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Validation of Human Papillomavirus as a Favourable Prognostic Marker and Analysis of CD8+ Tumour-infiltrating Lymphocytes and Other Biomarkers in Cancer of Unknown Primary in the Head and Neck Region

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Abstract. *Background: Human papillomavirus (HPV) is a favourable prognostic factor in oropharyngeal cancer. Moreover, we and others reported that HPV-positive cancer of unknown primary in the head and neck region (HNCUP) has better outcome than HPV-negative HNCUP. However, not all studies concord. Here, our previous finding was investigated in a new cohort and additional biomarkers were analyzed. Materials and Methods: A total of 19 HNCUPs diagnosed 2008-2013 were analyzed for HPV DNA by polymerase chain reaction assay (PCR) and p16 by immunohistochemistry (IHC). Thereafter, 69 HNCUPs diagnosed between 2000-2013 were analyzed for HPV16 mRNA by PCR (if HPV16DNA-positive) and cluster of differentiation 8 positive (CD8+) tumour-infiltrating lymphocytes (TILs) and human leukocyte antigen (HLA) class I-expression using IHC. Results: HPV DNA, alone and in combination with p16 overexpression, was validated as a favourable prognostic factor in HNCUP. HPV16 mRNA was present in most HPV16 DNA-positive cases, confirming HPV-driven carcinogenesis in HNCUP. High CD8+ TIL counts indicated favourable prognosis. Conclusion: HPV status is useful for the management of patients with HNCUP and the role of CD8+ TILs should be further explored.*

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Head and neck squamous cell carcinoma (HNSCC), including cancer of the pharynx, larynx, oral cavity, nose and nasal sinuses, often presents as a lump in the neck, and the specific site of the primary tumour is in general revealed after subsequent diagnostic procedures. However, in 2-9% of the cases the primary tumour is not found and the condition is denoted as cancer of unknown primary in the head and neck region (HNCUP) (1). Treatment of the latter has traditionally comprised of neck dissection followed by postoperative oncological treatment, *i.e.* radiotherapy, at times with the addition of platinum-based chemotherapy or cetuximab, while today in some cases only oncological treatment is given (2).

Human papillomavirus (HPV)-associated oropharyngeal SCC is a HNSCC subset dominated by tonsillar and base of tongue SCC (3, 4). HPV-positive tonsillar and base of tongue SCC have increased in incidence in the past decades in the developed world and have better clinical outcomes than HNSCC in general [~80% *vs.* 40% 5-year overall survival (OS)] (5-8). HPV is frequently found in HNCUP and such cases likely have an HPV-positive oropharyngeal SCC origin, which gives reasons for steering diagnostic procedures as well as radiotherapy towards the oropharynx (9-11).

Treatment de-escalation may be attainable in patients with HPV-positive HNCUP, similar to that discussed for patients with HPV-positive oropharyngeal SCC (12). Still, before HPV status of HNCUP can be used to guide treatment, more knowledge on the biology and clinical behaviour of the

disease is needed, especially due to the debate on whether CUP in different parts of the body share common traits or whether they behave more like normal metastases (13).

Recently, we and others have reported similarities in clinical behaviour between HPV-positive HNCUP and HPV-positive oropharyngeal SCC, with a significantly better clinical outcome for patients with HPV-positive HNCUP compared to those with HPV-negative HNCUP (11, 14-15). However, other surveys have not found an impact of HPV status on survival, leaving the role of HPV in prognosis in HNCUP unresolved (16, 17).

The aim of this study was, therefore, to validate our findings on the impact of HPV status on survival in patients with HNCUP who were diagnosed 2000-2007 in a new cohort of patients diagnosed in 2008-2013. In addition, the joint cohort was used to analyse the influence on survival of cluster of differentiation 8-positive ($CD8^+$) tumour-infiltrating lymphocytes (TILs) and HLA class I expression, two prognostic biomarkers in addition to HPV status in tonsillar and base of tongue SCC (3, 18-20).

