only showed weak p16^{INK4A} immunostaining. In a recent study on juvenile onset laryngeal papillomatosis progressing to carcinoma in one patient, the papilloma with episomal and the carcinoma with integrated HPV6 were all p16^{INK4A} negative.⁴³

All our tonsillar papilloma samples were HPV-negative, whereas 70% of the samples showed variable p16^{INK4A} positivity, indicating that p16^{INK4A} immunostaining appears of no use for predicting HPV-presence in these lesions. This is in accordance with a previous study on tumor-free palatine tonsils, all proven to be negative for HPV, but showing p16^{INK4A} overexpression in a quarter of these normal cases.²⁵ By using the block-type scoring system, however, most tonsillar and laryngeal papillomas and laryngeal dysplasias were

interpreted as being p16^{INK4A} block negative. This fits with the fact that these head and neck lesions are usually HPV-negative or contain LR-HPV types. Block-positivity is used to identify HR-HPV-containing precancerous anogenital lesions, which is in accordance with the block-positivity observed in our study in OPSCC and tonsillar dysplasias, being associated with HR-HPV.²³

Also at a subcellular level variability in p16^{INK4A} immunostaining patterns could be observed: in the HPV16-positive OPSCC and tonsillar dysplasias we predominantly found an equally strong p16^{INK4A} staining intensity in nuclei and cytoplasm, whereas in the other head and neck lesions the nuclear intensity was generally stronger than that of the cytoplasm.

The fact that studies on oral papillomas showed an HPV-positivity in 13–60%, predominantly HPV6 or –11,^{44–46} makes the absence of HPV in the tonsillar papillomas in our series remarkable. The lack of HPV in these samples might be explained by elimination of the virus by the immune system with persistence of the lesions, or by the fact that other viruses, yet to be discovered, play an etiological role in the development of the tonsillar papillomas. Also the mechanisms underlying the predilection of different HPV-types for different anatomical head and neck lesions remain to be further explored.

The highly variable p16^{INK4A} immunostaining patterns in the benign and premalignant head and neck lesions may be explained by several other factors. It is well described in the literature that in uterine cervical lesions LR-HPV6 results in a less intense p16^{INK4A} immunostaining pattern as compared to HPV16, which is caused by the fact that the affinity of the HPV6 E7 protein for cellular pRb is 10-fold lower than that of HPV16 E7.^{19,47,48} Therefore, it is surprising that our HPV6/11-containing benign and premalignant head and neck samples were often strongly positive, or completely negative for p16^{INK4A}.

Since a subgroup of HPV-negative lesions also shows overexpression of p16^{INK4A}, several factors have been proposed to explain this observation, which remain to be studied. These include infection with other viruses (*i.e.*, cytomegalovirus and adenovirus), which functionally inactivate pRb in a similar fashion as the HPV-oncogene E7,^{49,50} physiological stress, oncogene-driven senescence by functional overactivation of (proto)oncogenes including Ras, Raf, MEK and E2F or replicative senescence due to DNA-damage or oxidative stress.^{51–54}

In summary, our results indicate that a strong nuclear and cytoplasmic p16^{INK4A} immunostaining pattern can accurately predict the presence of HR-HPV16 in OPSCC and tonsillar dysplasias. Our data underscore the proposed cut-off level of 70% p16^{INK4A} positive cells, corresponding to block positive p16^{INK4A} immunopositivity, in these lesions as indicator for HR-HPV16-presence. In the other premalignant and benign head and neck lesions, however, caution is recommended when using this surrogate marker for HPV-infection.

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