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P21$^{Cip1/WAF1}$ expression is strongly associated with HPV-positive tonsillar carcinoma and a favorable prognosis

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Human papillomavirus is involved in the carcinogenesis of tonsillar squamous cell carcinomas. Here, we investigated the expression and the prognostic value of key cell cycle proteins in the pRb and p53 pathways in both human papillomavirus type 16-positive and -negative tonsillar squamous cell carcinomas. Using immunohistochemistry, 77 tonsillar squamous cell carcinomas with known human papillomavirus type 16 status and clinical outcome were analyzed for expression of Ki67, p16^{INK4A,} cyclin D1, pRb, p14^{ARF}, MDM2, p53, p21$^{Cip1/WAF1}$, and p27^{KIP1}. Results were correlated with each other and with clinical and demographic patient data. A total of 35% of tonsillar carcinomas harbored integrated human papillomavirus type 16 DNA and p16^{INK4A} overexpression, both being considered essential features for human papillomavirus association. These tumors also showed the overexpression of p14^{ARF} ($P<0.0001$) and p21$^{Cip1/WAF1}$ ($P=0.001$), and downregulation of pRb ($P<0.0001$) and cyclin D1 ($P=0.027$) compared with the human papillomavirus-negative cases. Univariate Cox regression analyses revealed a favorable survival rate for non-smokers ($P=0.006$), as well as for patients with T1-2 tumors ($P<0.0001$) or tumors showing low expression of cyclin D1 ($P=0.028$), presence of human papillomavirus and overexpression of p16^{INK4A} ($P=0.01$), p14^{ARF} ($P=0.02$) or p21$^{Cip1/WAF1}$ ($P=0.004$). In multivariate regression analyses, smoking and tumor size, as well as expression of cyclin D1 and p21$^{Cip1/WAF1}$, were found to be independent prognostic markers. We conclude that human papillomavirus positivity in tonsillar squamous cell carcinomas strongly correlates with p21$^{Cip1/WAF1}$ and p14^{ARF} overexpression and downregulation of pRb and cyclin D1. In particular p21$^{Cip1/WAF1}$ overexpression is an excellent favorable prognosticator in tonsillar squamous cell carcinomas.
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Head-and-neck squamous cell carcinoma is the sixth most prevalent malignancy in the world, contributing 6% of new cancer cases annually worldwide.[1,2] These tumors have a 5-year survival rate of approximately 50%, which has not improved in the last two decades.[3] Well-recognized risk factors in the etiology of head-and-neck squamous cell carcinomas are extensive tobacco and alcohol consumption in ~90% of cases, as well as oncogenic human papillomaviruses (HPVs), predominantly HPV type 16.[3,4] Interestingly, the association of HPV is strongest for tonsillar squamous cell carcinoma with a prevalence up to 50%.[5–8] The diagnosis of HPV positivity in head-and-neck squamous cell carcinomas appears to have significant prognostic implications. In one study,

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Table 1 Clinicopathological features and cell cycle protein expression in relation to HPV status

Characteristic	*All TSCC* n = *77 (%)*	*HPV-positive TSCC* n = *27 (35%)*	*HPV-negative TSCC* n = *50 (65%)*	*Fisher's exact test* P-*value*
Cell cycle proteins				
Ki67				
Positive	75 (97)	26 (96)	49 (98)	NS
Negative	2 (3)	1 (4)	1 (2)	
$p16^{INK4A}$				
Positive	29 (38)	27 (100)	2 (4)	<0.0001
Negative	48 (62)	0 (0)	48 (96)	
Cyclin D1 >5%				
Positive	33 (43)	7 (26)	26 (52)	0.027
Negative	44 (57)	20 (74)	24 (48)	
Cyclin D1 >50%				
Positive	12 (16)	2 (7)	10 (20)	NS
Negative	65 (84)	25 (93)	40 (80)	
pRb>20%				
Positive	62 (80)	22 (81)	40 (80)	NS
Negative	15 (20)	5 (19)	10 (20)	
pRb intensity in tumor≥ intensity in adjacent squamous epithelium				
Positive	41 (53)	4 (15)	37 (74)	<0.0001
Negative	36 (47)	23 (85)	13 (26)	
$p14^{ARF}$				
Positive	34 (44)	20 (74)	14 (28)	<0.0001
Negative	43 (56)	7 (26)	36 (72)	
MDM2				
Positive	23 (30)	10 (37)	13 (26)	NS
Negative	54 (70)	17 (63)	37 (74)	
p53				
Positive	39 (51)	10 (37)	29 (58)	NS
Negative	38 (49)	17 (63)	21 (42)	
$p21^{Cip1/WAF1}$				
Positive	32 (41)	17 (63)	15 (30)	0.008
Negative	43 (56)	10 (37)	33 (66)	
Unknown	2 (3)		2 (4)	
$p27^{KIP1}$				
Positive	25 (32)	9 (33)	16 (32)	NS
Negative	49 (64)	18 (67)	31 (62)	
Unknown	3 (4)		3 (6)	
Clinicopathological variables				
Gender				
Female	20 (26)	9 (33)	11 (22)	NS
Male	57 (74)	18 (67)	39 (78)	
Age (years)				
<60	41 (53)	13 (48)	28 (56)	NS
≥60	36 (47)	14 (52)	22 (44)	
Death due to TSCC				
Yes	41 (53)	8 (29)	33 (66)	0.002
No	33 (43)	18 (67)	15 (30)	
Unknown	3 (4)	1 (4)	2 (4)	
Death due to any cause				
Yes	48 (62)	10 (37)	38 (76)	<0.0001
No	26 (34)	16 (59)	10 (20)	
Unknown	3 (4)	1 (4)	2 (4)	
Smoking[a]				
Yes	64 (83)	17 (63)	47 (94)	<0.0001
No	12 (16)	10 (37)	2 (4)	
Unknown	1 (1)		1 (2)	
Alcohol[b]				
Yes	46 (60)	12 (44)	34 (68)	0.024
No	29 (38)	15 (56)	14 (28)	
Unknown	2 (2)		2 (4)	
Smoking and/or alcohol				
Yes	68 (88)	20 (74)	48 (96)	0.002
No	8 (10)	7 (26)	1 (2)	
Unknown	1 (1)		1 (2)	
Smoking and alcohol				
Yes	42 (54)	9 (33)	33 (66)	0.003
No	33 (43)	18 (67)	15 (30)	
Unknown	2 (3)		2 (4)	