Materials and Methods

Patients and tumours. The Swedish Cancer Registry was used to identify patients with HNCUP (ICD-code C77.0) from January 1st 2008 to April 30th 2013 at the Karolinska University Hospital, Stockholm, Sweden and the study was performed with permission of the Regional Ethical Committee in Stockholm numbers 2005/431-31/4 and 2009/1147-31. Clinical work-up consisted of fine-needle aspiration cytology of the neck mass, panendoscopy of the upper aerodigestive tract with biopsies from the base of tongue and nasopharynx, bilateral tonsillectomy, computed tomography (CT) or magnetic resonance imaging of the head and neck region and in some cases full body positron emission tomography (PET)-CT. Patients in whom a primary tumour was identified by the diagnostic work-up, patients with non-SCC, and patients receiving only palliative treatment were not considered for continued analysis. Of the residual 40 patients, an additional eight unfit for surgery, and seven treated with radiotherapy only, *i.e.* never submitted to neck dissection and thus lacking obtainable specimens, were not included in the analysis. Of the remaining patients, 23/25 had formalin-fixed paraffin-embedded metastases available, but four were excluded due to lack of additional tumour material for further immunohistochemistry (IHC) analysis. This resulted in 19 patients with HNCUP in the 2008-2013 cohort with a minimum follow-up time of 3 years and their characteristics and HNCUPs are presented in Table I. They were all treated with neck dissection and postoperative radiotherapy of up to 68 Gy. In addition, seven patients received chemotherapy (cisplatin) or cetuximab.

Fifty patients with HNCUP, selected by the same criteria (the 2000-2007 cohort) and described previously (11) are also described in Table I. Notably, in four patients with HPV-negative HNCUPs in this cohort, a primary tumour was found during follow-up. OS, *i.e.* days from date of diagnosis to date of death irrespective of cause of death, and disease-free survival (DFS), *i.e.* date of diagnosis to date of relapse, with patients dying without relapse censored at time of death and patients never free of tumour excluded, were calculated for the present and the joint cohorts.

Table I. *Patient characteristics and human papillomavirus (HPV) status in the different cohorts.*

Characteristic	2000-2007 cohort (n=50)	2008-2013 cohort (n=19)	2000-2013 cohort (n=69)	*p*-Value*
Gender, n (%)				
Male	37 (74)	15 (79)	52 (75)	0.763[a]
Female	13 (26)	4 (21)	17 (25)	
Mean age, years	65	60	64	0.138[b]
N Stage, n (%)				
1	14 (28)	7 (37)	21 (30)	0.356[c]
2	31 (62)	11 (58)	41 (59)	
3	5 (10)	1 (5)	7 (10)	
Smoking history, n (%)[#]				
Yes	38 (78)	16 (84)	54 (79)	0.742[a]
No	11 (22)	3 (16)	14 (21)	
HPV DNA, n (%)				
Positive	20 (40)	12 (63)	32 (46)	0.085[d]
Negative	30 (60)	7 (37)	37 (54)	
p16, n (%)				
Positive	21 (42)	12 (63)	33 (48)	0.116[d]
Negative	29 (58)	7 (37)	36 (52)	
HPV DNA/p16, n (%)				
$HPV^+/p16^+$	18 (36)	11 (58)	29 (42)	0.082[dd]
$HPV^+/p16^-$	2 (4)	1 (5)	3 (4)	
$HPV^-/p16^+$	3 (6)	1 (5)	4 (6)	
$HPV^-/p16^-$	27 (54)	6 (32)	33 (48)	
p53 expression				
0-60%	36 (72)	14 (74)	50 (72)	0.889[d]
61-89%	0 (0)	1 (5)	1 (1)	
90-100%	14 (28)	4 (21)	18 (26)	

*Comparing the 2000-2007 and 2008-2013 cohorts. [a]Fisher's exact test, [b]independent *t*-test, [c]Mann-Whitney, [d]chi-square test, [dd]chi-square comparing $DNA^+/p16^+$ *vs.* $DNA^-/p16^-$. [#]Smoking data missing for one patient in the 2000-2007 and 2000-2013 cohorts.

HPV DNA and RNA extraction. DNA and RNA were extracted from 15 μm sections of formalin-fixed paraffin-embedded metastases using the Roche High Pure RNA Paraffin Kit (Roche AB, Stockholm, Sweden) according to the manufacturer's instructions, however, omitting the DNAse-treatment step. A blank per sample was treated as HNCUP samples to control for cross contamination. Samples were diluted to 2 ng/μl and stored at −20˚C.

HPV DNA detection. A multiplex polymerase chain reaction (PCR) assay identifying 27 HPV types, with a β-globin housekeeping gene as an amplification control, was run as previously described, with 10 μl of each sample per reaction, and SiHa cells as positive and RNAs-free water as negative controls (11, 21-22).

