Oral HPV infection and persistence in patients with head and neck cancer

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Objective. To investigate the presence and persistence of human papillomavirus (HPV) infection in the oral mucosa of patients with head and neck squamous cell carcinoma (HNSCC), and its correlation with prognosis.

Study design. HPV infection was characterized in tumors and pre and posttreatment oral scrapings in 51 patients with HNSCC and matched controls using the SPF10 LiPA Extra assay. p16INK4A immunostain and in situ hybridization for high-risk HPV genotypes recognized transcriptionally active infection in tumor samples. The risk of infection was compared in patients and controls. The association of pretreatment HPV status with recurrence and survival and with posttreatment HPV persistence was assessed.

Results. Oral HPV infection risk was significantly higher in patients with HNSCC than in controls ($P < .001$). Oral HPV infection was associated with infection in the first posttreatment scrapings ($P = .015$), but did not affect recurrence or prognosis.

Conclusion. Oral HPV infection is frequent in patients with HNSCC and has no prognostic implications, suggesting that posttreatment polymerase chain reaction monitoring on oral cells is not effective to monitor patient recurrence risk.
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The human papillomavirus (HPV) is involved in the genesis of tumors of the upper digestive tract, particularly of squamous cell carcinomas (SCCs) arising in the tonsils and the oropharyngeal region.[1,2] Despite changes in behavioral exposure to traditional risk factors for head and neck SCC (HNSCC),[3] the worldwide incidence of oropharyngeal SCC has steadily increased over recent years, which has been attributed largely to the increasing number of HPV-associated tumors.[4] The identification of HPV in oropharyngeal carcinomas has prognostic significance, with longer survival and higher rate of response to therapy in cases positive for HPV.[5-7]

The natural history of HPV infection has been extensively detailed in the uterine cervix,[8,9] whereas less data is available on the different phases of HPV infection and oncogenesis in the head and neck. In the female genital tract, HPV persistence in the cervical mucosa is the strongest risk factor for high-grade intraepithelial and invasive SCC,[10,11] and increases the risk of tumor recurrence.[12,13] HPV infection and persistence is easily monitored on cervical exfoliated cells, which can be used for the molecular identification and typing of HPV.[14,15] It has been suggested that oral exfoliated cells could be similarly used to monitor HPV infection in the oral cavity and the risk of oropharyngeal SCC.[16] Several authors[17-20] reported that HPV infection is more common in oral mucosa cells of patients with HNSCC as compared with controls, although scarce information is available on the persistence of viral infection after tumor treatment[21] and on its correlation with tumor recurrence.[22]

Defining the role of HPV infection in head and neck cancerogenesis is further complicated by a wide range of analytical methods available for virus detection, each with different sensitivity and specificity and different targets. In particular, when compared with the gold standard of viral oncogene transcription,[23] widely

Statement of Clinical Relevance

The study sustains the hypothesis that incidental oral human papillomavirus infection and persistence are frequently observed in head and neck carcinoma and bear no prognostic implications, whereas oncogenic infection is rare and limited to a subset of oropharyngeal cancers.

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used consensus polymerase chain reaction (PCR)-based methods are not sufficiently specific, while p16INK4A (p16) immunostain and in situ hybridization (ISH) have low sensitivity.[23] Determination of p16 expression and ISH are nonetheless currently the methods of choice in most laboratories to identify HPV-associated oropharyngeal SCC,[20,21,23] but cannot easily be applied to oral cytology monitoring, where HPV DNA amplification is the most convenient method to assess the presence of the virus.[20,21]

We have recently demonstrated with a highly sensitive commercial PCR and reverse hybridization-based assay the presence of several low-risk (LR) and high-risk (HR) HPV genotypes in over 90% of paired HNSCC biopsies and oral mucosa samples, with an excellent agreement for HPV infection and genotype characterization between cancer and cytologic samples.[24] In the present study, we investigated the persistence of HPV infections in the oral cavity of patients with HPV-positive and HPV-negative HNSCC after cancer treatment by means of repeated oral mucosa scrapings during patient follow-up, to shed light on the natural history of HPV infection in the upper digestive tract and on its correlation with disease recurrence and survival.

MATERIALS AND METHODS

Patients and sampling

The study series consists of 51 consecutive patients presenting to our center between 2008 and 2011, who underwent an endoscopic biopsy for SCC of the mouth, tongue, oropharynx, larynx, or hypopharynx. Only patients with biopsy and cytologic samples adequate for the study were included.[24] The mean age of patients was 62.2 (standard deviation, SD 11.5) years and the male to female ratio was 43:8 (Table I). Inclusion criteria were the first presentation of a previously untreated invasive SCC, and intention to referring to our center for further therapy and follow-up. After biopsy, 23 (45%) patients were treated with surgery alone, 5 (9.8%) only with radiotherapy, 4 (7.8%), 4 (7.8%), and 1 (1.9%), respectively, with chemo and radiotherapy, surgery and radiotherapy, and surgery and chemotherapy, and 14 (27.4%) patients with surgery followed by chemo and radiotherapy (Table I). Biopsy samples were fixed in formalin and processed routinely for histopathologic study. The first cytologic sample (t0) was obtained from each patient 3-8 days after endoscopy via a gentle scraping of the mucosa of the cheeks with a plastic spatula. Follow-up cytologic samples were obtained in the same way during planned control visits to the outpatient clinic at 6 and 12 months after hospital discharge, and at any subsequent control visit until June 2012. Posttreatment scrapings were classified as t1-t5 according to the number of scrapings obtained from each patient. The scraped cells were suspended immediately in ThinPrep-Preserv-Cyt solution (Cytec Corporation, Marlborough, MA) and stored at 4 °C. All follow-up biopsies performed in the head and neck region and metastatic sites from the enrolled patients were included in the study. The negative control group consisted of patients who were seen at the otolaryngology clinic for benign conditions and who agreed to a buccal scrape. For each enrolled patient, 1 control was chosen after matching by age (within 5-year categories) and sex among the control group. The mean age of the control subjects was 61.6 (SD 11.1) years. The protocol was reviewed and approved by the Institutional Ethical Review Board and is in compliance with the Helsinki Declaration. Each subject enrolled in the project signed a detailed informed consent form.

