ANTICANCER RESEARCH *25*: 4375-4384 (2005)

P16^{INK4a} Correlates to Human Papillomavirus Presence, Response to Radiotherapy and Clinical Outcome in Tonsillar Carcinoma

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Abstract. *Background: Human papillomavirus (HPV) in tonsillar carcinoma is correlated with favourable clinical outcome. Here, p16^{INK4A}, in situ HPV DNA hybridisation (ISH) and HPV L1 capsid detection were evaluated in tonsillar carcinoma to predict the response to radiotherapy (RT) and prognosis. Materials and Methods: Fifty-one pre-treatment paraffin-embedded tonsillar cancer biopsies were analysed. Immunohistochemistry (IHC) was used for p16^{INK4A} and HPV L1 capsid analysis and PCR and ISH for HPV detection. Results: High-risk HPV DNA was detected by PCR in 49% of the tumours. P16^{INK4a} staining was correlated to HPV. In the high-grade p16^{INK4a} staining group, 94% had a complete RT response. High p16^{INK4a} staining as well as the HPV PCR-positive cases had a favourable prognosis. HPV DNA ISH and L1 IHC could not predict RT response or clinical outcome. Conclusion: P16^{INK4a} overexpression was correlated to HPV in tonsillar carcinoma and is useful for predicting RT response and prognosis in tonsillar carcinoma patients.*

Squamous cell carcinoma of the head and neck (HNSCC) is the eighth most common malignancy in the world (1). Smoking, heavy alcohol abuse, betel nut chewing and smokeless tobacco use are established aetiological factors (2, 3). However, 15-20% of the HNSCC patients have no known smoke or alcohol exposure (4, 5). A relationship between human papillomaviruses (HPVs) and HNSCC was suggested as early as 1983 (6). Currently, there is increasing evidence that high-risk types of HPV, similar to those observed in cervical cancer (7, 8), have an aetiological role in a subset of head and neck malignancies (5, 9-16). Tonsillar carcinoma is of particular interest, since it is the head and neck cancer where HPV is most commonly found [reviewed in (17-19)]. Approximately half of all tonsillar cancer cases are high-risk HPV-positive (14, 20-22). Moreover, recent studies have shown that HPV-negative and -positive tonsillar tumours have a different biology and that HPV is a favourable prognostic factor in tonsillar cancer (13, 14). Furthermore, there are indications that HPV-negative and -positive tonsillar tumours have different genotypes (23, 24).

It is also likely that HPV plays a role in the deregulation of the cell cycle in HPV-positive tonsillar cancer (13, 21, 24-28).

Progression through the cell cycle from G1 into S is a critical checkpoint controlled by cyclins and associated cyclin-dependent kinases (CDKs), such as cyclin D and CDK4 [reviewed in (29)]. A main function of the tumour suppressor retinoblastoma (pRb) protein is to repress transcription of essential DNA synthesis genes, thus inhibiting transition into the S-phase. PRb represses these genes by binding to its co-factor E2F [reviewed in (29)]. Accumulation of cyclin D-dependent kinases triggers phosphorylation of pRb, releasing EF2 from pRb and, thereby, cancelling pRb repression [reviewed in (29)]. The cyclin D-dependent kinases are, in their turn, inhibited by CDK inhibitors, one of these being the tumour suppressor protein p16^{INK4a} (30). Through the binding of p16^{INK4a} and other CDK inhibitors to cyclin D-dependent kinases, pRb remains hypophosphorylated and cell cycle progression into the S-phase is prevented (29, 30). In high-risk HPV, the E7 oncoprotein binds to pRb (31) leading to its degradation, releasing E2F and resulting in cell cycle progression into the S-phase (32) [for review see (33)].

High-levels of p16INKa have been found to correlate with inactive pRb and, in addition, p16INKa overexpression in pRb dysfunctional cells has been shown to reflect a loss of negative feedback by pRb on INK4a gene transcription (34, 35).

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Key Words: HPV, tonsillar carcinoma, p16, radiotherapy, L1 capsid, HPV ISH, prognosis.

Consequently, in high-risk HPV-positive and pRb dysfunctional cervical cancer overexpression of $p16^{INKa}$ is observed, while no $p16^{INKa}$ staining is seen in HPV-negative dysplasia or in low-risk HPV-infected dysplasia (36, 37). Thus, $p16^{INK4a}$ overexpression may be seen as a surrogate biomarker for the presence of high-risk HPV in cervical cancer (36, 37). In line with these studies, a correlation between HPV-16, pRb negativity and overexpression of $p16^{INK4A}$ in oropharyngeal carcinoma has also been reported (21, 27, 28, 38, 39).