HPV RNA detection. HPV16 DNA-positive samples were subjected to DNAse treatment using the RNeasy MiniElute Cleanup kit (Qiagen, Hilden, Germany) followed by cDNA synthesis using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Random hexamer primers were used. A PCR was run to amplify HPV16 *E2, E5, E6*I, E6*II* and *E7* mRNA, and the housekeeping gene *U1A* to control for

Table II. *Patient characteristics, p16 status and p53 expression according to human papillomavirus (HPV) DNA-status in the different cohorts.*

Characteristic	2008-2013 cohort (n=19)		*p*-Value	2000-2013 cohort (n=69)		*p*-Value
	HPV DNA+ (n=12)	HPV DNA− (n=7)		HPV DNA+ (n=32)	HPV DNA− (n=37)	
Gender, n (%)						
Male	9 (75)	6 (86)	1.000[d]	25 (78)	27 (73)	0.620[d]
Female	3 (25)	1 (14)		7 (22)	10 (27)	
Mean age, years	55	69	0.009[b]	59	67	0.005[b]
N Stage, n (%)						
1	5 (42)	2 (29)	0.382[c]	12 (38)	9 (24)	0.168[c]
2	7 (58)	4 (57)		18 (56)	23 (62)	
3	0 (0)	1 (14)		2 (6)	5 (14)	
Smoking history, n (%)[#]						
Yes	10 (83)	6 (86)	1.000[a]	25 (81)	29 (78)	0.818[d]
No	2 (17)	1 (14)		6 (19)	8 (22)	
p16, n (%)						
Positive	11 (92)	1 (14)	0.002[a]	29 (91)	4 (11)	<0.001[d]
Negative	1 (8)	6 (86)		3 (9)	33 (89)	
p53 expression						
0-10%	7 (58)	3 (43)	0.038[aa]	22 (69)	19 (51)	0.009[dd]
11-35%	4 (33)	0 (0)		5 (15)	1 (3)	
36-60%	0 (0)	0 (0)		1 (3)	2 (5)	
61-89%	0 (0)	1 (14)		0 (0)	1 (3)	
90-100%	1 (8)	3 (43)		4 (13)	14 (38)	

[a]Fisher's exact test, [aa]Fisher exact test comparing 0-60% p53 expression with 61-100% p53 expression, [b]independent *t*-test, [c]Mann-Whitney, [d]chi-square test, [dd]chi-square test comparing 0-60% p53 expression with 61-100% p53 expression. [#]Smoking data missing for one patient in the 2000-2013 cohort.

amplifiable RNA, and β-globin to verify that no DNA remained, as previously described for *E2, E5* and *E7* (19). The following primers were used for amplification of *E6*I* and *E6*II* cDNA: HPV16E6_194.F bp194-214 5'-GTGTACTGCAAGCAA CAGTTA-3', HPV16E6_565.R bp 565-545 5'-GC ATGATTACAGCTGG GTTTC-3', HPV16E6_445.R bp 445-425 5'-TTCTTCAGGACA CAGTGGCTT-3'. The multiplex assay described above was then performed including probes: E6*Ip 5'-AGTTAATACACCTCACGT-3' and E6*IIp 5'-TTGATGATCTCACGTCGC-3' for detection of *E6*I* and *E6*II* (20, 22).

Immunohistochemistry. As previously reported, a streptavidin–biotin peroxidase method was used for IHC, with: mAb p53 (clone: DO1; Santa Cruz Biotech, Santa Cruz, CA, USA), mAb CD8 (clone: 4B11; Leica Biosystems, Newcastle, UK) and mAb HC10/HLA class I (a kind gift from Dr Soldano Ferrone, University of Pittsburgh, Cancer Institute, PA, USA) (11, 18, 20). IHC evaluations were conducted as described previously (11, 18, 20). For detection of $p16^{INK4A}$, Ventana CINtec® p16 Histology (Roche AB, Stockholm, Sweden) was used and samples were considered positive if >70% of tumour cells showed strong staining (6, 23).

Statistical analysis. The Kaplan–Meier method was used to calculate 3-year OS and DFS and to generate survival curves, with differences between groups analysed using the log-rank test. Uni- and multivariate Cox regression was used to calculate hazard ratios. Fisher's exact test, Mann–Whitney, independent *t*-test and chi-square test were used as indicated in Tables I and II. IBM SPSS Statistics Software (Version 22.0; IBM Corp., Armonk, NY, USA) was used. Reported *p*-values are two-sided and values below 0.05 were considered significant.