HPV DNA detection and typing

DNA extraction from buccal scrapes was performed within 1 week of sampling by lysis and digestion with proteinase K. Briefly, pelleted cells from 1.5 mL of Preserv-Cyt solution were washed in phosphate buffered saline (PBS) and resuspended in 100 μL of lysis solution (KCL 50 mM, Tris−HCl 10 mM (pH 8.3), MgCl 2 2.5 mM, Tween 20 0.45%, NP40 0.45%, proteinase K 500 mg/mL) at 56 °C for 1 h. Following heat inactivation of proteinase K, 10 μL of the solution was used for PCR amplification of the HPV sequences from the L1 region using SPF10 primers in a final reaction volume of 50 μL for 40 cycles.

For DNA isolation from the formalin-fixed paraffin-embedded biopsies, 3-5 10-μm-thick sections were incubated in 200 μL of lysis solution (1 mg/mL proteinase K in Tris 50 mM, pH 8.0, ethylenediaminetetraacetic acid (EDTA) 1 mM, Tween 20 0.45%, and octylphenoxypolyethoxyethanol (IGEPAL) CA-630 0.45%) for 16-24 h at 56 °C. Proteinase K was inactivated with heat and the lysates were centrifuged to eliminate wax, purified with spin columns (Quiagen, Crawley, UK), and resuspended in 100 μL of TRIS-EDTA 0.1%. Ten microliters of the solution were used for the reaction. Positive and negative controls were introduced in each set of 12 reactions; these included DNA from SiHa and HeLa cell lines at a specified number of HPV copies and blank reagents throughout all steps of the procedure. Concurrent amplification of human *HLA-DPB1* gene was included in the assay as an internal control for DNA adequacy. HPV type-specific sequences were detected by the line probe INNO-LiPA HPV genotyping assay version Extra (Innogenetics NV, Ghent, Belgium) according to the manufacturer's instructions. The Extra version of the assay allows the simultaneous and separate detection of 18 HR (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82), 7 LR (6, 11, 40, 43, 44, 54, and 70), and

Table I. Demographic characteristics, tobacco and alcohol consumption, tumor characteristics, and HPV genotype distribution among patients with SCC and controls

Explanatory variable	*With FU*	*Without FU*	*Total*	*Controls*
No.	34	17	51	51
Sex (M/F)	28/6	15/2	43/8	43/8
Age − mean (SD)	67.1 (11.5)	59.9 (10.9)	62.3 (11.5)	61.6 (11.1)
Tumor site − *n* (%)				N/A
Oral cavity	5 (14.7)	0	5 (9.8)	
Tongue	6 (17.6)	2 (11.7)	8 (15.6)	
Oropharynx	8 (23.5)	1 (5.8)	9 (17.6)	
Larynx	10 (29.4)	11 (64.7)	21 (41.1)	
Hypopharynx	4 (11.7)	3 (17.6)	7 (13.7)	
Lymph node metastasis	1 (2.9)	0	1 (1.9)	
Clinical stage − *n* (%)				N/A
I	9 (26.4)	6 (35.2)	15 (29.4)	
II	7 (20.5)	1 (5.8)	8 (15.6)	
III	5 (14.7)	2 (11.7)	7 (13.7)	
IV	13 (38.2)	8 (47)	21 (41.1)	
Current smoker − *n* (%)	24 (70.5)	11 (64.7)	35 (68.6)	27 (52.9)
Alcohol abuse* − *n* (%)	5 (14.7)	6 (35.2)	11 (21.5)	0
Therapy − *n* (%)				N/A
Surgery	16 (47)	7 (41.1)	23 (45)	
Radiotherapy	2 (5.8)	3 (17.6)	5 (9.8)	
Chemo and radiotherapy	4 (11.7)	0	4 (7.8)	
Surgery and radiotherapy	1 (2.9)	3 (17.6)	4 (7.8)	
Surgery and chemotherapy	0	1 (5.8)	1 (1.9)	
Surgery, chemotherapy, and radiotherapy	11 (32.3)	3 (17.6)	14 (27.4)	
Tumor progression or recurrence − *n* (%)	11 (32.3)	6 (35.2)	17 (33.3)	N/A
HPV positive in t0 scraping − *n* (%)	29 (85.2)	16 (94.1)	45 (88.2)	8 (15.6)
Genotype distribution in T0 scraping − *n* (%)				
NT	7 (20.5)	1 (5.8)	8 (15.6)	7 (13.7)
LR	6 (17.6)	4 (23.5)	10 (19.6)	0
HR	16 (47)	11 (64.7)	27 (52.9)	1 (1.9)

FU, follow-up; *NT*, no specific type; *LR*, low risk; *HR*, high risk; *N/A*, not applicable.
*Consumption of ≥15 drinks per week.

