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Time trends in the prevalence of HPV in oropharyngeal squamous cell carcinomas in northern Spain (1990–2009)

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Recent studies support an important role for human papillomavirus (HPV) in oropharyngeal squamous cell carcinomas (OPSCC), although the incidence varies widely depending on the geographic location and time period studied. The aim of this study was to determine the proportion of HPV in a large cohort of OPSCC in northern Spain in the years 1990–2009. Clinical records and paraffin embedded tumor specimens of 248 consecutive patients surgically treated for OPSCC (140 tonsillar and 108 base of tongue) between 1990 and 2009 were retrieved. OPSCC cases were histomorphologically evaluated, and protein expression of p16 and p53 was analyzed by immunohistochemistry. Detection of high-risk HPV DNA was performed by GP5+/6+-PCR and *in situ* hybridization (ISH). Thirty cases (12%) were positive for p16 immunostaining, of which eight (3.2% of the total series) were found positive for HPV type 16 by genotyping of GP5+6+-PCR products. All HPV GP5+/6+-PCR-positive tumors were p53-immunonegative, seven had a basaloid morphology and seven were also positive by HPV ISH. Presence of HPV correlated inversely with tobacco and alcohol consumption ($p < 0.001$), but not with age of onset of OPSCC. Overall survival was better in the HPV-positive group, although not statistically significant ($p = 0.175$). OPSCC patients in northern Spain demonstrated a low involvement of HPV, increasing (although not significantly, $p = 0.120$) from 1.8% in 1990–1999 to 6.1% of cases in 2000–2009.

In contrast to squamous cell carcinoma (SCC) in other subsites within the head and neck region, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) is increasing.[1–3] This phenomenon is world-wide and also observed in Spain.[4] The north of Spain has the highest incidence within the country, both for head and neck SCC as well as OPSCC.[5] The opposite incidence trends for head and neck SCC and OPSCC are probably related to the two main etiological factors; while the habit of smoking tobacco is generally decreasing slowly, various studies have reported an increasing prevalence of human papillomavirus (HPV) in head and neck SCC, particularly in OPSCC.[2]

Reported prevalences of HPV positive OPSCC vary enormously in the literature, ranging from 4.4% up to even 93%. This variation has been ascribed to geographical differences, the variation in the time period of OPSCC diagnosis, anatomical subsite, and differences in HPV detection techniques. In North America, the proportion of HPV-positive OPSCC ranges between 60 and 70%,[6] in Australia it is reported to be 46%, in Asia 25–50%, and in South America 4.4%.[7–9] In Western European countries including United Kingdom, France, and Germany, reported proportions vary between 40 and 60%, and The Netherlands appeared to have the lowest proportion of about 20% in 2000–2006, but apparently increasing to 30% in 2010.[8,10,11] Studies in Northern Europe have reported higher proportions, up to 93% in tonsillar carcinomas in Sweden.[8,12] There are few data on the prevalence in Southern Europe. In a systematic worldwide review by Herrero et al.,[7] studies from Spain and Italy were included and indicated a proportion of around 13%, and in a revision of the global burden of cancer cases attributable to infectious agents, De Martel et al.[13] estimated the prevalence of HPV infection in oropharyngeal cancer in Southern Europe to be 17%. The aim of this study was to determine the proportion of HPV infection in a large cohort of OPSCC in Asturias (northern Spain).

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What's new?

Despite the decreasing prevalence of tobacco smoking across much of the developed world, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) continues to rise. In some areas, the rise has been accompanied by an increasing prevalence of human papillomavirus (HPV) in OPSCC, though prevalence rates vary widely. Here, low HPV prevalence is reported for tumor material collected from a subset of OPSCC patients in northern Spain over the period 1990–2009. OPSCC incidence is notably high in that region of the country, but HPV rates increased only modestly, from 1.8% to 6.1%, over the years studied.