Results

Presence of HPV DNA, HPV mRNA and p16 expression in HNCUP. All 19 metastases in the 2008-2013 cohort contained amplifiable DNA and 12/19 (63%) were HPV DNA-positive (10 HPV16, and one each of HPV33 and HPV35) compared to 20/50 (40%) in the 2000-2007 cohort, possibly indicating an increasing trend in the proportion of HPV-positive HNCUP (p=0.085, Table I). In the combined cohort, 46% were HPV DNA positive (29 HPV16, two HPV33 and one HPV35) (Table I). p16 overexpression was observed in 12/19 (63%) samples in the 2008-2013 cohort and 33/69 (48%) in the combined 2000-2013 cohort (Table I), and in 11/12 (92%) and 29/32 (91%) in the HPV-DNA samples of these respective cohorts (Table II).

Evaluation of HPV mRNA succeeded (as indicated by presence of *U1A*) in 25/29 (86%) of the HPV16 DNA-positive samples, with 23/25 (92%) being positive for HPV16 *E6*I* and HPV16 *E7* mRNA. Of these, 20/23 (87%) were positive for HPV16 *E2* mRNA (data not shown). p16

Table III. *CD8+ tumour-infiltrating lymphocyte (TIL) counts and human leukocyte antigen (HLA)-class I expression in relation to human papillomavirus (HPV) and p16 status in the 2000-2013 cohort.*

	HPV DNA+/p16+	HPV DNA−, [a]	*p*-Value	HPV DNA+	HPV DNA−	*p*-Value	p16+	p16−	*p*-Value
CD8 TILs	n=24	n=29		n=27	n=29		n=26	n=30	
Mean	69.2	35.8	0.03[a]	63.5	35.8	0.06[a]	67.8	33.1	0.02[b]
Median	43.3	23.5		33.4	23.5		43.6	23.5	
Interquartile range	21.4-93.4	8.4-52.0		20.3-82.9	8.4-52.0		21.9-86.4	9.1-51.2	
Range	0.1-303.8	0.0-148.3		0.1-303.8	0.0-148.3		0.1-303.8	0.0-148.3	
HLA class I	n=29	n=34		n=32	n=34		n=31	n=35	
Absent	7 (24)	4 (12)	0.079[b]	9 (28)	4 (12)	0.168[b]	8 (26)	5 (14)	0.107[c]
Weak	5 (17)	3 (9)		5 (16)	3 (9)		5 (16)	3 (12)	
High	17 (58)	27 (79)		18 (56)	27 (79)		18 (58)	27 (77)	

[a]Regardless of p16 status, [b]independent *t*-test, [c]Mann–Whitney test.

overexpression was observed in 22/23 (97%) of the HPV16 *E6* and *E7* mRNA-positive samples (including three cases lacking *E2* mRNA expression) and 2/2 of the HPV DNA-positive, HPV16 mRNA-negative samples (data not shown).

p53 expression evaluated by IHC is shown in Tables I and II. Absence or low p53 expression was correlated to being HPV DNA+/p16+ and HPV DNA+.

A comparison between patients with HPV DNA+ and HPV DNA− HNCUP is shown for the 2008-2013 cohort and the 2000-2013 cohorts in Table II. Patients with HPV DNA+ HNCUP were significantly younger than those with HPV DNA+ HNCUP.

CD8-positive TIL counts and HLA class I expression. Evaluation of CD8+ TILs was possible in 56/69 samples in the combined cohort (Table III). HPV DNA+/p16+ HNCUP had significantly higher numbers of TILs than HPV DNA− HNCUP (p=0.034) and a similar trend was observed for HPV DNA+ compared to HPV DNA− HNCUP (p=0.066) (Table III). In addition, p16+ HNCUP had significantly higher numbers of CD8+ TILs than p16− HNCUP (p=0.020) (Table III).

It was possible to evaluate HLA class I expression in 66/69 samples, with the remaining three having insufficient tumour material. The data are shown for the same groups as described above for CD8+ TILs in Table III.

HPV status in relation to 3-year DFS and OS in the 2008-2013 cohort. In the 2008-2013 cohort, patients with HPV DNA+ HNCUP had significantly better 3-year DFS and OS than those with HPV DNA− HNCUP, with 100% *vs.* 66.7% (p=0.045) and 91.7% *vs.* 42.9% (p=0.028) 3-year DFS and OS, respectively. A similar trend was observed when comparing patients with HPV DNA+/p16+ samples with those with HPV DNA−/p16+/− samples, with 100% *vs.* 66.7% (p=0.056) and 90.9% *vs.* 42.9% (p=0.039) 3-year DFS and OS, respectively. Using overexpression of p16 alone in HNCUP as compared to non-overexpressing p16 HNCUP resulted in a 100% *vs.* 71.4% 3-year DFS (p=0.079) and 83.3% *vs.* 57.1% 3-year OS (p=0.299).