2 unclassified HPV types (69/71 and 74). Hybridization patterns were analyzed with the specific software Line reader and Analysis Software (LiRAS) for LiPA HPV and the results were confirmed by 2 independent readers.[25]

HPV DNA in situ hybridization

DNA ISH was performed on paraffin tissue sections with the INFORM HPV III family 16 and INFORM HPV II family 6 probes (Ventana Medical Systems Inc, Tucson, AZ) using the ISH I View Blue Plus Detection Kit according to the manufacturer's instructions, on a Ventana BenchMark XT automated stainer. The probes hybridize, respectively, with HR HPV genotypes 16, 18, 33, 35, 45, 51, 52, 56, and 66 and with LR genotypes 6 and 11. Cases were read by the study head and neck pathologist (P.M.). Any definitive nuclear staining in the tumor cells was considered positive. Cases were classified in a binary manner as either positive or negative.

p16 Immunostain

For the immunohistochemical detection of p16 protein, the CINtec Histology Kit (MTM Laboratories AG, Heidelberg, Germany) was used according to the manufacturer's instructions. Samples of high-grade cervical intraepithelial lesions were used as positive controls. Cases were read by the study head and neck pathologist (P.M.). Brown staining of tumor cell nuclei and cytoplasm was interpreted as positive. Cases with p16 immunostain that showed intense diffuse, continuous, and confluent nuclear and cytoplasmic staining of tumor cells were considered as positive according to previously published criteria.[26]

Statistical analysis

The Shapiro−Wilk test was used to test the normal distribution of quantitative variables. When quantitative variables were normally distributed, the results were expressed as mean values and SD, otherwise

median and interquartile range (IQR; 25th-75th percentile) were reported; comparisons between groups were performed with the appropriate parametric or nonparametric tests. Qualitative variables were summarized as counts and percentages, and patient groups were compared using χ^2 test or Fisher's exact test, as appropriate.

Logistic regression models were used to find the odds of HPV infection among controls and patients. Results were expressed as odds ratio (OR, crude or adjusted for age, sex, smoke, and alcohol) with 95% confidence interval (CI). Disease-specific survival (DSS) and disease-free survival (DFS) were derived with the use of the Kaplan−Meier product-limit method and the log-rank test was used to analyze comparisons among groups. Univariate and multivariate Cox proportional hazard models were fitted to investigate the associations between DSS and DFS and covariates. Results were expressed as hazard ratios (HRs) with 95% CI. All tests were 2 sided. Data were analyzed with the STATA statistical package (release 10.0, 2009, Stata Corporation, College Station, TX).

RESULTS

t0 Cytologic samples

HPV DNA was present in 45 cytologic samples (88.2%). Twenty-seven (52.9%) of them hosted 1 or more HR HPV genotypes (range 1-5), 10 (17.8%) hosted only LR genotypes, and in 8 (15.6%) no specific viral type could be identified. In the control population, 1 (1.9%) sample was positive for HR HPV, and in 7 (13.7%) no specific viral type could be identified. Case and control series significantly differed in the proportion of cases positive for HPV and HR HPV, and in the distribution of LR, HR, and non−type-specific infections ($P < .001$) (Table I).

Tumor biopsy samples

SPF10 LiPA assays of 46 (90.1%) tumor biopsies were positive for HPV DNA. Twenty-seven (52.9%) of them hosted 1 or more HR HPV genotypes, 11 (21.5%) hosted only LR genotypes, and in 8 (15.6%) no specific viral type could be identified. HPV was present in all SCCs of the mouth and of the hypopharynx (5 and 7, respectively), in 18 (85.7%) of 21 SCCs of the larynx, in 7 (87.5%) of 8 SCCs of the tongue, in 8 (88.8%) of 9 SCCs of the oropharynx, and in the lymph node metastasis. HR genotypes were present in 2 (40%) SCCs of the mouth, in 5 (71.4%) SCCs of the hypopharynx, in 12 (57.1%) SCCs of the larynx, in 3 (37.5%) SCCs of the tongue, and in 5 (62.5%) SCCs of the oropharynx. The immunohistochemical stains showed that p16 was expressed in at least 75% of the neoplastic cells in 4 SCCs (2 from the oropharynx, 1 from the larynx, and 1 from the tongue), all positive for HPV DNA (Figure 1). ISH documented the presence of integrated and episomal HR HPV in the nuclei of the 2 p16-positive oropharyngeal tumors, whereas all other samples and ISH for LR HPV were negative (Figure 1). Fisher's exact test showed a significant correlation between p16 and ISH results ($P = .004$). No correlation was observed between the presence of HPV and HR HPV DNA in biopsies and p16 expression or ISH results.

Comparison of HPV status in t0 scrapings and tumor biopsy samples

In 38 (74.5%) patients there was complete agreement for type-specific infections and infection profile or absence of infection between t0 scrapings and tumor biopsy samples. In 8 (15.6%) cases, biopsy and cytologic samples shared 1 or more HR genotypes, but differed for further type-specific infections: in 4 cases, 1 genotype (2 HR and 2 LR) was only present in the biopsy; in 3 cases, 1 genotype (2 HR and 1 LR) was only present in the scraping; in the last case, 1 HR and 1 LR genotypes were only present, respectively, in the biopsy and in the scraping. In 5 patients no correlation was found between biopsy and scraping. In 2 of them, only the biopsy result was positive (1 HR, 1 non−type-specific infection); in 1, only the scraping was positive for a non−type-specific HPV infection; in 1, the biopsy result showed LR infection, and the scraping non−type-specific HPV DNA; in the last, the biopsy result showed a non−type-specific infection, and the scraping an HR genotype.