The first aim of this study was, therefore, to further assess whether $p16^{INK4A}$ could be used as a biomarker for high-risk HPV in tonsillar carcinoma by comparing it to HPV detection by PCR. In parallel, *in situ* HPV DNA hybridisation (ISH) and immunohistochemical (IHC) detection of the HPV L1 capsid protein were also evaluated for specificity and sensitivity of HPV detection, in comparison to HPV detection by PCR. A second aim was to determine if any of these methods could be used to distinguish between radiotherapy (RT)-sensitive or -resistant tonsillar cancer cases. Finally, we aimed to investigate if any of these methods alone could substitute for HPV analysis by PCR in predicting a prognostic favourable clinical outcome in tonsillar cancer (14, 40).

Materials and Methods

Patients, materials, treatment and clinical data. Archival tissues from 51 patients, diagnosed with tonsillar squamous cell carcinoma at the Karolinska University Hospital, Stockholm, Sweden between 1983 and 1999, were analysed and the study was conducted in accordance with the local ethical committee. The records of the patients were collected from the files at Karolinska University Hospital. The mean age of the patients was 63 years at diagnosis (41-88) and 76% of the patients were male (Tabel I). TNM stage classification was done according to the International Union Against Cancer (UICC) (Tabel I). All biopsies were obtained at the time of diagnosis, prior to RT. Consecutive serial sections were used for the assays, with histological confirmation of the lesion in initial and final sections. Forty-nine patients underwent treatment, while 3 patients declined treatment or were not in a condition for such treatment. Thirty-four patients underwent surgery, none of them as single treatment, thus 49 underwent RT given with a daily fraction of 2 Gy, 5 days weekly, to a total dose of 64 Gy. Twenty-nine of the patients received preoperative RT, 6 patients had postoperative RT and 14 patients had RT as a single treatment modality. Complete response (CR) to RT was defined as no tumour found one month after completion of RT at evaluation by clinical examination and, in non-surgical cases when required, also in combination with biopsy at the primary tumour site. The follow-up time including all patients ranged from 4-120 months (mean 35 months), while those who were classified as tumour-free or survivors were followed for at least 36 months (mean 47 months). The survival end-points were defined from the time of diagnosis and only deaths due to tonsillar cancer were included (disease-specific survival).

HPV detection by polymerase chain reaction. The biopsies had previously been analysed and reported with regard to HPV status (14,41), but had not been analysed by immunohistochemistry for $p16^{INK4a}$ or the HPV L1 capsid, or by *in situ* hybridisation for the presence of HPV DNA. PCR assays were performed on DNA extracted from tissue sections of each primary tonsillar carcinoma. Forty of the biopsies were formalin-fixed paraffin-embedded tissues and 11 of the cases were fresh-frozen biopsies. Three 20-μm sections of fresh-frozen biopsies or 5 pieces of 5-μm-thin sections of formalin-fixed, paraffin-embedded tissue were cut. To check that HPV was not contaminating the following block, control sections from tissue-free blocks were collected between each biopsy. After DNA extraction by standard phenol-chloroform and proteinase-K digestion, the samples were screened for HPV by a broad-spectrum PCR, using consensus primers $GP5^+/GP6^+$ (located in L1) (42). To minimize false-negative results, all the samples that were found negative with the $GP5^+/GP6^+$ primer pair were tested with the consensus primers CPI/IIG (located in E1) (43). Conditions for the $GP5^+/6^+$ and for the CPI/IIG PCRs have been described previously (41). HPV typing was done by direct cycle sequencing of the purified PCR products from the consensus primers using the Big Dye Terminator Cycle Sequencing Kit, performed in ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Both DNA strands were sequenced and aligned to those available at NCBI BLAST GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). The samples were also tested with HPV-specific primers for HPV-16, -18 and -33, as previously described (44). To rule out false-negative results, the HPV-negative samples were assayed for human DNA with primers GH26/27 of the HLA DQ locus (45).

Immunohistochemical analysis of $p16^{INK4a}$ and of the L1 capsid of HPV. Immunohistochemistry was performed with the $p16^{INK4a}$ primary monoclonal mouse anti-human $p16^{INK4a}$ antibody (clone E6H4, DakoCytomation A/S, Carpinteria, CA, USA) (37). The staining was performed in an automated deparaffinization and staining machine (BenchMark® XT, Ventana Medical Systems, Tucson, AZ, USA). Serial 5-μm sections of formalin-fixed and paraffin-embedded tissue were used with a final haematoxylin and eosin-stained section to confirm the histopathological diagnosis and the representativeness of the cut-off levels. The sections were processed in BenchMark® XT for deparaffinization (98˚C, for 30 min) and then re-hydrated through graded alcohol. Epitope retrieval was performed by heating and then the sections were treated with peroxidase blocking reagent. The slides were incubated for 32 min (42˚C) with 100 μl of the monoclonal antibody $p16^{INK4a}$, followed by incubation with visualisation reagent. The reaction was developed using chromogen solution (DAB) and counterstained with haematoxylin. As a negative antibody control, monoclonal mouse IgG2a was used.