HPV status in relation to 3-year DFS and OS in the 2000-2013 cohort. In the entire 2000-2013 cohort all 69 patients were included in the analysis, and 3-year DFS and OS were significantly higher in the HPV DNA+/p16+ and HPV DNA+ groups when compared to the HPV DNA−/p16+/− group and in the p16+ group when compared to the p16− group, see Figure 1 for details. This was confirmed in a multivariate analysis for HPV DNA+ status and for 3-year OS for HPV DNA+/p16+ status (with a similar trend for DFS), but neither for 3-year OS nor DFS for p16+ status, when controlling for age, sex and smoking habit (Table IV).

CD8+ TILs in relation to 3-year OS and DFS. It was possible to assess clinical outcome according to numbers of CD8+ TILs for 56 patients, and this was evaluated separately for the HPV DNA+/p16+, HPV DNA+ groups compared to the HPV DNA− group, and the p16+ group compared to the p16− group. The numbers of CD8+ TILs were divided into quartiles and evaluation was determined by comparing survival in patients with tumours with the three highest quartiles *vs.* those with tumours with the lowest quartile as performed previously by Nordfors *et al.* (18).

For the HPV DNA+/p16+, HPV DNA+ and p16+ groups there were no significant differences in 3-year DFS or OS when comparing patients with the three highest CD8+ TIL quartiles as compared to those with the lowest CD8+ TIL quartile (data not shown). For HPV DNA− HNCUP, 3-year DFS was significantly higher among patients with the three highest CD8+ TIL quartiles as compared to the lowest CD8+ TIL quartile (70% *vs.* 33.3%, p=0.046), while statistical significance was not reached for 3-year OS (54.5% *vs.* 42.9%, p=0.484).