Follow-up cytologic samples

Thirty-four (66.6%) patients had 1 or more scrapings (range 1-5, mean 2.0, SD 1.1) performed during the follow-up. Four patients did not enter the follow-up program and died of progressive disease 7-8 months after the diagnosis. Three of them were positive for HR HPV in both tumor and scraping, the last one had LR HPV in both samples. Thirteen patients chose to refer to a different outpatient clinic. There was no difference in HPV status ($P = .650$), HR HPV status ($P = .372$), and genotype distribution ($P = .426$) in t0 scrapings obtained from patients with and without follow-up. These 2 groups of patients did not differ in age, sex, smoking habit, stage, and tumor site distribution (Table I). The number of patients that fulfilled each follow-up scraping, the proportion of cases for positive HPV, and the genotype distribution in the follow-up cytologic samples are detailed in Table II. HPV status, HR HPV status, and genotype distribution in scrapings obtained at each follow-up point were significantly different ($P < .001$) from the control series, whereas

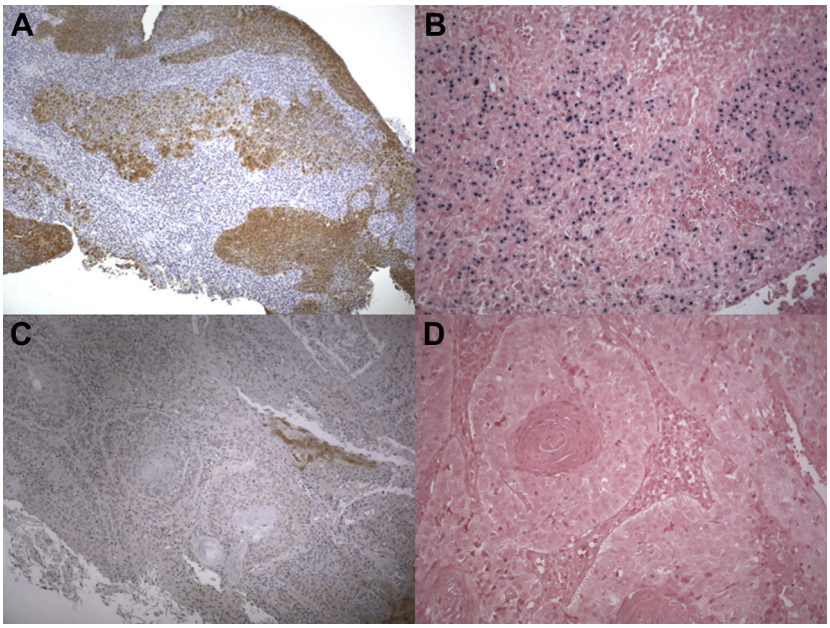

Fig. 1. Photomicrographs showing p16INK4A diffuse immunoreactivity (**A**) and HR HPV ISH nuclear dot-like signal (**B**) in an HPV-associated nonkeratinizing oropharyngeal SCC, and the absence of p16 expression (**C**) and of ISH signal (**D**) in a keratinizing SCC of the tongue (**A**, **C**: diaminobenzidine tetrahydrochloride chromogen and hematoxylin nuclear counterstain; **B**, **D**: nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate chromogen and red counterstain II; magnification: **A**, **C** ×10; **B**, **D** ×20).

Table II. Number of patients, timing, and HPV distribution in baseline and follow-up oral scrapings from patients and controls

Explanatory variable	*t0*	*t1*	*t2*	*t3*	*t4*	*t5*	*Controls*
No. of patients	51	34	21	11	4	1	51
Median (IQR) time from diagnosis	//	9 (6-12)	19.5 (15-24)	27 (27-30)	36 (31-39)	33 (—)	//
HPV DNA (%)	46 (90.1)*	29 (85.2)*	17 (80.9)*	9 (81.1)*	3 (75.0)	1 (100)	8 (15.6)
HR HPV (%)	27 (52.9)*	16 (47.0)*	8 (38.0)*	7 (63.6)*	2 (50.0)	0	1 (1.9)
LR HPV (%)	10 (19.6)*	9 (26.4)*	5 (23.8)*	0	0	0	0
NT HPV (%)	9 (17.6)*	4 (11.7)*	4 (19.0)*	2 (18.1)	25 (50.0)	1 (100)	7 (13.7)

IQR, interquartile range; *HPV*, human papillomavirus; *HR*, high risk; *LR*, low risk; *NT*, no specific type.
*Significantly different from controls ($P < .001$).

there was no difference between follow-up scraping groups for these parameters.