Material and Methods

Patients and tissue specimens

Surgical tissue specimens from 248 consecutive patients with OPSCC (140 of tonsillar and 108 of base of tongue origin; there were no cases originating from the soft palate) who underwent surgical treatment at the Hospital Universitario Central de Asturias between 1990 and 2009 were retrospectively collected, following institutional review board guidelines. Informed consent was obtained from all patients. Our hospital is a center of referral for our region and the vast majority of OPSCC patients in Asturias are treated in our institution. Representative tissue sections were obtained from archival, formalin-fixed and paraffin-embedded (FFPE) blocks and the histological diagnosis was confirmed by an experienced pathologist (MFF). The series included 113 well, 90 moderately and 44 poorly differentiated tumors, determined according to the degree of differentiation of the tumor (Broders' classification).

All patients had a single primary tumor and received no treatment prior to surgery. Only nine patients were women, and the mean age was 58 years (range 30–85 years). All but seven patients were habitual tobacco smokers, 113 moderate (1–50 pack-years) and 105 heavy (>50 pack-years), and 234 were alcohol drinkers. The stage of the tumors was determined according to the TNM system of the International Union Against Cancer (7th Edition): six tumors were stage I, 21 stage II, 41 stage III, 153 stage IVa and 27 stage IVb. No patient had distant metastases at the time of diagnosis. 166 (67%) of 248 patients received postoperative radiotherapy. An overview of all clinicopathological and follow-up data is given in Table 1.

Tissue microarray construction and DNA extraction

Five morphologically representative areas were selected from each individual tumor paraffin block: two for DNA isolation and three for the construction of a tissue microarray (TMA). To avoid cross-contamination, two punches of 2 mm diameter were taken first, using a new, sterile punch (Kai Europe GmbH, Solingen, Germany) for every tissue block, and stored in eppendorf tubes at room temperature. Subsequently, three 1 mm cylinders were taken to construct TMA blocks, as described previously.[14] A total of 10 TMAs were created, containing three tissue cores of each of the 248 OPSCC. In addition, each TMA included three cores of normal epithelium (tonsil) as an internal negative control and three cores of a HPV-positive cervix carcinoma as a positive control.

Special care was taken to obtain high-quality DNA from the formaldehyde-fixed, paraffin-embedded tissues. DNA extracted from archival material can be partly degraded and cross-linked, the extent of which depends on the pH of the formaldehyde and the time of the fixation before paraffin embedding. We applied an elaborate extraction protocol especially for paraffin embedded tissues,[15] which includes thorough deparaffinization with xylene, methanol washings to remove all traces of the xylene, and a 24-hr incubation in 1 mol/L sodium thiocyanate to reduce cross-links. Subsequently, the tissue pellet was digested for 3 days in lysis buffer with high doses of proteinase K (final concentration, 2 μg/μL, freshly added twice daily). Finally, DNA extraction was done using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

Immunohistochemistry

The TMAs were cut into 3-μm sections and dried on Flex IHC microscope slides (DakoCytomation, Glostrup, Denmark). Immunohistochemistry was performed using an automatic staining workstation (Dako Autostainer, Dako Cytomation, Glostrup, Denmark) with the Envision system and diaminobenzidine chromogen as substrate. The following primary antibodies were used: anti-p53 clone DO-7 (DAKO, Glostrup, Denmark) and anti-p16 clone E6H4 (Roche mtm laboratories AG, Heidelberg, Germany).

P16 and p53 immunostainings were evaluated by two independent observers (MFF and JPR). P16 immunostaining was scored as negative (0), weak to moderate staining (1+) or moderate to strong diffuse nuclear and cytoplasmic positive staining (2+). Scores $\geq$ 1 were considered as p16-positive expression. P53 immunostaining was evaluated as positive when $>$ 10% of the malignant cells showed nuclear staining.