Immunohistochemistry for detection of the HPV L1 capsid protein was performed with a monoclonal mouse anti-HPV, (clone K1H8; DakoCytomation A/S), which binds to a non-conformational internal epitope of the major capsid protein (L1) of HPV-1. It has been found to stain nuclei of cells infected by HPV -6, -11, -16, -18, -31, -33, -42, -52, -56 and -58. The procedure was performed in the same way as with $p16^{INK4a}$ described above.

Immunohistochemical staining was evaluated by light microscopy and a strong nuclear staining of the L1 capsid was considered to be a positive staining (Figure 1). The $p16^{INK4a}$ staining was scored on a semi-quantitative scale as follows: negative (<10% of the tumour cells were positive), 1+ (10-50% of the cells were positive), 2+ (50-90% of the cells were positive) and 3+ (>90% of the cells were positive) (Figure 1). A positive tissue control section from a cervical carcinoma *in situ* was included with all samples.

HPV DNA detection by in situ hybridisation. The DNA ISH technique to detect high-risk HPV types as well as low- risk HPV types was performed by using two separate fluorescent-labelled probe cocktails (both from Ventana Medical Systems). These were INFORM HPV Tissue High Risk Probe, containing a mix of probes hybridising with HPV-16, -18, -31, -33, -35, -45, -51, -52, -56, -58, -59, -68 and -70 and INFORM HPV Tissue Low Risk Probe, containing probes hybridising with HPV-6, -11, -42, -43 and -44. Five-μm sections were processed in the automated system BenchMark® XT (Ventana Medical Systems), as described by the manufacturer. Sections were deparaffinized briefly by proteolytic digestion (ISH-protease 2, Ventana Medical Systems) for 2 min, followed by denaturation of DNA. Thereafter, the probe mixture was added and, after hybridisation with the probe mix for 2 hours, visualisation was performed (Figure 1).

Statistical analysis. The comparison between the four methods and the prediction properties of the methods for response to RT, being tumour-free after three years or dead from tonsillar carcinoma, were evaluated using odds ratios by exact logistic regression or Fisher's exact two-tailed test. The results on the correlation between the method and clinical outcome were then adjusted for factors such as age at diagnosis, gender, differentiation grade of the tumour and TNM stage by exact logistic regression. The statistical software's used for the analysis were LogXact 5 (Cytel software corporation, Cambridge MA, USA) and SAS 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Detection of HPV and type by PCR. HPV DNA was detected in 25/51 (49%) of the tonsillar cancers by PCR (Table I). Of the HPV-positive tumours, 24 typed HPV-16 and one tumour typed HPV-33. The 25 HPV-positive tumours were all detected with the GP5+/6+ primers and no additional HPV-positive tumours were found using the CPI/IIG primers.

P16^{INK4a} as a biomarker of HPV in comparison to detection of HPV by PCR. Twenty-seven of the 51 (53%) tumours were p16^{INK4a}-positive (Table I, exemplified in Figure 1). More specifically, 20/25 (80%) HPV PCR-positive cases and 7/26 (27%) HPV-negative cases were p16^{INK4a}-positive (80% sensitivity), which indicates significantly more p16^{INK4a}-positive tumours among the HPV PCR-positive cases (Table I, $p<0.001$; logistic regression). Thus, 20/27 p16^{INK4a}-positive cases were HPV PCR-positive (73% specificity).

Specificity was also correlated to the grading of the p16^{INK4a} staining; the higher the grading the better the specificity: 5/11 (45%) p16^{INK4a} 1+ cases were HPV PCR-positive (76% specificity) ($p=0.257$; logistic regression), 7/8 (88%) p16^{INK4a} 2+ cases were HPV PCR-positive (95% specificity) ($p=0.003$; logistic regression) and 8/8 (100%) p16^{INK4a} 3+ cases were PCR-positive (100% specificity) ($p<0.001$; logistic regression).

The odds for HPV PCR-positive cases to be p16^{INK4a} 2+ or 3+ (15/25) was 49 times more likely than being p16^{INK4a}-negative (5/25) (OR=49, $p< 0.001$; logistic regression). The odds for HPV PCR-positive cases to be p16^{INK4a} 2+ or 3+ (15/25) was 16 times more likely than being p16^{INK4a} 1+ (5/25) (OR=16, $p=0.017$; logistic regression).