Natural history of type-specific HPV infections

Three (8.8%) patients tested negative for HPV in all posttreatment scraping samples. One of them had non—type-specific HPV DNA in the tumor sample, the others had negative biopsy results. Sixty-seven individual type-specific infections, defined as the presence of a specific viral type in 1 or more consecutive samples, and 12 infections not sustained by a specific viral type were identified in the other 31 patients and are listed in Table III. Briefly, 25 persistent type-specific infections (17 HR, 68% and 8 LR, 32%), defined as the presence of a specific HPV type in at least 2 consecutive samples,[11] were present since t0 in 18 patients. All but one (96%) were observed also in the patient biopsy sample; the only discordant case was a persistent HR oral infection whose tumor biopsy result was positive for non—type-specific HPV DNA. The mean duration of observed infections was 15.8 months (6-42, SD 8.4). Twelve HR and 7 LR infections (76%) persisted in all follow-up scrapings. Remission was observed in 5 HR and 1 LR infection after 12-19 months (mean 13.8, SD 4.1), but could not be documented for the 19 infections that persisted until the last follow-up scraping. Four HR infections recurred after remission. In 7 patients, 12 (8 HR, 75% and 4 LR, 25%) type-specific infections were present at t0 but were not found in the next follow-up scrapings. Ten (83.3%) of these infections, including a coinfection sustained by 5 different HR genotypes, were also present in the tumor biopsy. One HR and 1 LR infection were not present in the biopsy, which however

Table III. Natural history of oral HPV infections during the follow-up of patients with HPV-positive SCC

		No. of specific infections			*No. of persistent infections since t0 (months of persistence)*			*No. of resolved infections at t0*			*No. of infections recurred during FU*			*No. of infections appeared during FU (months of persistence)*		
Case	*FU (months)*	*HR*	*LR*	*NT*	*HR*	*LR*	*NT*	*HR*	*LR*	*NT*	*HR*	*LR*	*NT*	*HR*	*LR*	*NT*
1	6			1			1 (6)									
2	9		1			1* (9)										
3	42	2	2		1* (42)									1	2†	
4	12	1												1*		
5	9	1	1					1*							1	
6	15	1		1			1* (9)							1		
7	9	4			2* (9)			1						1		
8	24	3	2		1* (24)				1			1		1,1 (12)		
9	33		1	2						1*			1		1	
10	21	2	1		2* (9,21)	1* (21)										
11	27	2	3		1* (12)				1*		1				2†	
12	24		1			1* (24)										
13	18		1	2						1*			1		1	
14	38			1												1 (26)
15	42	2	2		1* (12)	1* (12)					1				1 (24)	
16	12	2			2* (12)											
17	30	1	1			1* (30)								1		
18	27	2						1*			1					
19	6	1	1		1* (6)	1* (6)										
20	12	1			1* (12)											
21	36	2	3						2*			1		2 (12)†		
22	27	5		1				5*								1
23	36	7			2* (19,19)						2			2,1 (30)		
24	9		1			1* (9)										
25	15		1			1* (15)										
26	15		1	1			1* (6)								1	
27	15	1			1* (15)											
28	18		1	1						1					1*	
29	12	1			1* (12)											
30	24	1		2						1*			1	1		
31	6	1			1 (6)											

FU, follow-up; *HR*, high risk; *LR*, low risk; *NT*, no specific type.
*The same type-specific infection was present in tumor biopsy.
†The same viral type was present in 2 nonconsecutive scrapings.

hosted other HR genotypes also found as persistent oral infections. Three infections (1 HR and 2 LR) recurred in later nonconsecutive scrapings. Twenty-three (13 HR and 10 LR) type-specific de novo infections were observed in follow-up scrapings from 16 patients. In 3 cases, the same viral type was responsible for 2 different infection episodes in nonconsecutive scrapings from the same patient. Two (1 HR and 1 LR) (8.6%) of these 23 type-specific infections had been present in the tumor biopsy but not in the t0 scraping. Eleven were observed in the last available scraping from the patient; of the other 12, 4 persisted and were documented in consecutive scrapings. Globally, 37 (55.2%) of 67 individual infections were present at t0; 11 (29.7%) of them underwent permanent remission, 19 (51.3%) persisted, and 7 (18.9%) recurred after remission. Thirty infections (44.7%) were contracted during the follow-up (7 recurrent and 23 new infections).

In 7 patients, non−type-specific HPV infections were observed at t0. In 5 patients, all with non−type-specific HPV DNA in the tumor biopsy, a consecutive series of scrapings positive for non−type-specific infections (mean follow-up 21 months, SD 7.6, range 15-33) was interrupted by a single type-specific (2 HR and 3 LR) infection. In 1 case, the non−type-specific viral infection persisted throughout the entire follow-up (6 months), but the biopsy result was negative for HPV; in the last patient, the results of both tumor biopsy and t1 scraping were positive for LR HPV infection. Two non−type-specific infections were acquired de novo during the follow-up; one, presenting in a previously negative patient, persisted for 24 months, the other appeared in the last follow-up scraping of a patient who hosted 5 different HR type-specific infections in the biopsy and t0 scraping, all resolved in the next scraping.

Correlation between tumor and oral HPV infection

The odds of an oral HPV or HR HPV infection at the time of diagnosis were compared among patients and

controls. Patients were significantly more likely than controls to have any oral HPV infection (OR adjusted for sex, age, smoke, alcohol 87.38, 95% CI 15.81-483.02, $P < .001$) or HR HPV infection (adjusted OR 79.54, 95% CI 8.44-749.60, $P < .001$). Fisher's exact test showed a significant association of HPV status between tumors and t0 scrapings; no association was observed with scrapings obtained at t1, t2, and t3. Tumor HR HPV status was significantly associated with t0 and t1 scrapings. A significant association was observed for HPV and HR HPV status also between t0 and t1 but not t2 or t3, and between t1 and t2 but not t3 scrapings (Table IV).