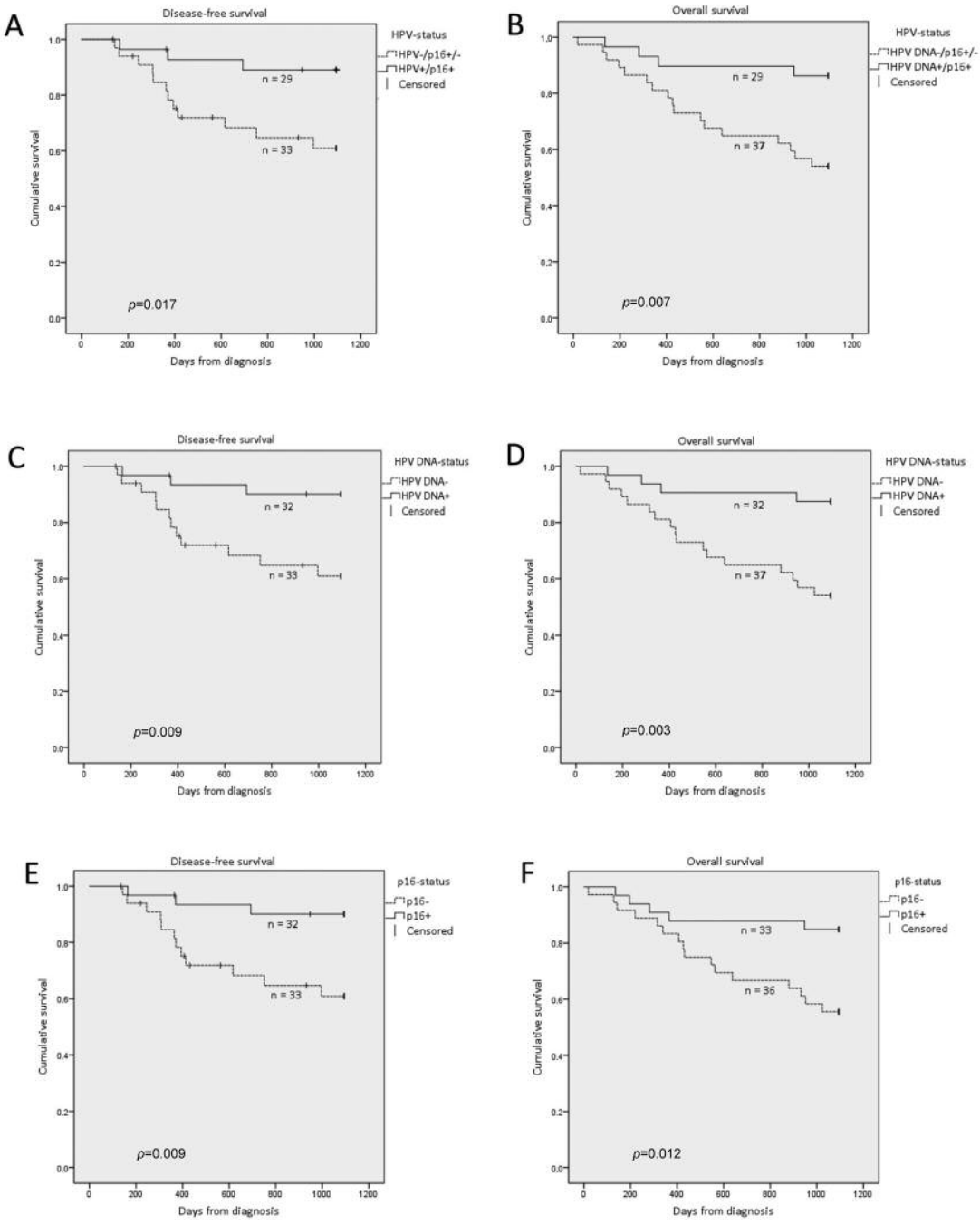

Figure 1. *Disease-free (DFS) (A, C, E) and overall (OS) (B, D, F) survival for the 2000-2013 cohort of patients with cancer of unknown primary in the head and neck region according to combined human papillomavirus (HPV) DNA and p16 status (A, B), HPV DNA status (C, D) and p16 status (E, F). 3-Year DFS and OS rates for patients with HPV DNA$^+$/p16$^+$ vs. HPV DNA$^-$/p16$^{+/-}$ status were 89.7% vs. 63.6% and 86.2% vs. 54.1% respectively, while for those with HPV DNA$^+$ vs. HPV DNA$^-$ they were 90.6% vs. 63.6% and 84.8% vs. 55.6%, respectively, and for those with p16$^+$ vs. p16$^-$ they were 90.6% vs. 63.6% and 87.5% vs. 54.1%, respectively.*

Data for p16$^-$ HNCUP were analogous to those obtained for HPV DNA$^-$ HNCUP (data not shown). When dividing the entire HNCUP cohort into quartiles irrespective of HPV and p16 status, patients in the three highest CD8$^+$ TIL quartiles had significantly higher 3-year DFS and OS compared to patients in the lowest quartiles (85.0% *vs.* 53.8%, p=0.006 for DFS and 76.2% *vs.* 50.0%, p=0.044 for OS).

Table IV. *Multivariate cox regression of head and neck cancer of unknown primary according to HPV DNA, and p16 status and patient characteristics for the 2000-2013 cohort.*

			3-Year overall survival			3-Year disease-free survival		
Regression model	Characteristic	Comparison	HR	95% Cl	*p*-Value	HR	95% Cl	*p*-Value
Model 1	HPV DNA status	Positive *vs*. negative	0.24	0.081-0.74	0.013	0.163	0.035-0.749	0.020
	Gender	Male *vs*. female	2.38	0.769-7.33	0.113	0.672	0.221-2.04	0.482
	Age	Additional year of age	1.08	1.02-1.14	0.015	1.03	0.961-1.10	0.420
	Smoking history	Ever *vs*. never smoker	10.6	1.76-64.5	0.010	5.71	0.623-52.3	0.123
Model 2	HPV DNA/p16 status	+/+ *vs*. –/–	0.309	0.101-0.941	0.039	0.213	0.044-1.02	0.053
	Sex	Male *vs*. female	2.22	0.719-7.01	0.164	0.641	0.210-1.96	0.436
	Age	Additional year of age	1.08	1.02-1.15	0.009	1.03	0.965-1.11	0.346
	Smoking history	Ever *vs*. never smoker	11.9	1.93-73.2	0.008	6.07	0.654-56.4	0.113
Model 3	p16 status	Positive *vs*. negative	0.407	0.144-1.15	0.089	0.232	0.048-1.13	0.071
	Sex	Male *vs*. female	2.19	0.717-6.68	0.169	0.463	0.145-1.48	0.194
	Age	Additional year of age	1.07	1.01-1.13	0.030	0.997	0.931-1.07	0.942
	Smoking history	Ever *vs*. never smoker	7.62	1.35-43.1	0.022	3.31	0.415-26.5	0.258

HR: Hazard ratio, CI: confidence interval.