In situ HPV DNA hybridisation in comparison to detection of HPV by PCR. Ten out of the 51 (20%) tumours were found to be high-risk HPV-positive using the ISH kit with high–risk probe mix (Table I, exemplified in Figure 1). None of the tonsillar carcinomas were found low-risk HPV-positive in ISH low-risk probe mixture analysis, in accordance with the PCR finding of only high-risk types (HPV-16, HPV-33). Hence, significantly more HPV PCR-positive cases were high-risk ISH-positive, 10/25 (40% sensitivity), compared to HPV PCR-negative cases positive for ISH, 0/26, ($p<0.001$; logistic regression). All of the 10 high-risk ISH-positive cases were HPV-16-positive according to PCR (100% specificity).

ICH detection of the HPV L1 capsid protein in comparison to detection of HPV by PCR. The L1 capsid protein could be detected in 4/51 (8%) tonsillar tumours (Table I, exemplified in Figure 1). Three of 4 L1 capsid IHC-positive cases were HPV-positive by PCR (12% sensitivity), while the remaining L1 capsid IHC-positive case was HPV PCR-negative ($p=0.580$; logistic regression), yielding a specificity of 95%.

Prediction of response to radiotherapy (RT) using different detection methods. Forty-nine out of the 51 patients could be evaluated regarding response to radiotherapy, since 3 patients did not receive full-dose RT, and 33/49 patients had a CR (Table II).

Twenty out of the 25 HPV PCR-positive cases had a complete RT response compared to 13/24 out of the HPV-negative cases, indicating a tendency towards a better, but not a statistically significantly better, response to RT in the HPV PCR-positive cases (Table II, $p=0.066$; Fisher exact test, $p=0.103$; logistic regression).

Likewise, 20/27 (74%) of the p16^{INK4a}-positive cases had a complete RT response compared to 13/22 (59%) of the p16^{INK4a}-negative cases (Table II, $p=0.420$; logistic regression).

However, a high-grade (2+ and 3+) of p16^{INK4a} staining was correlated to a better response to RT (Table II). Although not statistically significant, 8/8 of the p16^{INK4a} 3+ group and all but one (7/8) of the p16^{INK4a} 2+ group had a complete RT response, compared to 5/11 of the p16^{INK4a} 1+ group and 13/22 out of the p16^{INK4a}-negative cases. Furthermore, combining the two high (2+ and 3+) p16^{INK4a} staining groups, 15/16 tonsillar cancer patients had a statistically significantly better complete RT response, compared to the p16^{INK4a} 1+ group (5/11) and to the p16^{INK4a}-negative cases [(13/22), $p=0.017$ and $p=0.018$ respectively; logistic regression, see Table II)]. Furthermore,