Table 1 Continued

Characteristic	*All TSCC* n = *77 (%)*	*HPV-positive TSCC* n = *27 (35%)*	*HPV-negative TSCC* n = *50 (65%)*	*Fisher's exact test* P-*value*
TNM classification				
Stage 0–3	39 (51)	13 (48)	26 (52)	NS
Stage 4	37 (48)	14 (52)	23 (46)	
Unknown	1(1)		1 (2)	
T classification				
<4 cm (T 1–2)	37 (48)	17 (63)	20 (40)	NS
≥ 4 cm (T 3–4)	39 (51)	10 (37)	29 (58)	
Unknown	1 (1)		1 (2)	
Tumor grade[c]				
Poor/moderate	63 (82)	24 (89)	39 (78)	NS
Well	10 (13)	2 (7)	8 (16)	
Unknown	4 (5)	1 (4)	3 (6)	
Lymph node metastasis				
Positive	55 (71)	22 (81)	33 (66)	NS
Negative	21 (27)	5 (19)	16 (32)	
Unknown	1 (1)		1 (2)	
Recurrent disease				
Yes	16 (21)	4 (15)	12 (24)	NS
No	32 (41)	16 (59)	16 (32)	
Never disease free	29 (38)	7 (26)	22 (44)	

HPV, human papillomavirus; NS, not significant; TSCC, tonsillar squamous cell carcinoma.

[a]Patiens were classified as daily tobacco smokers (≥ 1 cigarette, pipe, and/or cigar per day) or non-smokers (never smokers or patients who had stopped smoking more than 10 years before the diagnosis of TSCC).

[b]Patients were classified as drinkers (consumption of >2 whiskey equivalents per day (one whiskey is equivalent to ~ 10 g alcohol).

[c]Tumor grade was scored as well, moderately, or poorly differentiated according to the criteria of the World Health Organization.

these patients had $<50\%$ chance of dying from the disease compared with HPV-negative tumors.[9]

It has been shown that there are several differences between HPV-positive and -negative head-and-neck squamous cell carcinomas. Despite the fact that HPV positivity in head-and-neck squamous cell carcinomas is an indicator for favorable prognosis, from a clinical point of view these tumors are often poorly differentiated[4,6,10–12] and metastasized to lymph nodes at presentation.[10,11] Furthermore, HPV-positive tumors are often smaller at first diagnosis (diameter ≤ 4 cm),[13] and associated with low/no exposure to alcohol and tobacco.[10,11] At the molecular level, the functional inactivation of two key tumor suppressor proteins, ie, p53 and pRb by the HPV-derived oncoproteins E6 and E7, often result in the downregulation of p53, pRb, cyclin D1, and a strong upregulation of $p16^{INK4A}$ in HPV-positive tumors.[5,7,14–16] HPV-negative tumors, in contrast, often show inactivation of $p16^{INK4A}$, p53 overexpression as a result of gene mutations, cyclin D1 gene amplification and overexpression, as well as EGFR accumulation.[3,17–19]

The literature, however, shows conflicting data with respect to HPV-associated characteristics and clinical outcome of head-and-neck squamous cell carcinomas. First, although many studies describe a significant association between HPV presence and favorable prognosis, some studies did not find such a correlation.[20–22] Second, HPV has also been identified in head-and-neck squamous cell carcinomas of smokers, significantly reducing its favorable effect on clinical outcome.[13] Furthermore, despite the fact that regional lymph node metastasis is considered as the most important prognostic factor in head-and-neck squamous cell carcinomas,[23] this parameter seems to be unreliable in tonsillar squamous cell carcinomas.[24,25] Finally, some studies reported overexpression and/or p53 mutations almost exclusively in HPV-negative tumors,[20] whereas others have found that HPV infection and p53 alterations can coexist.[26] This may have a strong effect on survival, because it has been indicated that tumors with intact p53 are still capable of inducing apoptosis in response to radiation therapy, which results in a favorable clinical outcome.[27]

This study was undertaken to investigate the expression of key cell cycle proteins in the pRb pathway ($p16^{INK4A}$ cyclin D1, $p27^{Kip1}$ pRb) and the p53 cascade ($p14^{ARF}$ MDM2, p53 and $p21^{Cip1/WAF1}$), using a series of 77 tonsillar squamous cell carcinomas for which the HPV16 status and the clinical follow-up data were available. Tonsillar squamous cell carcinomas show the highest prevalence of oncogenic HPV and are thus ideally suited to search for molecular and clinicopathological differences induced by either HPV, or tobacco and alcohol consumption.

Table 2 Primary antibody characteristics and used evaluation criteria

Antibody characteristics and tissue pretreatment							*Evaluation criteria for positivity*	*References*
Antigen	*Antibody clone*	*monoclonal/ polyclonal*	*Raised in*	*Isotype*	*Dilution*	*Tissue pretreatment[a] (min)*	*Company[b]*	*If % tumor cells with nuclear staining (+, ++, +++)[c]*	
Ki67	Ki-67	Mono	Mouse	IgG	1:50	3 × 5	Dako A/S	> 20%	29–31
$p16^{INK4A}$	E6H4	Mono	Mouse	IgG	1:25	40	Dako A/S	> 25% and/or > 25% cytoplasmatic staining	10,15,32
Cyclin D1	SP4	Mono	Rabbit	IgG	1:50[d]	3 × 5	Labvision	(1) > 5%, and (2) > 50%	29,33,34
pRb	84-B3–1	Mono	Mouse	IgM	1:50[d]	3 × 5	Novocastra	(1) Intensity in tumor ≥ intensity in adjacent squamous epithelium, and (2) > 20%	7,16,33
$P14^{ARF}$		Poly	Rabbit	IgG	1:100	3 × 5	Labvision	> 30% and/or any nucleolar staining	31
MDM2	IF2	Mono	Mouse	IgG	1:50[e]	10	Zymed	> 10%	35
p53	DO-7	Mono	Mouse	IgG	1:50	3 × 5	Dako A/S	> 10%	7,30,31,34,35
$p21^{Cip1/WAF1}$	70	Mono	Mouse	IgG	1:100	3 × 5	BD Biosciences	> 10%	29,30,35,36
$p27^{Kip1}$	1B4	Mono	Mouse	IgG	1:20	3 × 5	Monosan	> 10%	30,36

[a]Microwave heating in 0.01 M citrate buffer (pH 6.0).
[b]Companies: Dako A/S, Glostrup, Denmark; Labvision, Fremont, USA; Novocastra, Newcastle upon Tyne, UK; Zymed, South San Francisco, USA; BD Biosciences, San Jose, USA; Monosan, Uden, The Netherlands.
[c]+ positive, ++ strongly positive, +++ extremely positive.
[d]Primary antibody incubation overnight instead of 1-h period.
[e]Detection of primary antibody by Powervision (Dako) instead of avidin–biotinylated peroxidase complex.