HPV infection in follow-up biopsy samples

Sixteen posttreatment biopsies were obtained from 15 patients, all with previous HPV-positive cytologic samples. Biopsies included 7 local recurrences, 1 second tumor, 2 lymph nodes, and 1 pulmonary metastasis, and 5 non-neoplastic tissue samples from the site of the treated primary tumor. The mean time from the initial diagnosis was 10.2 months (SD 13.8, range 1-33) for the non-neoplastic samples, 3 of which were obtained after surgical treatment and 1 after radiotherapy, 20.8 months (SD 12.1, range 9-38) for local recurrences, which occurred in 3 cases after surgery and chemoradiotherapy, in 2 after chemoradiotherapy, and in 2 after surgery alone, 11 months for the second tumor, presenting after chemoradiotherapy, 16 and 24 months for the lymph node metastases, both occurring after surgery, and 26 months for the pulmonary metastasis, which presented in a patient treated with surgery and chemoradiotherapy. HPV DNA was found in all biopsies, independently of their site, time interval, type of treatment, and diagnosis (negative, tumor, or metastasis). The same type-specific infections observed in the temporally closest scraping were present in all samples; in 4 of them, 1 further viral type not observed in the scraping was present in the biopsy, and in 1 case 1 of the viral types present in the scraping was absent in the biopsy. None of the posttreatment biopsies were positive by p16 or ISH.

Clinical follow-up

Median follow-up time was 28 months (IQR 19-42, range 6-51). Thirteen patients had 1 or more local recurrences ($n = 7$), a second SCC in the upper aerodigestive tract ($n = 1$), metastasis to the laterocervical lymph nodes ($n = 3$), and lung metastasis ($n = 2$), at a median time of 17 months (IQR 9-23, range 3-36) after the initial diagnosis and response to therapy. At 12, 24, and 36 months the rate of patients free from disease was 87.0% (95% CI 73.3-93.2), 76.6% (95% CI 60.6-86.7), 65.6% (95% CI 47.0-79.1), respectively; DFS ranged from 3 to 51 months (median 26, IQR 15-40). Eleven (19.6%) patients died. Four of them did not respond to treatment or died of surgical complications: 2 patients of 52 and 79 years with stage IV tumors suffered disease progression despite surgery and adjuvant chemo and radiotherapy, 1 (73 years old, with stage IV tumor) died of surgery-related complications, and 1 (77 years old, stage II) did not respond to radiotherapy, while surgery and chemotherapy had been excluded for severe concomitant diseases. Six other patients died of their disease, including 4 with disease recurrence, 1 with lung metastasis, and 1 with laterocervical metastasis. One patient died of myocardial infarction with no evidence of tumor. One patient with a second tumor, 1 with recurrent tumor, 1 with lung metastasis, and 1 with laterocervical metastasis were alive with disease at the time of the last follow-up; 2 local recurrences and 1 laterocervical metastasis were successfully treated with surgery. At 12, 24, and 36 months the rate of patients who were alive was 92.0% (95% CI 80.1-96.9), 85.2% (95% CI 71.3-92.7), 73.1% (95% CI 56.0-84.4), respectively. None of the controls, including the 1 with HR HPV infection, were diagnosed with HNSCC at our center after enrollment in the control group.

Correlation of HPV infection with outcome and survival

HPV DNA was present in the biopsies and in oral scrapings of 15 (88.2%) of 17 patients who suffered disease progression, recurrences, second tumors, or metastases, and in 30 (88.2%) of 34 patients who did not. Eleven (64.7%) and 16 (47%) infections, respectively, were sustained by HR HPV in the biopsies; 10 (58.8%) and 14 (41.1%) in the cytologic samples. HPV was present in the tumors and in oral scrapings of all 11 patients with unfavorable outcome (8 HR in both types of sample) and of all 4 patients who were alive with disease (3 HR in the biopsies and 2 in the scrapings); of the 36 patients with favorable outcome, 31 (86.1%) had HPV-positive tumors and 32 (88.8%) had HPV-positive scrapings; HR genotypes were present in 16 (44.4%) tumors and in 17 (47.2%) cytologic samples. Twenty-eight patients were treated with protocols including chemo and/or radiotherapy: HPV and HR HPV infections were present, respectively, in 12 (92.6%) and 9 (63.2%) tumors of the 13 patients of this group that experienced disease recurrence or progression, and in 13 (86.6%) and 6 (40%) tumors of the 15 patients that did not ($P > .05$). Oral HPV infection was present in 11 (84.6%) patients with recurrence or progression and in 12 (80%) patients without; HR genotypes were present in 8 (66.6%) and 7 (46.6%) cases, respectively. In these 28 patients, HPV was present in the tumors and in the oral cavity of all 7 patients with unfavorable outcome

Table IV. Concordance (%) and significance levels (*P*) of Fisher's exact tests for HPV and HR HPV infection of each sample series compared with the following series

	HPV biopsy	*HPV t0*	*HPV t1*	*HPV t2*	*HR HPV biopsy*	*HR HPV t0*	*HR HPV t1*	*HR HPV t2*
t0	91.2% (<0.001)				96.1% (<0.001)			
t1	85.3% (0.094)	88.2% (0.015)			70.6% (0.037)	70.6% (0.037)		
t2	85.7% (0.080)	85.7% (0.080)	90.5% (0.012)		66.7% (0.203)	61.9% (0.038)	76.2% (0.032)	
t3	90.9% (0.182)	100% (0.182)	72.7% (0.491)	72.7% (0.491)	81.8% (0.088)	81.8% (0.088)	72.7% (0.242)	63.6% (0.545)

HPV, human papillomavirus; *HR*, high risk.