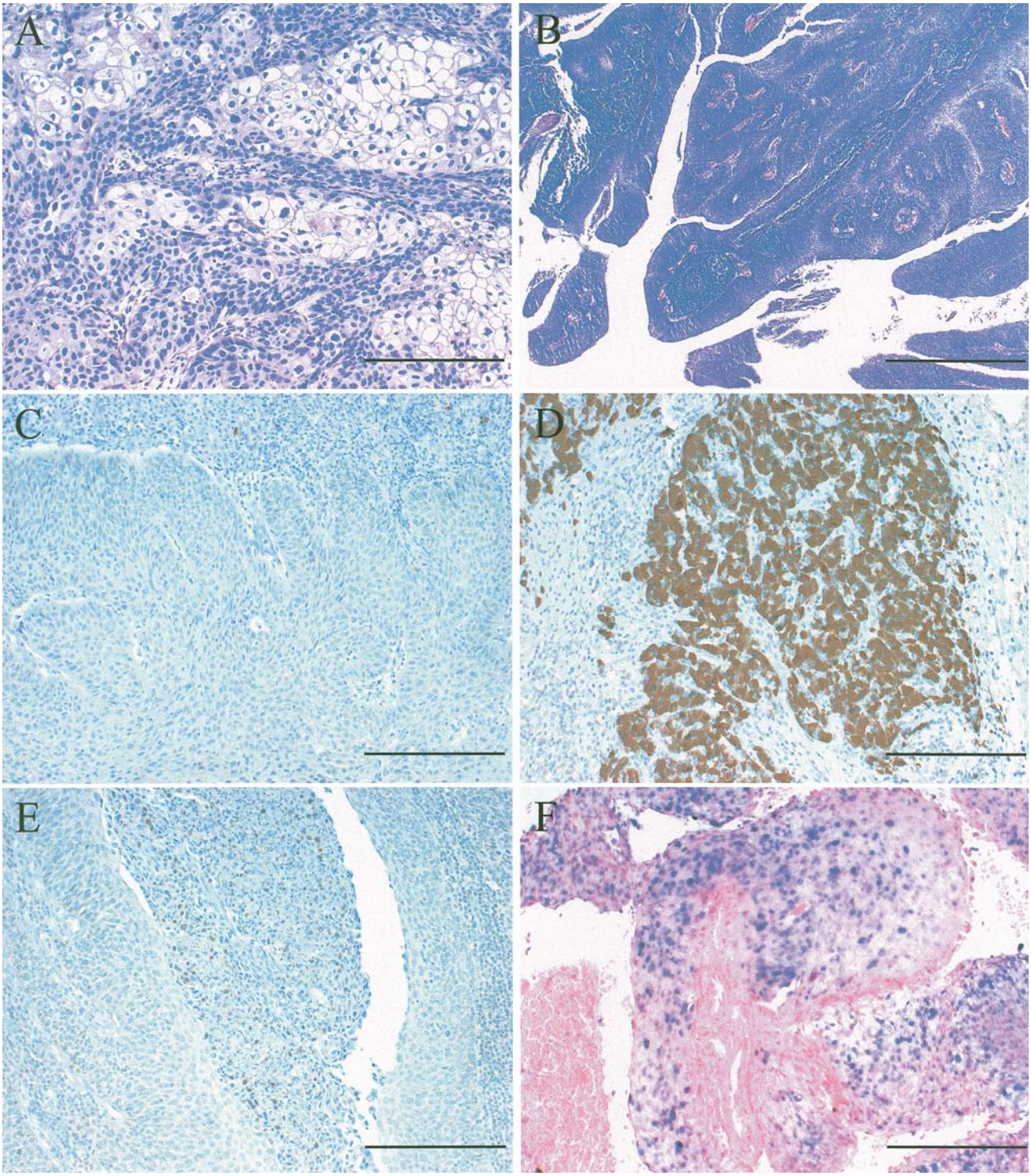

Figure 1. *(A) Moderately-differentiated non- keratinized squamous cell carcinoma of "bowenoid-condylomatous" type. Notice single cells with large atypical hyperchromatic nuclei. There is also a pronounced koilocytic change with multiple bizarre nuclei in vacuolated cytoplasm (20X). Scale bar=200 μm. (B) Papillary subtype of moderately-differentiated non-keratinized squamous cell carcinoma. Characteristic multiple finger-like projections with fibrovascular cores (4X). Scale bar=400 μm. (C) Immunohistochemistry for weak $p16^{INKa}$ 1+ staining (20X). Scale bar=200 μm. (D) Immunohistochemistry for strong $p16^{INKa}$ 3+ staining (20X). Scale bar=200 μm. (E) HPV L1 capsid protein immunohistochemistry. Positive nuclear staining in exfoliated degenerative tumour cells (20X). Scale bar=200 μm. (F) High-risk HPV DNA detection by in situ hybridisation. Moderate nuclear staining positivity. Positive staining is usually noted in "superficial" maturating tumour cells with larger nuclei (20X). Scale bar=200 μm.*

Table I. *Patient data, tumour characteristics, HPV status by PCR correlated to HPV detection markers and p16^{INK4}.*

	HPV PCR pos (%)	p16^{INK4} pos[1] (%)	HR ISH pos[2] (%)	LI capsid pos[1](%)
All patients	25/51 (49%)	27/51 (53%)	10/51 (20%)	4/51 (8%)
Sex:				
Male	17/39 (44%)	18/39 (46%)	9/39 (23%)	4/39 (4%)
Female	8/12 (67%)	9/12 (75%)	1/12 (8%)	0/12 (0%)
Age:				
<60 years	17/22 (77%)	15/22 (68%)	6/22 (27%)	3/22 (14%)
≥60 years	8/29 (28%)	12/29 (41%)	4/29 (14%)	1/29 (3%)
TNM stage[3]:				
I	3/3 (100%)	2/3 (67%)	2/3 (67%)	1/3 (33%)
II	3/7 (43%)	2/7 (29%)	0/7 (0%)	0/7 (0%)
III	5/14 (36%)	9/14 (64%)	2/14 (14%)	1/14 (7%)
IV	14/27 (52%)	14/27 (52%)	6/27 (22%)	2/27 (7%)
Histology[4]:				
Well-differentiated	1/2 (50%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
Moderately-differentiated	14/26 (54%)	13/26 (50%)	7/26 (27%)	3/26 (12%)
Poorly-differentiated	10/22 (45%)	12/22 (55%)	2/22 (9%)	0/22 (0%)
HPV status (PCR):				
Positive		20/25 (80%)	10/25 (100%)	3/25 (12%)
Negative		7/26 (27%)	0/26 (0%)	1/26 (4%)

[1]P16^{INK4} and L1 major capsid detected with IHC. [2]High-risk HPV types detected with DNA ISH. [3]TNM, tumour stage according to International Union Against Cancer. [4]One tumour with unknown histology.

the significantly better RT response in the combined high (2+ and 3+) p16^{INK4a} staining group remained, when including factors such as gender, tumour stage, differentiation grade and age (p=0.032).