Materials and methods

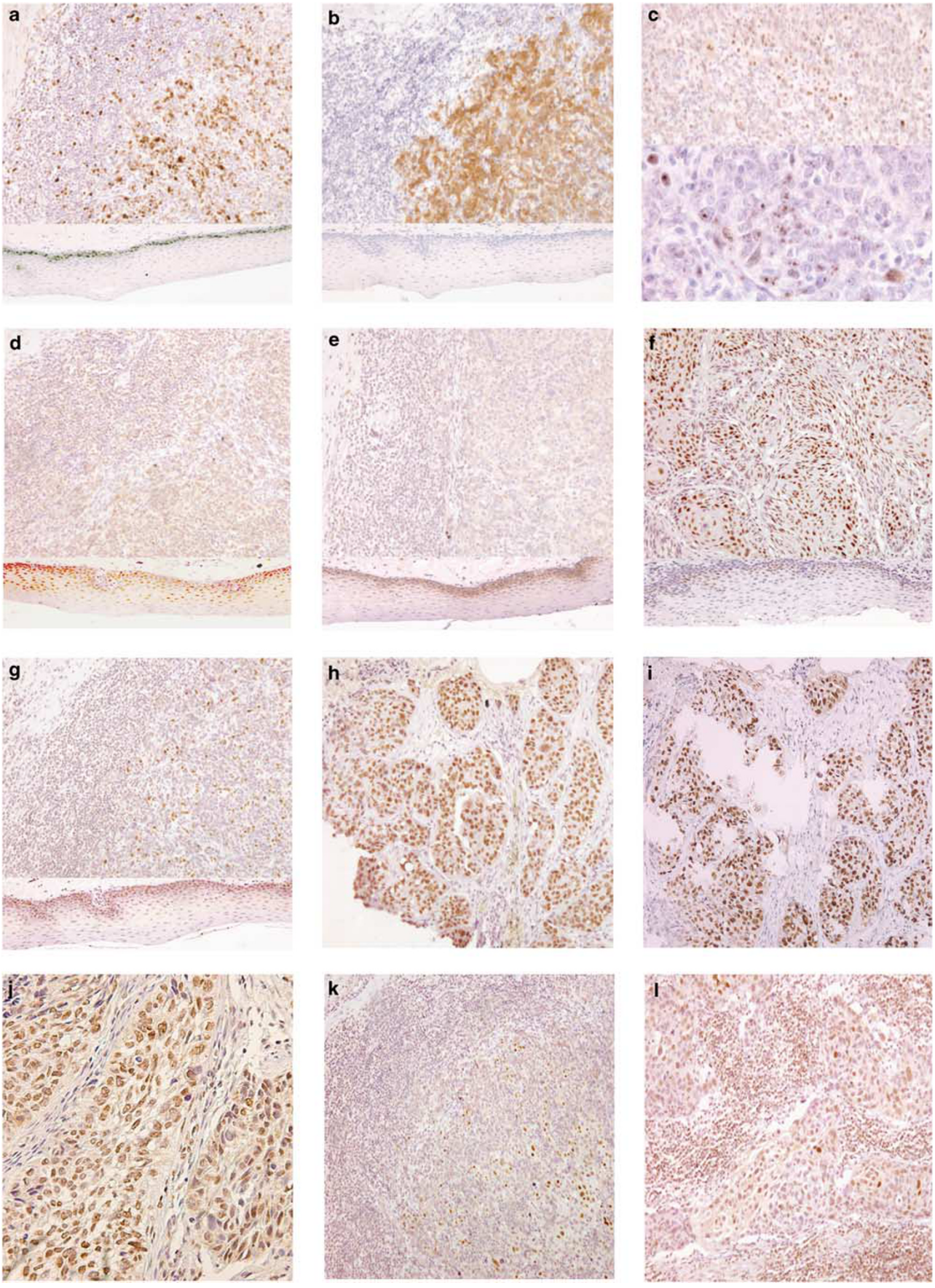
Tumor Material and Patient Data

Formaldehyde-fixed, paraffin-embedded archival biopsy and resection material of primary tonsillar squamous cell carcinomas from 77 patients was selected from the archives of the Department of Pathology, Maastricht University Medical Center, The Netherlands. This material had been previously examined for HPV-specific DNA by means of PCR and FISH, and only showed the presence of oncogenic HPV16.[13] Demographic data, including age at diagnosis, gender, alcohol and tobacco exposure, treatment modality, and date and cause of death were obtained from the medical records. Tumor site, degree of differentiation (ie, well, moderately, or poorly differentiated), and TNM classification were determined from review of pathological, radiological, and surgical reports. All patients were treated by surgery, radiotherapy, chemotherapy, or a combination, irrespective of HPV status. The study protocol was approved by the institutional ethical committee, and all patients gave informed consent. Table 1 provides demographic and clinical features of the 77 patients included in this study. Fifty-seven patients were male and 20 patients were female. The mean age at diagnosis was 58.8 (range 39–87) years. Data on smoking and alcohol intake were obtained from 76 and 75 patients, respectively. A total of 64 (84%) of 76 patients were smokers (≥1 cigarette, pipe, and/or cigar per day) and 46 (61%) of 75 patients were classified as drinkers (consumption of >2 whiskey equivalents per day (one whiskey equivalent is ~10 g alcohol)). Forty-two (55%) patients used both tobacco and alcohol, whereas only eight (10%) patients had not been subjected to these intoxications. Thirty-nine (51%) patients had a tumor with a diameter ≥4 cm, and 55 (71%) had lymph node metastasis at the time of diagnosis. Tumor grade was poor or moderate in 63 (82%) patients, well differentiated in 10 (13%) patients, unavailable in 3 patients (4%), and 1 patient (1%) had a carcinoma *in situ*. Following primary treatment, 29 (38%) patients never became disease-free, 16 (21%) developed a recurrent disease (locoregional, regional, or distant), and 32 (42%) patients remained disease-free after primary treatment.

A series of 4-μm thick sections was cut from the specimens for hematoxylin–eosin staining and a detailed histopathological classification (FJB) was given according to the criteria of the World Health Organization.[28] Furthermore, we applied immunohistochemistry to visualize Ki67, $p16^{INK4A}$ cyclin D1, pRb, $p14^{ARF}$ MDM2, p53, $p21^{Cip1/WAF1}$, and $p27^{Kip1}$ expression.