HPV DNA detection and genotyping by GP5+/6+-PCR-enzyme-immuno-assay and luminex assay

High-risk HPV DNA detection and genotyping were performed as described previously.[11,16] In short, isolated DNA was subjected to GP5+/6+-PCR with an enzyme-immunoassay (EIA) read-out for detection of 14 high-risk HPV types (*i.e.,* HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). Subsequent genotyping of the infection was performed by bead-based array on the Luminex platform. When GP5+/6+ PCR was positive, type-specific PCR for HPV16 was performed using primers located in the E7 gene as

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Table 1. Clinicopathological features according to HPV-G5+6+ PCR status

		p16-neg	p16-pos and HPV-pos	*p*-Value	
Total number		240	8		
Age (years)		58 (30–85)	56 (38–80)	0.326	*t*-test
Gender	Male	231	8	1.000	Fisher's exact
	Female	9	0		
Localization	Tonsil	134	6	0.472	Fisher's exact
	Base of tongue	106	2		
Tobacco	No	5	2	<0.001	Pearson
	1–50 pack-year	125	6		
	>50 pack-year	105	0		
	Missing	5	0		
Alcohol	No	6	3	<0.001	Pearson
	<50 gr/day	15	2		
	51–100 gr/day	59	3		
	>100 gr/day	155	0		
	Missing	5	0		
Stage	I	5	1	0.121	Pearson
	II	19	2	0.045	FE (I + II *vs*. III + IVa + IVb)
	III	40	1		
	IVa	149	4		
	IVb	27	0		
T	1	17	1	0.407	Pearson
	2	56	4	0.113	FE (T1 + 2 *vs*.T3 + 4a + 4b)
	3	80	2		
	4a	84	1		
	4b	3	0		
N	0	59	3	0.416	Fisher's exact
	1–3	181	5		
Grade	Well	109	4	0.209	Pearson
	Moderate	89	1		
	Poor	41	3		
	Missing	1	0		
Radiotherapy	No	78	4		
	Yes	162	4		
Recurrence	No	95	4	0.717	Fisher's exact
	Local	47	0		(no *vs*. yes)
	Regional	18	1	0.986	Mantel-Cox
	Distant metastasis	37	1		
	Local + regional	29	2		
	Local + regional + metastasis	14	0		
Second Primary	No	212	8	0.603	Fisher's exact
	Yes	28	0		
Clinical course	Alive without tumor	54	5	0.175	Mantel-Cox
	Died of disease	131	3		
	Died of other causes	47	0		
	Lost to follow-up	8	0		

previously described.[15] Following the algorithm of Smeets et al.,[16] only those cases showing positive p16 immunostaining were analyzed.

HPV DNA detection by *in situ* hybridization

In situ hybridization (ISH) with biotinylated HPV DNA probes considered to react with HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (Y1443, DakoCytomation, Glostrup, Denmark) was performed on all 248 OPSCC using 3 μm FFPE tissue sections of the TMAs, according to the manufacturer's instructions. The results were evaluated by two independent observers (MFF and JPR). Focal DAB staining in the tumor nuclei indicated the presence of HPV.

Statistical analyses

Univariate analysis by Pearson χ^2 and Fisher's exact tests was used for comparison between categorical variables and Student's *t*-test for parametric continuous variables. For time-to-event analysis, Kaplan-Meier curves were plotted. Differences between survival times were analyzed by the log-rank method. Patients developing second primary tumors were censored at the incidence date of the second tumor. All tests were two-sided. The values of $p \leq 0.05$ were considered statistically significant.

Results

Thirty of 248 cases (12%) were p16-immunopositive, 15 with a 1+ score, and 15 with a 2+ score. P53 was scored positive in 145 of 246 cases (58%). Eleven (4%) cases showed a p16-positive 2+ score and p53-negative immunostaining. HPV DNA detection by GP5+/ 6+ PCR was performed on the 30 cases that showed either 1+ or 2+ p16-immunopositivity, and resulted in a total of eight positive cases (Table 2). Six were located in the tonsil and two in the base of tongue. All contained HPV type 16 and were 2+ p16-immunopositive and p53-immunonegative. Seven of them were also HPV-positive by ISH, which was performed on all 248 cases. Histologically, seven of them showed a basaloid-like differentiation pattern (Table 2). In addition, ISH showed the presence of HPV DNA in one other case not scored positive by GP5+6+ PCR-EIA.