Lastly, neither the *in situ* DNA hybridisation detection method of HPV, nor the L1 capsid IHC method correlated to response to RT (Table II, p=0.578 and p=1.000, respectively; logistic regression).

Prediction of staying disease-free 3 years after diagnosis and survival using different detection methods. Forty-three patients were included in the analysis of being free of tonsillar cancer 3 years after diagnosis. Disease status could not be estimated for 8 patients, who died of unknown or other causes than tonsillar cancer within 3 years after diagnosis or who did not undergo treatment. In the disease-specific survival analysis, only death due to tonsillar cancer was included. Forty-one patients were included in the survival analysis. Ten patients were not included, who died of other causes than tonsillar cancer or of unknown reasons or who did not undergo treatment.

A significantly higher number of patients (17/22) in the HPV PCR-positive tumour group stayed disease-free 3 years after diagnosis compared to the HPV PCR-negative tumour group (6/21) (Table II, p=0.003; logistic regression). Furthermore, the disease-specific survival was significantly better in patients with HPV PCR-positive tonsillar cancer (17/22) compared to patients with HPV-negative tonsillar cancer [(5/19) p=0.002; logistic regression, see Table II)]. The better prognosis in staying disease-free as well as the higher survival rate for the HPV PCR-positive group was not influenced by gender, tumour stage, differentiation grade or age (p=0.004 and p=0.004, respectively; logistic regression).

There was no statistically significant difference in staying disease-free 3 years after diagnosis between the p16^{INK4a}-positive and the p16^{INK4a}-negative cases (Table II, p=0.178; logistic regression).

However, a high grade of p16^{INK4a} staining was correlated to a better disease-free survival. In both the p16^{INK4a} 3+ group and the p16^{INK4a} 2+ group, 86% (6/7 in respective group) of the cases were disease-free, compared to 33% (3/9) of the cases in the p16^{INK4a} 1-positive group and 40% (8/20) of the cases in the p16^{INK4a} -negative group (Table II). The better outcome for the p16^{INK4a} 2+ and 3+ groups individually was, however, not statistically significant compared to the p16^{INK4a}-negative cases and the p16^{INK4a} 1+ group p16^{INK4a} (p=0.958; logistic regression and p=0.096; Fisher exact test, respectively). Combining the high staining (2+ and 3+) p16^{INK4a} groups, 12/14 (86%) of the patients were disease-free after three years, which was statistically significantly better than that observed in the p16^{INK4a} -negative group 40% (8/20) and the p16^{INK4a} 1+ group 33% (3/9) (p=0.018 and p=0.033, respectively; logistic regression).

Table II. *Different detection markers correlated to complete response to radiotherapy, disease status after three years[1] and disease-specific survival[2].*

Detection method	No. (%) CR to RT, p3 value	No. (%) tumour-free pat.[2], *p* value	No. (%) survived[2], *p* value
HPV PCR status:			
Positive	20/25 (80%), $p=0.103$	17/22 (77%), $p=0.003$	17/22 (77%), $p=0.002$
Negative	13/24 (54%)	6/21 (29%)	5/19 (26%)
$P16^{INK4}$ status[4,5]:			
Positive (all grades)	20/27 (74%), $p=0.420$	15/23 (65%), $p=0.178$	15/23 (65%), $p=0.173$
Negative	13/22 (59%)	8/20 (40%)	7/18 (39%)
1+	5/11 (45%), $p=1.000$	3/9 (33%), $p=1.000$	3/9 (33%), $p=1.000$
2+	7/8 (88%), $p=0.096$	6/7 (86%), $p=0.957$	6/7 (86%), $p=0.914$
3+	8/8 (100%), $p=0.096$	6/7 (86%), $p=0.957$	6/7 (86%), $p=0.914$
High-grade (2-3+)[6,7]	15/16 (94%), $p=0.018$[6]	12/14 (86%), $p=0.018$[6]	12/14 (86%), $p=0.018$[6]
$p=0.017$[7]	$p=0.033$[7]	$p=0.033$[7]	
HR ISH status[8]:			
Positive	8/10 (80%), $p=0.578$	6/9 (67%), $p=0.611$	6/9 (67%), $p=0.617$
Negative	25/39 (64%)	17/34 (50%)	16/32 (50%)
LI capsid status[4]:			
Positive	3/4 (75%), $p=1.000$	2/3 (67%), $p=1.000$	2/3 (67%), $p=1.000$
Negative	30/45 (67%)	21/40 (53%)	20/38 (53%)
All patients	33/49 (67%)	23/43 (54%)	22/41 (54%)