Immunohistochemical Staining

Immunohistochemical protein staining on 4-μm thick formaldehyde-fixed, paraffin-embedded tissue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sections was carried out as described earlier.[15] Briefly, sections were deparaffinized and subsequently pretreated with 2% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. Antigen retrieval was carried out by microwave heating in 0.01-M citrate buffer (pH 6.0). The primary antibodies used to detect Ki67, $p16^{INK4A}$ cyclin D1, pRb, $p14^{ARF}$ MDM2, p53, $p21^{Cip1/WAF1}$, and $p27^{Kip1}$ are listed in Table 2. After incubation with a biotinylated secondary antibody, immunohistochemical detection was performed by an avidin–biotinylated peroxidase complex (ABC) procedure (Vectastain-Elite-ABC kit; Vector, Burlingame, USA). Peroxidase activity was detected using 0.5 mg/ml diaminobenzidine/2% H_2O_2. Sections were counterstained with hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). In each analysis, negative and positive controls were included. Analysis was carried out by three independent observers (JJM, EJMS, and SMHC), and in case of interobserver variations, consensus was reached by combined examination of the slides. Both staining intensity ($-$, $+/-$, $+$, $++$, $+++$) and the percentage of stained tumor cells were scored. Evaluation criteria for positive scoring of each of the cell cycle proteins are listed in Table 2.

Statistical Analysis

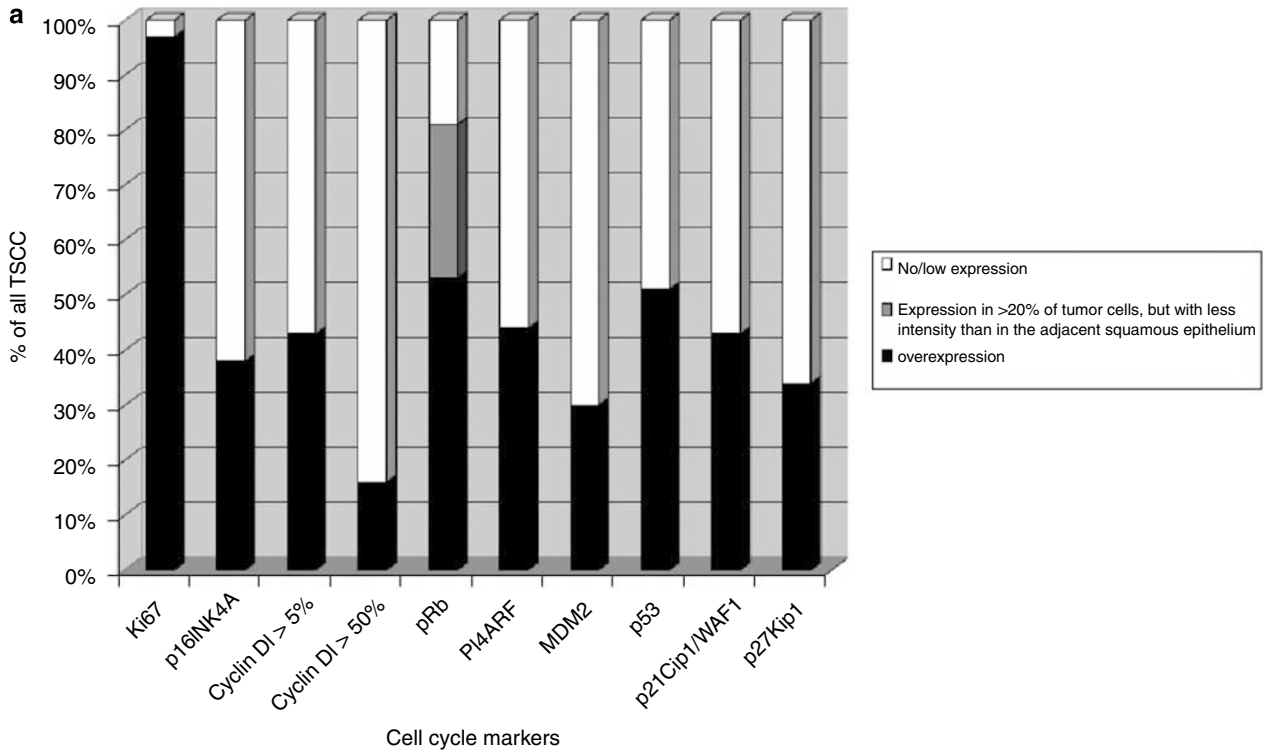
The study population consisted of 77 patients with a tonsillar squamous cell carcinoma diagnosed between 1992 and 2001. Tumors were considered to be HPV-associated if they showed HPV16 presence by *in situ* hybridization analysis, in addition to the overexpression of $p16^{INK4A}$, as detected by immunohistochemistry. Factors associated with HPV status were selected on cross-tabulations, which were analyzed by the use of the two-tailed Fisher's exact test and/or χ^2-test. The maximum significance levels are indicated for all analyses ($P \leq 0.05$). Disease-specific survival curves were calculated using the Kaplan–Meier method. Survival was calculated from the date of diagnosis until patient's death or until the last date the patient was known to be alive (this ranged from 16 to 141 months). Patients who died of other causes than tonsillar carcinoma were considered censored observations in the disease-specific survival analyses. Disease-free survival 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 at the date of death. The statistical significance of differences between survival times as determined by the log-rank test in univariate analysis. Multivariate analyses were performed using the Cox proportional hazards model. Variables in the multivariate model included HPV association, T classification, smoking, and cyclin D1-, $p14^{ARF}$- and $p21^{Cip1/WAF1}$ expression. Variables remained in the model if their *P*-values were <0.10. All calculations were performed by the use of the SPSS Base System version 12.0.1.