Table 2. Results of HPV-genotyping, HPV-ISH, p16 and p53 immunohistochemistry and histological examination of HPV G5+6+ PCR-positive cases

		GP5+/6+ PCR		IHC		
	T/BT	HPV type	ISH	p16	p53	Histology
1	T	16	Pos	2+	0	B
2	T	16	Pos	2+	0	B
3	T	16	Pos	2+	0	B
4	T	16	Pos	2+	0	B
5	T	16	Neg	2+	0	B/K
6	T	16	Pos	2+	0	B
7	BT	16	Pos	2+	0	B
8	BT	16	Pos	2+	0	K

Abbreviations: T/BT: tonsillar/base of tongue; ISH: *in situ* hybridization; IHC: immunohistochemistry; B: basaloid differentiation; K: keratinizing differentiation.

Relation to clinicopathological and follow-up data

The relationship of HPV GP5+6+ PCR status with clinicopathological data and follow-up is shown in Table 1. All HPV-positive patients were men. No difference in the mean age at diagnosis or the oropharyngeal subsite was observed between HPV positive and negative cases. Presence of HPV correlated inversely with tobacco and alcohol consumption ($p < 0.001$). Three of eight (37.5%) HPV-positive cases were early stage tumors (I-II), whereas among the HPV-negative cases only 24 of 240 (10%) were early stage ($p = 0.045$). No significant correlation was found with T-classification, lymph node involvement or grade of differentiation. With regard to follow-up data, the development of recurrent disease did not significantly differ between HPV-positive (4/4, 50%) and negative (145/240, 61%) cases ($p = 0.717$). However, disease-specific survival was better in the HPV-positive group, although this did not reach significance due to the low number of cases ($p = 0.175$). Of note, none of the HPV-positive patients developed a second primary tumor.

Finally, to analyze possible time-trends, patients were categorized in two groups according to the time of diagnosis: 166 patients diagnosed between 1990 and 1999 and 82 patients between 2000 and 2009. The proportion of HPV-positive cases showed an increase from 1.8 to 6.1% ($p = 0.120$), while heavy tobacco use decreased from 49 to 29% ($p = 0.009$). Heavy alcohol intake remained stable at 60–64%, and the proportion of tonsillar tumors dropped from 63 to 44% ($p = 0.006$) (Table 3). Age at diagnosis, disease stage, T-classification, N-classification, recurrences and second primary tumors did not show a time-trend.

Discussion

Reported proportions of HPV-positive OPSCC vary largely in the literature, ranging from 17% up to even 93%. Apart from differences in geographical distribution and subsites in the oropharynx, worldwide the prevalence appears to be increasing over time,[6,10–12] making the years in which series of tumor samples were collected an important parameter to take into consideration when comparing different studies from the literature. Besides case selection, also the applied HPV detection test plays a role. The most reliable test for HPV is RT-PCR to detect E6/E7 mRNA transcripts. However, detection of mRNA is technically more challenging on archival FFPE samples because mRNA from FFPE is often of poor quality, and detection is type-specific. No single detection method seems to give reliable results on FFPE. Smeets et al.[16] proposed a combination of p16 IHC followed by HPV-DNA GP5+/ 6+ PCR on the p16-positive cases, which has recently been validated by comparing E6/E7 RT-PCR and

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Table 3. Distribution of p16-positive and HPV G5+6+ PCR-positive OPSCC, heavy tobacco and alcohol use and tonsillar localization over two decades: 1990–1999 and 2000–2009