HLA class I expression in relation to 3-year OS and DFS. Analysis of absent/weak *vs*. high HLA class I expression in correlation to clinical outcome in the HNCUP 2000-2013 cohort, was performed for 66 patients and as described previously for tonsillar/base of tongue SCC (20). All analyses were performed separately for patients in the HPVDNA$^+$/p16$^+$, HPV DNA$^+$ groups *vs*. the HPVDNA$^-$ group, and the p16$^+$ group *vs*. the p16$^-$ group. No significant differences in 3-year DFS or OS were observed in correlation to HLA class I expression in any of the groups (data not shown).

Nodal status, smoking and sex in relation to 3-year OS and DFS. In the 2000-2013 cohort, lower N-stage was correlated to a better 3-year OS (N1=90.5%, N2=63.4%, N3=42.9%, p=0.019) and 3-year DFS (N1=95.0%, N2=72.5%, N3=40.0%, p=0.025) with similar trends when stratifying for HPV (data not shown). Notably, 3-year OS and DFS was 100% for patients with HPV DNA$^+$ HNCUP and N1 disease. Age was correlated to 3-year OS (HR=1.040, 95% CI=1.005-1.077, p=0.026) but not 3-year DFS (HR=1.014, 95% CI=0.974-1.056, p=0.502). Never-smokers tended to have better prognosis than ever smokers (85.7% *vs*. 64.8% 3-year OS, p=0.130, 92.9% *vs*. 74.0% 3-year DFS, p=0.133). No differences in OS or DFS were seen between sexes.

Discussion

In this study, patients with HPV DNA$^+$ HNCUP, diagnosed between 2008 and 2013, had significantly better clinical outcome than patients with HPV DNA$^-$ HNCUP. This result confirms findings from a previous report by us in a cohort diagnosed between 2000 and 2007 (11). Notably, in the present 2008-2013 cohort, 3-year DFS was 100% in HPV DNA$^+$ HNCUP. It was possible to analyse HPV16 mRNA in 25/29 HPV16 DNA$^+$ HNCUP diagnosed 2000-2013 and *E6* and *E7* mRNA expression was found in the great majority of HPV16 DNA$^+$ samples, indicating that these HNCUP are indeed driven by HPV. Finally, in the 2000-2013 cohort, not taking HPV status into account, high CD8$^+$ TIL numbers correlated to positive clinical outcome. It was not possible to evaluate CD8$^+$ TILs and HLA class I expression in the subgroups with HPV DNA$^+$/p16$^+$, HPV DNA$^+$ or p16$^+$ tumours due to too few events.

The definition of HNCUP and the scope of the diagnostic work-up before deciding on a HNCUP diagnosis may vary between studies and can affect the obtained data (15-17). In studies where bilateral tonsillectomy, a procedure often revealing the primary tumour, was part of the work-up, some reports show that HPV$^+$ HNCUP, defined by presence of HPV DNA or overexpression of p16, have better clinical outcome than HPV$^-$ HNCUP (14, 15). Other studies have however not been able to demonstrate a survival difference in a similar HNCUP setting (16, 17). Here, in a HNCUP cohort from 2008-2013, the survival benefit for patients with HPV DNA$^+$ HNCUP previously found for patients with HNCUP diagnosed 2000-2007 was confirmed (11).

Since the definition of HPV$^+$ status is still under some debate, all analyses were performed separately for patients in the groups of HPV DNA$^+$ *vs*. HPV DNA$^-$, HPV DNA$^+$/p16$^+$ *vs*. HPV DNA$^-$/p16$^{+/-}$ and p16$^+$ *vs*. p16$^-$ tumours. In the 2008-2013 cohort, HPV DNA$^+$ and HPV DNA$^+$/p16$^+$ were superior to p16 in determining a 3-year OS benefit, while differences were smaller for 3-year DFS. The

analysis was repeated in the entire 2000-2013 cohort, giving us one of the largest HNCUP cohorts reported to date and here the above three ways of defining HPV status gave significant results for both 3-year DFS and OS.

The proportion of HPV+ HNCUP was 40% 2000-2007 compared to 63% in the 2008-2013, possibly indicating an increasing trend, but the difference was not statistically significant. Nevertheless, the proportion of HPV+ tumours in the 2008-2013 was relatively similar to that reported for tonsillar and base of tongue SCC during the same period (7, 24).