Results

Cell Cycle Protein Expression

Seventy-seven tonsillar squamous cell carcinomas were examined for expression of the cell cycle proteins Ki67, $p16^{INK4A}$ cyclin D1, pRb, $p14^{ARF}$ MDM2, p53, $p21^{Cip1/WAF1}$, and $p27^{Kip1}$, using the primary antibodies and evaluation criteria stated in Table 2. Representative immunostaining results in normal epithelium, and tumor tissues are shown in Figure 1. The frequency of tumors exhibiting the expression of the respective proteins is presented in Figure 2a. In all, 75 out of 77 (97%) tonsillar squamous cell carcinomas showed strong, nuclear Ki67 expression in 30–90% of the tumor cells (Figure 1a). Also nuclear pRb staining was often observed in tonsillar carcinomas (62 out of 77 cases, 81%), although two subsets were identified with a difference in staining intensity (ie, tumors showing $<$ or $\geq$ intense immunostaining than present in the adjacent squamous epithelium. Figure 1d and j, respectively). A significant association was seen between the overexpression of pRb (a higher nuclear staining intensity in tumor cells than in the adjacent normal squamous epithelium) and p53 accumulation ($P = 0.017$). Both overexpression of p53 and $p21^{Cip1/WAF1}$ correlated independently with the accumulation of MDM2 (Figure 1k; $P = 0.030$ and

Figure 1 Representative examples of immunohistochemical staining on tissue sections for (**a**) Ki67, (**b**) $p16^{INK4A}$ (**c**) $p14^{ARF}$ (**d**, **j**) pRb, (**e**, **i**) cyclin D1, (**f**) $p21^{Cip1/WAF1}$ (**g**, **h**) p53, (**k**) MDM2, and (**l**) $p27^{Kip1}$. Sections are from HPV-positive tonsillar squamous cell carcinomas (**a–g**), HPV-negative tonsillar squamous cell carcinomas (**h–l**) and adjacent squamous epithelium (**a**, **b**, **d–g**; lower image). Evaluation criteria as described in Materials and methods section. (**a**) Strong nuclear Ki67 staining in tumor cells and parabasal epithelial cells; (**b**) strong and diffuse $p16^{INK4A}$ staining in tumor cells, predominantly cytoplasmatic; epithelium negative; (**c**) strong and diffuse nuclear $p14^{ARF}$ staining (higher image). Some tumors show a rather nucleolar-like immunostaining (lower image). (**d**) Weak/no nuclear pRb staining in tumor cells in comparison with adjacent normal squamous epithelium. (**e**) Weak/no nuclear cyclin D1 staining in tumors cells; parabasal epithelial cells weakly positive; (**f**) strong nuclear $p21^{Cip1/WAF1}$ staining in tumor cells; some (para)basal epithelial cells weakly positive; (**g**) low frequency of tumor cells positive for nuclear p53; some (para)basal epithelial cells weakly positive; (**h**) high frequency of tumor cells positive for nuclear p53; (**i**) high frequency of tumor cells positive for nuclear cyclin D1; (**j**) strong nuclear pRb staining in tumor cells; (**k**) low frequency of tumor cells showing strong and nuclear MDM2 staining; (**l**) weak to strong nuclear immunostaining for $p27^{Kip1}$ in tumor cell areas as well as in adjacent lymphocyte areas. Magnification: $\times 40$ (**a–c** (higher image), **d–i**, **k–l**), $\times 120$ (**j**) and $\times 200$ (**c**; lower image).



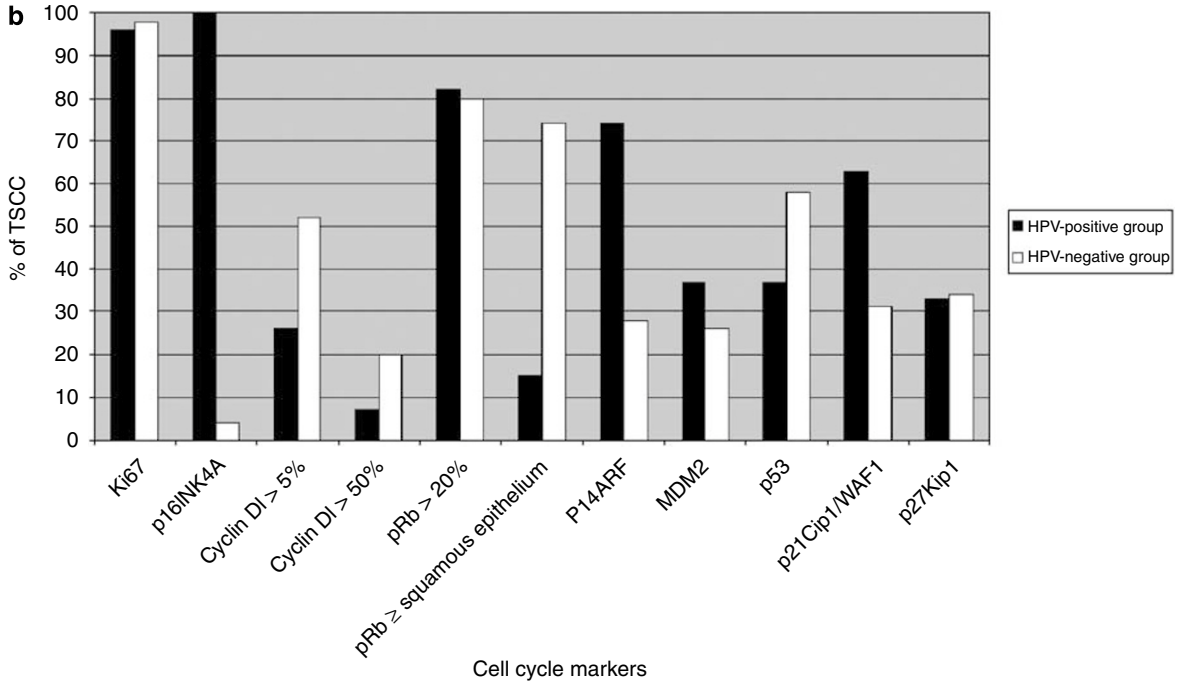


Figure 2 (**a**) Cell cycle protein expression in tonsillar squamous cell carcinomas according to the evaluation criteria presented in Table 2. (**b**) Fraction of HPV-positive and HPV-negative tonsillar squamous cell carcinomas showing expression of individual cell cycle markers (see Table 2 for the used criteria for positivity of each individual cell cycle marker).

0.004, respectively), but not with each other (data not shown; Supplementary Table 1). In addition, there was a significant association between the overexpression of $p16^{INK4A}$ and that of both $p14^{ARF}$ ($P<0.0001$) and $p21^{Cip1/WAF1}$ ($P=0.027$; Figure 1b, c and f). In case of $p14^{ARF}$ overexpression in the tumor, a diffuse nuclear staining pattern was more often observed than nucleolar staining (Figure 1c, above and below, respectively). Furthermore, $p16^{INK4A}$ accumulation strongly correlated with both downregulation of pRb ($P<0.0001$) and cyclin D1 ($P=0.035$; Figure 1d and e).

Staining of normal tonsillar squamous epithelium was seen in the basal or parabasal cell layers for Ki67, cyclin D1, pRb, $p21^{Cip1/WAF1}$, and p53, varying from low to high intensities (Figure 1a, b, d–g). This squamous epithelium, present in the individual sections, was used as an internal positive control. In $p14^{ARF}$- and $p27^{Kip1}$-stained sections, adjacent lymphocyte infiltration often showed a strong cytoplasmatic and nuclear staining, respectively, and therefore served as the internal positive control (Figure 1l).