(5 HR in both types of sample); of the 4 patients who were alive with disease, 4 had HPV-positive tumors (all HR) and 3 had HPV-positive oral scrapings (2 HR); of the 17 patients with favorable outcome, 14 (82.3%) had HPV-positive tumors and 13 (76.4%) HPV-positive scrapings; HR genotypes were present, respectively, in 7 (41.1%) and in 8 (47%) samples. Fisher's exact test did not show a significant association of HPV and HR HPV infection and outcome in all evaluated groups ($P > .05$).

The Cox proportional hazard analysis did not show any correlation between DFS or DSS and the presence of HPV or HR HPV infection in tumor or oral mucosa cells at t0. Only clinical stage IV was significantly correlated with shorter DSS and DFS (Figure 2). In patients with cytologic follow-up, Fisher's exact test did not show any association between HPV or HR HPV infection at t1, t2, and t3 and tumor recurrence.

DISCUSSION

The present study reports that oral HPV infections at the time of tumor diagnosis and their persistence during the first months of follow-up are significantly more frequent in subjects with HPV-positive SCC than with HPV-negative SCC, and that the risk of hosting HPV in the oral mucosa is significantly higher in subjects with head and neck carcinomas than in controls. The high prevalence of oral HPV infection that we observed in HNSCC as compared with the general population suggests that the presence of the tumor might favor the localization and persistence of the virus in the tumor cells[27,28] and in adjacent mucosa,[17,20] possibly mediated by immune suppressive mechanisms. It has recently been shown that SCCs activate several strategies in order to evade the host immune response,[29] which could favor viral infection and persistence in both tumor and oral mucosa. An increased risk of HPV oral infection because of immunologic dysfunction has indeed been documented in patients positive for HIV,[30,31] and in non-HIV tobacco smokers.[32,33]

Detecting HR HPV DNA in tumor tissue, while supporting an association between virus and cancer, does not distinguish whether HPV is transcriptionally active and thus able to promote carcinogenesis (the so-called driver infection), or inactive, probably representing incidental or passenger infection. To determine the oncogenic activity of HPV, direct or indirect methods that assess viral integration or viral oncogene transcription are necessary.[23] In the present study, an oncogenic HR HPV infection was demonstrated by means of validated p16 immunostain and ISH in only 2 oropharyngeal SCCs, which is the site where HPV carcinogenesis has been most consistently documented,[1,2] whereas most of the infections recorded in tumors by PCR did not show evidence of viral integration or transcription. The markedly different rates of HPV positivity that we obtained with PCR and ISH in our tumor series likely depend on the different analytical sensitivities of the 2 tests: SPF10 LiPA has the highest sensitivity among HPV typing assays, because of the short amplified DNA fragment (65 base pairs).[34] SPF10 LiPA sensitivity differs for different HPV genotypes, but is at least 1-10 copies per 10^3 cells,[35] whereas the ISH estimated sensitivity threshold is 1-2 viral copies per cell.[36] We assume that, in PCR-positive and ISH-negative tissue samples from the present series, the viral load was lower than this threshold, as observed by Chaturvedi et al.[37] who documented with quantitative (q)PCR that 22.5% and 50%, respectively, of SPF10 LiPA HPV16- and HPV non−16-positive tumors had less than 1 viral genome per cell. The fact that HPV transcriptional activity has been associated with a viral load of at least 0.5 copies per cell[38,39] supports the hypothesis that PCR-positive p16- and/or ISH-negative infections observed in the present study carry no oncogenic activity. However, on the basis of previous literature data[40-43] we cannot definitely exclude oncogene transcription in these infections. A conclusive clarification of this aspect would require the amplification of HPV E6/E7 messenger RNA, which could not be performed in our series because of the lack of frozen tumor samples. Indirect evidence from prognostic and survival associations suggests that most infections identified in the present study are passenger infections, as opposed to the strong prognostic correlations recently reported for oropharyngeal SCCs associated with HR HPV infection,[5-7] we did not find any differences in the rates of disease progression or recurrence, nor in outcome or survival of patients positive for HPV or HR HPV DNA. No correlation was found even when we considered only those patients who had been treated with protocols