HPV mRNA has been demonstrated in metastases from HPVDNA+ tonsillar and base of tongue SCC, while to our knowledge there are only two small studies investigating HPV mRNA in HNCUP, finding HPV mRNA in 10/22 and 2/3 HNCUP respectively (25-27). Additionally, the latter two studies did not compare HPV mRNA and HPV DNA status. Here, HPV16 *E6*I* and *E7* mRNA were detected in the great majority of HPV16 DNA+ HNCUP, showing that HPV is indeed actively transcribed in HPV DNA+ HNCUP. Lack of HPV16 *E2* mRNA expression, previously shown as a negative prognostic factor in HPV16+ tonsillar and base of tongue SCC, could however not be evaluated as a prognostic factor in this study, since only three patients had this feature.

Having a high number of CD8+ TILs has been shown to correlate to a favourable clinical outcome in several cancer types, including tonsillar and base of tongue SCC (18, 28-30). Here this also applied to the combined 2000-2013 HNCUP cohort. When dividing the cohort into subgroups depending on HPV status, statistically significant results were obtained for 3-year DFS in the HPV DNA− group, but not for other subgroups. This was likely due to small sample sizes and the very few events, especially in the HPV DNA+/p16+, HPV DNA+ and p16+ groups. Notably, the latter groups had higher numbers of CD8+ TILs than the corresponding HPV DNA− and p16− groups, not surprising given the viral component of the disease, and the corresponding findings for HPV+ and HPV− tonsillar and base of tongue SCC (18).

While, HPV DNA+ HNCUP had a similar mean number and median value of CD8+ TILs as HPV DNA+ tonsillar and base of tongue SCC (median of 33.4 *vs*. 36.0 and mean of 63.53 *vs*. 56.32 respectively, p=0.565), HPV DNA− HNCUP had significantly more CD8 TILs than HPV DNA− tonsillar and base of tongue SCCC (median of 23.5 *vs*. 6.2 and mean of 35.85 *vs*. 16.14, p=0.004) (18).

Similar to other HNSCC, having high expression of HLA class I was recently shown to correlate to better outcome in HPV− tonsillar and base of tongue SCC, while surprisingly having absent/weak expression correlated to a better prognosis in HPV+ tonsillar and base of tongue SCC (20, 31-32). Here, the corresponding correlations were not found. Whether this was due to a small cohort with few HPV+ and HPV− cases, or the very few events in the HPVDNA+/p16, HPVDNA+ and p16+ groups needs to be further investigated. Lack of p53 expression by IHC in HNCUP was previously shown to correlate to good prognosis (11). Here, absent/low p53 IHC expression correlated to presence of HPV hence the separate role of p53 on clinical outcome was not pursued.

One limitation of the study is the small sample size of the 2008-2013 cohort, particularly when sub-dividing the cohorts, making survival analysis difficult. This is especially true for the HPV DNA+/p16+, HPV DNA+ and p16+ groups with their excellent prognosis. Larger cohorts in such a rare entity as HNCUP are however difficult to obtain and it is worth noting that despite this several significant data were gained.

Clearly, HPV DNA+ HNCUP resembles HPV DNA+ tonsilIar/base of tongue SCC. HPV mRNA is expressed in most HPV DNA+ HNCUP and patients in the latter group have a remarkably better prognosis than those with HPV DNA− tumours, similar to data on HPV DNA+ and HPV DNA− tonsilIar/base of tongue SCC during the same time period in Sweden (8). Furthermore, HPV DNA+ HNCUP generally overexpresses p16, has higher numbers of CD8+ TILs and patients are younger as compared to patients with HPV DNA− HNCUP, similar to that observed for tonsillar and base of tongue SCC (18, 20). Thus, this study strengthens the hypothesis that HPVDNA+ HNCUP has an HPV DNA+ tonsilIar/base of tongue SCC as its origin and that HPV status of the HNCUP could positively influence the management of patients with HNCUP.

In conclusion, patients with HPV DNA+/p16 or HPV DNA+ HNCUP have an excellent clinical outcome, and much better 3-year OS and DFS than patients with HPV DNA− HNCUP. HPV DNA+ HNCUP expresses HPV mRNA and behaves much like HPV DNA+ tonsillar and base of tongue SCC. HPV status of HNCUP is therefore highly useful in the management of these patients. CD8+ TILs are promising as a prognostic biomarker in HNCUP and should be studied further for clinical use.

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