Correlations Between HPV Status, Expression of Cell Cycle Proteins, and Clinicopathological Variables

The correlations between each cell cycle marker and HPV status are shown in Table 1 and Figure 2b. HPV-associated tonsillar squamous cell carcinomas showed significantly more often overexpression of $p14^{ARF}$ ($P<0.0001$) and $p21^{Cip1/WAF1}$ ($P=0.008$), and downregulation of pRb ($P<0.0001$) and cyclin D1 ($P=0.027$) than HPV-negative tumors. P53 accumulation tended to be associated with the absence of HPV ($P=0.079$).

The male/female ratio and the age distribution were similar for the HPV-positive and HPV-negative subgroups. Smoking and alcohol abuse were seen significantly more often in the HPV-negative patient group ($P<0.0001$ and $P=0.024$, respectively). HPV-positive tumors tended to be smaller than HPV-negative tumors ($P=0.065$). Male patients were more often diagnosed with a T3–4 tumor ($P=0.006$), and were more often smokers ($P=0.011$).

Indicators for Disease-Specific Patient Survival

To determine whether or not cell cycle protein expression patterns and clinicopathological parameters can be used as indicators of prognosis, we correlated these with the disease-specific survival data of patients with tonsillar squamous cell carcinoma (Table 3). Two patients died postoperatively, due to bleeding and aspiration, and from one patient, no follow-up data were available. These patients were excluded from the analyses. Follow-up time ranged from 0 to 141 months, with a mean of 30 months. A total of 41 (55%) of 74 patients died as a consequence of tonsillar carcinoma. The survival after 5 years was 31% for patients with a HPV-negative tumor and 69% for patients with a HPV-positive carcinoma (Hazard ratio (HR) = 0.4; 95% confidence interval (CI) = 0.2–0.8). Besides the absence of HPV, the following parameters were also significantly associated with a shorter disease-specific survival according to Univariate Cox regression analysis: (1) smoking (HR = 5.8; 95% CI = 1.4–24.1), (2) a tumor diameter $\geq$ 4 cm (HR = 3.1; 95% CI = 1.6–6.0), (3) development of recurrent disease (HR = 14.1; 95% CI = 3.9–51.1), (4) no/low expression of either $p14^{ARF}$ (HR = 2.2; 95% CI = 1.1–4.3) or $p21^{Cip1/WAF1}$ (HR = 2.7; 95% CI = 1.3–5.5), and (5) positive cyclin D1 immunostaining in >50% of tumor cells (HR = 2.2; 95% CI = 1.1–4.7). Gender, age at diagnosis, alcohol use, tumor grade, TNM-stage, lymph node status, and the remaining cell cycle markers were not related to disease-specific survival.

The parameters that were significantly correlated with disease-specific survival in the univariate analysis, ie, HPV status, tumor size, smoking, and immunostaining of $p14^{ARF}$ $p21^{Cip1/WAF1}$, and cyclin D1, were included in the multivariate Cox regression analysis. Development of recurrent disease was not included for multivariate analysis, because this factor cannot be predicted at time of diagnosis, so the clinical impact is of less importance. Table 4 shows that four of the six parameters, ie, tobacco consumption, tumor diameter $\geq$4 cm, no/low $p21^{Cip1/WAF1}$ immunostaining, or strong cyclin D1 immunostaining, were the most optimal indicators of cancer-specific death, with tumor size and $p21^{Cip1/WAF1}$ immunostaining being the most significantly correlated. In Figure 3, the Kaplan–Meier curves for these four parameters are shown.

Discussion

In this study, we examined the expression of cell cycle-related constituents in a series of 77 tonsillar squamous cell carcinomas with the goal to determine their role in HPV-dependent and HPV-independent carcinogenesis. Furthermore, their prognostic value was evaluated by correlating the results with clinical follow-up data. Our results show that HPV16-positive tumors exhibit $p14^{ARF}$ and $p21^{Cip1/WAF1}$ overexpression and downregulation of pRb and cyclin D1 in contrast to HPV16-negative tumors. Secondly, tumor size and $p21^{Cip1/WAF1}$ positivity are the strongest independent indicators for a favorable outcome in patients with tonsillar squamous cell carcinoma.

All tonsillar squamous cell carcinomas, except one HPV-positive and one HPV-negative tumor, showed a high expression of Ki67, indicating that almost all tumors contained a high percentage of proliferative cells, which is in agreement with other studies on head-and-neck squamous cell carcinomas.[37] In addition, high expression levels of inhibitors of apoptosis, such as $BclX_L$ and survivin, have been reported in head-and-neck squamous cell carcinomas.[38–41] Expression of pRb was detected in 81% of the tumors. Approximately half of all tumors showed strong nuclear expression in the tumor cells, with expression levels equal to or higher than the adjacent normal squamous epithelium, whereas pRb downregulation was observed in the remaining cases. In our study, p53 overexpression was observed in little more than half of all tonsillar carcinomas, which is consistent with other

Table 3 Influence of HPV-related and clinicopathologic parameters on disease-specific survival in 74 patients with TSCC, as determined by univariate Cox proportional hazard regression analysis