[1]Tumour-free patients 3 years after diagnosis. [2]Patients with disease-specific survival, 36-120 months follow-up. [3]All *p* values above are according to logistic regression. [4]$P16^{INK4}$ and L1 major capsid detected with IHC. [5]The *p* value for $p16^{INK4}$-positive cases (all grades) and grades 1+, 2+ 3+ respectively is compared to $p16^{INK4}$-negative cases. [6]The *p* value for high-grade (2-3+) $p16^{INK4}$ cases compared to $p16^{INK4}$-negative cases and [7]to $p16^{INK4}$ 1+ cases respectively. [8]High-risk HPV types detected with DNA ISH.

There were no significant differences in survival rate between the $p16^{INK4a}$-positive and the $p16^{INK4a}$-negative cases ($p=0.173$; logistic regression) either, or between the four $p16^{INK4a}$ staining groups separately (Table II).

Discussion

In line with previous publications, HPV DNA of high-risk types with a clear dominance of HPV-16 was detected by PCR in 49% of the 51 primary tonsillar squamous cell carcinomas in this study [reviewed in (17-19)].

Furthermore, 53% of the tonsillar carcinomas were $p16^{INK4a}$-positive by IHC and there was a significant correlation between $p16^{INK4a}$ overexpression and being HPV PCR-positive, since 80% of the HPV-positive cases were $p16^{INK4a}$-positive compared to 27% of the HPV-negative cases. This correlation was even stronger in the $p16^{INK4a}$ high-grade staining groups, since 88% of the high-grade (2+) $p16^{INK4a}$ staining cases were HPV PCR-positive, and all of the very high-grade (3+) $p16^{INK4a}$ staining cases were HPV PCR-positive (together 94%). Thus, there is a strong correlation in tonsillar carcinoma between $p16^{INK4a}$ staining and the presence of HPV, indicating an HPV-related pRb dysfunctional status similar to that in cervical cancer (36, 37).

Similarly, previous reports have shown a strong correlation between presence of $p16^{INK4a}$ staining and high-risk HPV (21, 27, 28, 39, 46). In the study by Andl *et al*., there was a correlation between the presence of HPV and $p16^{INK4A}$ overexpression with simultaneous pRb downregulation in 23 analysed tonsillar carcinomas (21). Likewise, in a separate study of 34 tonsillar carcinomas, 16/18 HPV-positive (types 16 and 33) also demonstrated an overexpression of $p16^{INK4A}$, whereas only 1/16 HPV-negative tonsillar carcinomas displayed $p16^{INK4a}$ staining (28). In a third study, the presence of HPV was correlated with overexpression of $p16^{INK4a}$, as well as reduced expression of cyclin-D and pRb, in 67 tonsillar carcinomas (39). Importantly, recent data have shown that E6/E7 expression has been linked to high-risk HPV DNA detection by PCR in oropharyngeal carcinoma, but not to HPV DNA detection in carcinomas in other sites of the head and neck(16, 24, 38) Moreover, in the study of Wiest *et al*., E6/E7 expression in predominantly oropharyngeal cancers was correlated to $p16^{INK4a}$ overexpression as well as reduction of pRb expression (38).

All 10 high-risk *in situ* hybridisation-positive cases were HPV-16-positive according to PCR (100% specificity), yielding no false-positive cases. However, the sensitivity of 40% (10/25) gives false-negatives in 37% (15/41) of the cases in comparison to HPV PCR. The fact that no low-risk types were found using *in situ* hybridisation further supports the high specificity of the method.

The finding that only 8% of all cases contained the HPV L1 major capsid protein by IHC was not surprising since L1 expression in fully developed cancer lesions is proposed

to be down-regulated (47). The lower sensitivity of ISH and IHC of L1 in our study confirms the findings of other large studies (48, 49).