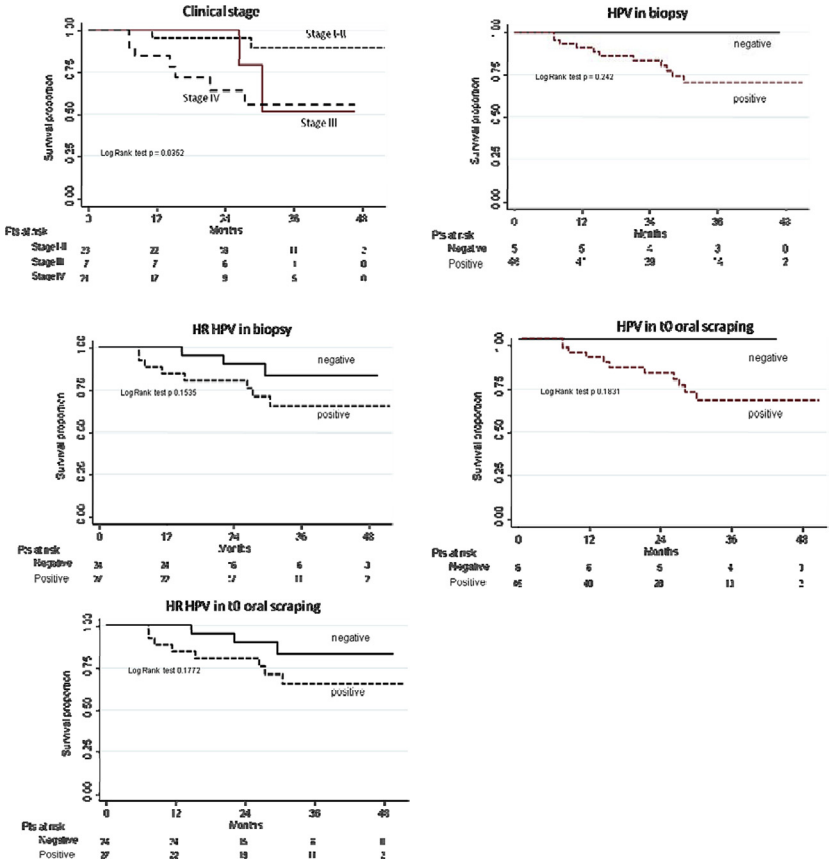

Fig. 2. Kaplan–Meier survival curves showing a significant association between clinical stage IV and DSS; the presence of HPV and HR HPV infections in tumor biopsies and in oral scrapings did not influence patient survival.

including chemo and/or radiotherapy, which tend to be more effective in HPV-associated tumors.[7]

Very few studies have so far addressed the issue of HPV persistence in the oral mucosa after tumor treatment. Recently, Agrawal et al.[21] demonstrated that posttreatment oral HPV infections were significantly more frequent among individuals diagnosed with HPV16-positive HNSCC than with HPV16-negative HNSCC, and that not only HPV16 but also other HR and LR HPV types were more common in the oral mucosa of patients with positive tumors. These observations are in agreement with ours regarding HPV persistence in the oral cavity after tumor treatment. We also documented that over two-thirds of the type-specific oral infections present at diagnosis persisted in the follow-up for at least 6 months, with a maximum of 42 months. Moreover, several infections appearing posttreatment were sustained by viral types responsible for previously resolved infections, suggesting the reactivation of a latent infection or re-infection from a site of persistence. Interestingly, the strength of association between tumor and oral positivity for HPV and HR HPV progressively reduced with increasing follow-up time. When we compared cytologic sample groups, the strongest correlation was found between the closest scraping series (t0 and t1, t1 and t2), suggesting that the infection profile changes over time, with both resolutions and new infections.

We observed several persistent infections in which a specific viral type could not be identified. In most of these patients, a specific genotype was characterized in a single follow-up sample. It is possible that the non–type-specific positive results were because of the presence of a number of viral genomes lower than the threshold of detection of the linear array, and that the genotype was recognized only when the viral load increased above this threshold (as a consequence of active replication or of sampling issues). As an alternative hypothesis, it is recognized that consensus primers amplifying the HPV L1 region (including SPF10) may fail to identify fully integrated viruses, yielding false-negative[44] or non–type-specific results (Morbini et al., personal communication). This phenomenon fully explains our observation of a persistent HR HPV oral infection in a patient with non–type-specific viral DNA in the biopsy, and could be true for the persistent non–type-specific infections positive for HR HPV in a single scraping. It seems, however,

unlikely for the LR infections with a similar behavior, given that integration is not commonly observed in LR HPV genotypes. Finally, SPF10 LiPA amplifies but does not recognize cutaneous HPV genotypes, which have been previously found in the oral mucosa.[45] The eventual presence of a genital type-specific infection in these patients renders this possibility unlikely. Quantitative, genotype-specific PCR on cytologic samples will help to clarify this aspect.

As far as prognosis is concerned, we could not find any association between HPV persistence in the oral mucosa and tumor recurrence, as previously reported.[22] On the contrary, patients with and without oral HPV infection or persistence showed similar recurrence rates, and all posttreatment biopsies including normal mucosa from sites of previous tumors hosted the virus. The prognostic relevance of oral infections, especially persistent ones, is probably correlated with their oncogenic potential. No direct assumption could be drawn on the nature (passenger versus driver) of the oral infections recorded with scrapings, since viral transcriptional activity was not determined in oral cytologic samples. However, all persistent infections documented in posttreatment biopsies did not show transcriptional activity. Literature data offer indirect evidence that oral infections are probably passenger: qPCR studies reported a median viral load of 13.0 HPV genomes per 10^3 oral exfoliated cells in patients with oropharyngeal cancers,[20] which is lower than the loads associated, respectively, with oncogene transcription,[38,39] and with replicative infections in the cervical mucosa (50-100 copies per cell).[9]

The lack of prognostic implications for the presence and persistence of HPV DNA in the oral mucosa suggests that consensus PCR on oral scraping is not effective for posttreatment monitoring, whereas its results may bear severe psychosocial implications for patients and families. As in tissue samples used for cancer diagnosis, we need to establish parameters that more accurately discriminate between passenger and potentially oncogenic infections in the oral mucosa, to make oral cytology an effective instrument for patient screening and monitoring.[46]

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