Variable	*Total*	*Death (%)*	P-*value*[a]	*Unadjusted HR (95% CI)*
Cell cycle proteins				
Ki67				
Positive	72	40 (56)	NS	1.5 (0.2–10.9)
Negative	2	1 (50)		1 (referent)
p16^{INK4A}				
Positive	28	10 (36)	0.029	0.5 (0.2–0.9)
Negative	46	31 (67)		1 (referent)
Cyclin D1 >5%				
Positive	31	20 (65)	0.060	1.8 (1.0–3.4)
Negative	43	21 (49)		1 (referent)
Cyclin D1 >50%				
Positive	11	9 (82)	0.028	2.2 (1.1–4.7)
Negative	63	32 (51)		1 (referent)
pRb >20%				
Positive	61	35 (57)	NS	1.5 (0.6–3.5)
Negative	13	6 (46)		1 (referent)
pRb intensity in tumor≥ intensity in adjacent squamous epithelium				
Positive	40	25 (62)	NS	1.5 (0.8–2.9)
Negative	34	16 (47)		1 (referent)
p14^{ARF}				
Positive	33	12 (36)	0.020	0.5 (0.2–0.9)
Negative	41	29 (71)		1 (referent)
MDM2				
Positive	22	10 (45)	NS	0.8 (0.4–1.7)
Negative	52	31 (60)		1 (referent)
p53				
Positive	37	22 (59)	NS	1.2 (0.7–2.2)
Negative	37	19 (51)		1 (referent)
p21$^{Cip1/WAF1}$				
Positive	30	10 (33)	0.004	0.4 (0.2–0.8)
Negative	43	31 (72)		1 (referent)
Unknown	1			
p27^{KIP1}				
Positive	25	14 (66)	NS	1.2 (0.6–2.3)
Negative	47	26 (55)		1 (referent)
Unknown	2			
Clinicopathological variables				
Gender				
Male	55	33 (60)	0.084	1 (referent)
Female	19	8 (42)		1.9 (0.9–4.2)
Age (years)				
<60	41	24 (58)	NS	1 (referent)
≥60	33	17 (52)		0.8 (0.4–1.5)
Smoking[b]				
No	11	2 (18)	0.006	1 (referent)
Yes	63	39 (62)		5.8 (1.4–24.1)
Alcohol[c]				
No	29	16 (55)	NS	1 (referent)
Yes	45	25 (56)		1.1 (0.6–2.0)
Smoking and/or alcohol				
No	8	1 (12)	0.015	1 (referent)
Yes	66	40 (61)		7.9 (1.1–57.7)
Smoking and alcohol				
No	32	17 (53)	NS	1 (referent)
Yes	42	24 (57)		1.3 (0.7–2.3)
TNM-classification				
Stage 0–3	38	20 (53)	NS	1 (referent)
Stage 4	36	21 (58)		1.5 (0.8–2.8)
T-classification				
<4 cm (T 1–2)	35	14 (40)	<0.001	1 (referent)
≥4 cm (T 3–4)	39	27 (69)		3.1 (1.6–6.0)
Tumor grade[d]				
Poor/moderate	61	37 (61)	NS	1 (referent)
Well	10	3 (30)		0.4 (0.1–1.4)
Unknown	3			
Lymph node metastasis				
Positive	54	29 (54)	NS	1.0 (0.5–1.9)
Negative	20	12 (60)		1 (referent)

Table 3 Continued

Variable	*Total*	*Death (%)*	P-*value*[a]	*Unadjusted HR (95% CI)*
Recurrent disease				
Yes	16	13 (81)	<0.0001	1 (referent)
No	32	3 (9)		14.1 (3.9–51.1)
Never disease free	26	25 (96)		
HPV-association				
Yes	26	8 (31)	0.010	0.4 (0.2–0.8)
No	48	33 (69)		1 (referent)

HPV, human papillomavirus; HR, hazard ratio; NS, not significant; TSCC, tonsillar squamous cell carcinoma.
[a]*P*-values based on the log-rank test.
[b]Patiens were classified as daily tobacco smokers (≥1 cigarette, pipe, and/or cigar per day) or non-smokers (never smokers or patients who had stopped smoking more than 10 years before the diagnosis of TSCC).
[c]Patients were classified as drinkers (consumption of >2 whiskey equivalents per day (one whiskey is equivalent to ~10 g alcohol).
[d]Tumor grade was scored as well, moderately, or poorly differentiated according to the criteria of the World Health Organization.

Table 4 Multivariate analysis, according to Cox proportional hazard regression analysis, of the patient and tumor characteristics related to disease-specific mortality

Characteristic	*HR*	*95% CI*	P-*value*
Smoking: yes *vs* no	4.06	0.95–17.26	0.058
Tumor size: T3–4 *vs* T1–2	2.58	1.32–5.07	0.006
$p21^{Cip1/WAF1}$: positive *vs* negative	0.38	0.18–0.79	0.009
Cyclin D1: >50 *vs* ≤50%[a]	2.19	1.02–4.69	0.044

CI, confidence interval; HR, hazard ratio.
[a]Nuclear staining.

studies.[7,42] Immunostaining of all other cell cycle proteins was evident in less than 50% of tumors.

We noticed that the tumors with the low expression levels of pRb showed the overexpression of $p16^{INK4A}$. Accumulation of this latter protein has been strongly associated with the presence of oncogenic HPV in oropharyngeal carcinomas,[7,10,14,15] which was also evident in the underlying study. These HPV16-positive tonsillar squamous cell carcinomas furthermore showed the downregulation of cyclin D1, next to the accumulation of $p14^{ARF}$ and $p21^{Cip1/WAF1}$. In addition, p53 expression was less profound in these HPV-positive tumors.

In HPV-associated tumors, the oncoprotein E7 interacts with pRb, resulting in its degradation. As a result, $p16^{INK4A}$ is upregulated and cyclin D1 is downregulated.[43] This is in agreement with Andl *et al*,[5] who suggested that E7 might overcome the need for cyclin D1 in the G1 phase of the cell cycle, because it interacts with the cyclin D1-binding site on pRb.[7,44] Indeed, high expression levels of cyclin D1 were predominantly observed in HPV-negative tonsillar carcinomas, most probably pointing to cyclin D1 gene amplification in these cases.

The $p14^{ARF}$ gene is a target for the transcription factor E2F, which promotes its expression particularly in the HPV-positive tumors, as has become evident in this study. In the HPV-negative tumors both $p14^{ARF}$ as well as $p16^{INK4A}$ were often undetectable, which is in accordance with the fact that their expression is downregulated in most head-and-neck squamous cell carcinomas due to gene inactivation at the 9p21 locus.[45,46] In normal tissue cells, $p14^{ARF}$ upregulation might lead to p53 upregulation, but in HPV-positive tonsillar squamous cell carcinomas, this is usually counteracted by means of functional inactivation of wild-type p53 by the viral E6 protein. In contrast and as observed in our study, p53 is frequently upregulated in HPV-negative tumors due to mutations in the TP53 gene as a result of exposure to tobacco and/or alcohol.[10,16,20,27,47]

Although $p21^{Cip1/WAF1}$ is known to be a downstream effector of p53,[48,49] it was surprising to find overexpression in HPV-positive tumors harboring low or no detectable levels of p53. Such observations have also been reported by Milde-Langosch *et al*[50] in HPV-associated uterine cervical tumors and suggest that also p53-independent mechanisms may lead to $p21^{Cip1/WAF1}$ accumulation as described previously.[51,52] Indeed, in HPV-positive cancer cells, $p21^{Cip1/WAF1}$ expression appears to be inducible,[53] although it remains unclear why under these conditions E7 cannot inactivate $p21^{Cip1/WAF1}$ by direct interaction.[14]