A second aim of this study was to investigate if any of the HPV detection methods or the proposed HPV surrogate marker p16^{INK4a} could predict response to RT. Similar to previous results, 80% of the HPV PCR-positive cases compared to 52% of the HPV-negative cases had a complete RT response, indicating a tendency, but not a statistically significantly better response among the HPV PCR-positive cases (50, 51). Of the p16^{INK4a}-positive cases, 74% had a complete RT response compared to 59% of the p16^{INK4a}-negative cases, which indicates a difference, although not statistically significant. However, by combining the two high-grade (2+ and 3+) p16^{INK4a} staining groups, 94% of the patients had a complete RT response, which is notably high and statistically significantly better compared to both the p16^{INK4a}-negative and the p16^{INK4a} 1+ groups.

Although the p16^{INK4a} high staining (2+ and 3+) groups only identified 15/33 (45%) of the patients that obtained CR to RT, the results indicated that the patients identified by high staining p16^{INK4a} had a very high (94%) chance of being disease-free one month after RT. Since a CR to RT is an important factor for cure of tonsillar cancer, a method that can predict which patients respond to RT, may also indicate which patients can be spared a more aggressive treatment (52). In this study, 3 years after therapy, 85% of all patients with CR to RT stayed tumour-free compared to only 12.5% of the patients with no or partial response after RT. Notably, where 3- to 10-year follow-up data were available, 12/13 of the patients, with high-grade (2+ and 3+) p16^{INK4a} staining and CR to RT, also stayed tumour free.

A final aim of this study was to analyse if p16^{INK4a} staining, similar to the presence of HPV detected by PCR in tonsillar cancer, is a prognostic favourable factor for tumour control and survival independent of response to RT. Indeed, the combined high (2+ and 3+) staining p16^{INK4a} group showed a statistically significantly higher number of patients staying disease-free after 3 years, as well as having a better survival, in comparison to the p16^{INK4a}-negative and the p16^{INK4a} 1+ groups. Furthermore, similar to previous reports, a significantly higher number of patients in the HPV PCR-positive tonsillar cancer group stayed disease-free and had a better disease-specific survival, compared to the HPV PCR-negative tonsillar cancer group (13, 14).

Which of the methods (HPV PCR or high-grade p16^{INK4a}) is best correlated to staying recurrence-free is presently unknown. The *in situ* HPV DNA hybridisation detection and the HPV L1 IHC methods could not be correlated to response to RT, recurrence or survival.

For scoring p16^{INK4a}-positive, >10% of the tumour cells had to exhibit staining, while to be regarded as high (2-3+) grade p16^{INK4a}-positive, > 50% of the tumour cells had to exhibit staining. However, the selected cut-offs may not be optimal for identifying response to RT or survival rate. In different studies on p16^{INK4a}, HPV and oropharyngeal carcinoma, different p16^{INK4a} antibodies were used, and the cut-off levels for scoring a sample p16^{INK4a} as positive varied from 5% - 30% of the tumour cells (21, 27, 28, 39, 46). For example, in the study of Klussman *et al.*, a significant correlation between p16^{INK4a} overexpression and time to recurrence was observed, setting the cut-off at >25% p16^{INK4a} staining of tumour cells to score p16^{INK4a}-positive (28). Correlation with RT was not done in this study, however. In another study, with 67 cases of tonsillar carcinoma, the presence of p16^{INK4a} was correlated to lower risk of recurrence and better survival and, in that study, the tumours were regarded as p16^{INK4a}-positive if >5% of the tumour cells showed p16^{INK4a} staining (39). Notably, in our study, an attempt to optimise the immunohistochemical staining method was only done from a technical perspective, since titration of reaction sensitivity by varying the antibody concentration or the use of amplifying visualisation techniques were not biologically validated. In the future, the cut-off level for p16^{INK4a} staining best suited for predicting response to RT should be evaluated even more systematically.

In conclusion, p16^{INK4a} overexpression was correlated with HPV status in tonsillar carcinoma, which further supports high-risk HPV as an aetiological risk factor for tonsillar carcinoma. Furthermore, all but one patient, with high-grade (2+ and 3+) p16^{INK4a} staining, had a complete RT response. Finally, both the presence of HPV by PCR, as well as high-grade p16^{INK4a} IHC, were statistically significantly correlated to staying disease-free and having disease-specific survival. Taken together, the results suggest p16^{INK4a} may, in the near future, prove to be a useful tool when designing treatment strategies for patients with tonsillar carcinoma.

Acknowledgements

The Swedish Cancer Foundation, the Swedish Research Council, the Stockholm Cancer Society, the Stockholm City Council, the Laryngeal Foundation as well as the Karolinska Institute, Sweden, are acknowledged for their financial support. Furthermore, Ventana Medical Systems, Tucson, AZ, USA, contributed with *in situ* reagents.

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Received June 29, 2005
Accepted July 22, 2005