Despite the strong association of $p21^{Cip1/WAF1}$ overexpression with HPV positivity in most tonsillar squamous cell carcinomas, we found $p21^{Cip1/WAF1}$ protein accumulation also in some HPV-negative tumors, which is in accordance with a study of Li *et al*.[54] These authors examined 67 tonsillar squamous cell carcinomas for HPV involvement, but did not find $p21^{Cip1/WAF1}$ expression being associated with HPV positivity. An explanation for this paradox might be on the one hand the use of a different $p21^{Cip1/WAF1}$-specific primary antibody and different criteria to assess $p21^{Cip1/WAF1}$ upregulation (>20% of positive nuclei *versus* >10%) in comparison with our and other studies,[29,30,35,36] and on the other hand the use of only PCR to determine the presence of HPV. To assess a firm association between virus and tumor cells, namely, it is recommended to carry out additional tests, such as $p16^{INK4A}$ immunostaining

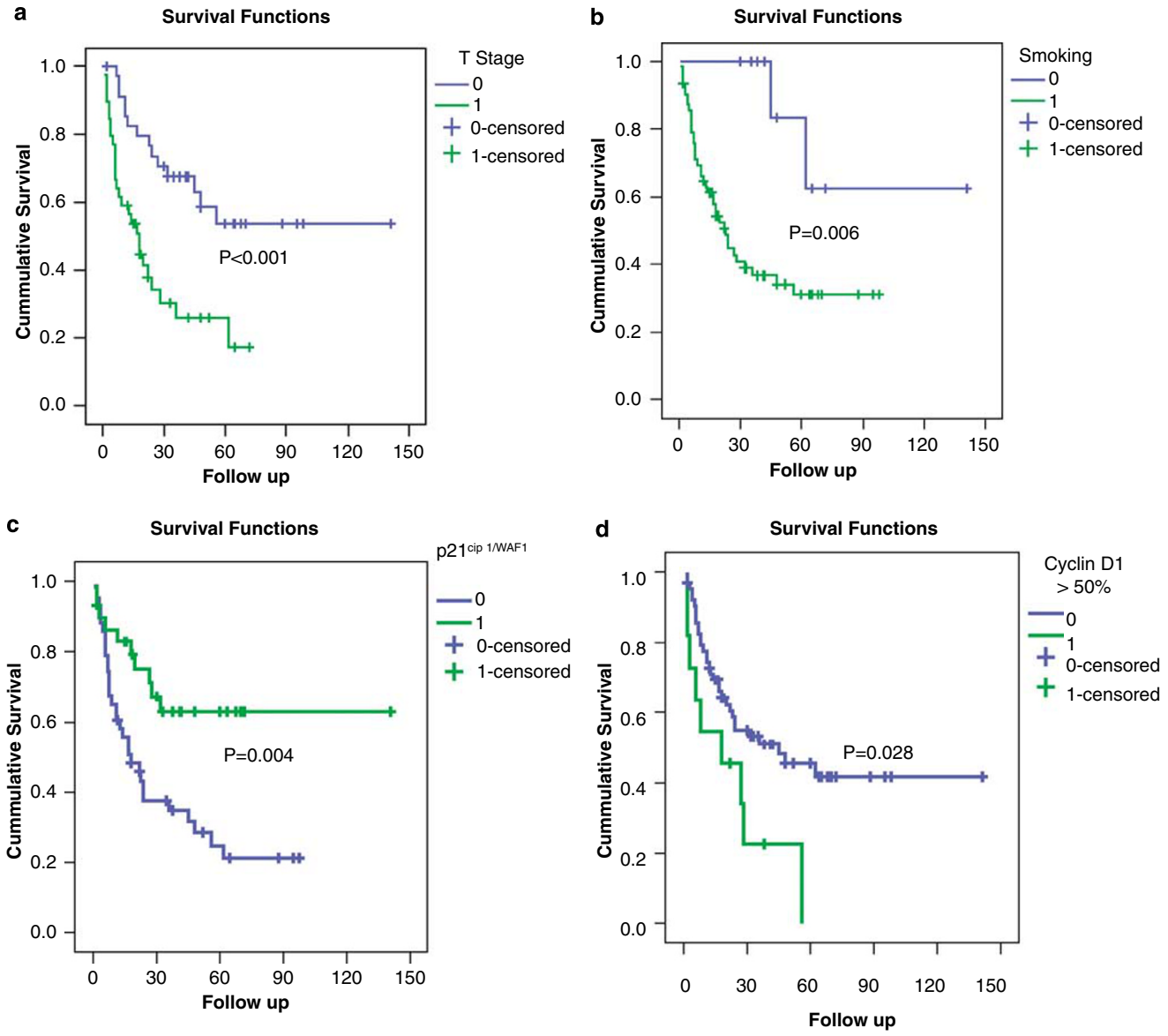


Figure 3 Kaplan–Meier survival curves according to (**a**) tumor size, (**b**) smoking status, (**c**) $p21^{Cip1/WAF1}$ expression, and (**d**) strong cyclin D1 expression.

and/or FISH, which has been applied in our study.[55,56]

In the univariate and multivariate statistical analyses, we found that $p21^{Cip1/WAF1}$ overexpression was a highly significant indicator of favorable prognosis in tonsillar squamous cell carcinomas independent of HPV status. Expression of $p21^{Cip1/WAF1}$ has also been associated with a favorable survival in patients with tongue squamous cell carcinomas, and in patients with ovarian, superficial bladder, gastric, colorectal, and esophageal cancers.[29,43,57,58] In one other study on tonsillar squamous cell carcinomas and two studies on laryngeal squamous carcinomas, no correlation was reported,[36,54,59] and in one study on head-and-neck squamous cell carcinomas derived from multiple head-and-neck localizations even a negative correlation with survival was described,[19] which may be explained by the heterogeneous tumor population. Other strong indicators for a favorable prognosis in our study include tumor diameter <4 cm, low/no expression of cyclin D1, and low/no tobacco consumption. In accordance with most other studies on HPV-related oropharyngeal carcinomas, we also found a favorable disease-specific survival in patients with HPV16-positive tonsillar squamous cell carcinomas,[12,46,60–64] and $p14^{ARF}$ overexpression,[31] although with less significance than the indicators described above. We noticed that lymph node status, which is generally considered as the most important prognostic factor in head-and-neck squamous cell carcinomas,[23] had little value in our series of tonsillar squamous cell carcinomas, which is in concordance with other studies.[24,25]

In summary, we can conclude that HPV16-positive tonsillar squamous cell carcinomas exhibit overexpression of $p14^{ARF}$ and $p21^{Cip1/WAF1}$ as well as downregulation of pRb and cyclin D1, and that strong immunostaining for $p21^{Cip1/WAF1}$ appears to be one of the most potent indicators for favorable prognosis in these tumors.

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