	1990–1999 $n = 166$	2000–2009 $n = 82$	*p*-value	
HPV positive	3 (1.8%)	5 (6.1%)	0.120	Fisher's exact
Tobacco > 50 pack-year	81 (49%)	24 (29%)	0.009	Pearson
Alcohol > 100 gr/day	106 (64%)	49 (60%)	0.991	Pearson
Tonsillar localization	104 (63%)	36 (44%)	0.006	Fisher's exact

p16 IHC/HPV-DNA PCR on both frozen and FFPE tissue samples of 82 patients, showing a concordance of 98%.[11] Other studies advocated a triple method using p16 IHC, HPV-DNA PCR and HPV ISH[17] or p16 IHC and histological evaluation of basaloid differentiation.[18]

This study evaluated all the parameters advocated in the above mentioned algorithms and, following the algorithm by Smeets et al.,[16] detected an overall 3.2% proportion of HPV GP5+/6+ PCR-positive cases in a series of 248 OPSCC, diagnosed between the years 1990 and 2009 in Asturias, northern Spain. Also the ISH, which was performed on all 248 cases, showed a total of eight HPV-positive cases, seven of which concurring with the GP5+/ 6+ PCR. When comparing the 2 decades, an increase from 1.8 to 6.1% was observed (Table 3). As the ENT Department in the Hospital Universitario Central de Asturias is a center of referral for our region and the vast majority of OPSCC patients in Asturias are treated in our institution, we do not believe that the increase is biased by different referral patterns over time. Still, this prevalence is among the lowest reported thus far in the literature.[6–12] Only two HPV studies from Spain have been published, one including patients from Barcelona, Granada and Seville,[7] and another on 15 patients from Oviedo.[19] Both studies reported a presence of HPV type 16 in 13.5% of the cases. Probably, the actual proportion is lower, because of the tests applied in those studies. It is not likely that the findings in our present series reflect an underestimation, as the different analyses proved to be concordant (Table 2). A possible explanation for the low proportion may lie in a lower exposure to HPV, either due to a lesser presence of HPV in the general population in the north of Spain or to different sexual behavior. Another reason may be the composition of patients; this series differs in some ways from other published series: the proportion of men versus women is much higher, the heavy use of tobacco and alcohol is more elevated, and tumors demonstrate a relatively higher T and N classification (overall survival is similar). Particularly the elevated proportion of heavy smoker and drinker patients may cause a high absolute number of smoking-related OPSCC, which reduces the proportion of HPV-related OPSCC. Nevertheless, the absolute number of HPV-related tumors is also very low. Comparing patients according the year of diagnosis showed an increase (but not significant) in HPV infection and a significant decrease in heavy tobacco use (Table 3). Since a universal HPV vaccination program including all woman at the age of 14 years has recently implemented in Spain, a stabilization or even reduction in HPV-related oropharyngeal cancer may be expected in the next years due to the reduction of HPV-16 infections.

HPV-positive OPSCC have been described to exhibit unique characteristics, including a younger age at diagnosis, less exposure to tobacco and alcohol, lower T-classification, higher N-classification, poorer tumor differentiation, less frequent second primaries, and above all, a better prognosis.[20] It may be that the higher survival is due to a better response to treatment, which is radiotherapy with or without chemotherapy.[21] However, also with surgery alone, HPV-positive oral SCC patients were shown to have a more favorable clinical course.[22]

Statistical analysis in our series is hampered by the imbalance in HPV-positive ($n = 8$) and HPV-negative ($n = 240$) cases, but our data showed less tobacco and alcohol consumption, lower tumor stage, better disease-specific survival, and less second primary tumors in HPV-positive patients, which is in agreement with the literature. In addition, seven of the eight HPV-positive cases showed the basaloid histological pattern which has been associated with HPV infection.[18] Our results did not confirm previous reports that HPV-positive cases would have younger age or higher N-classification.

Better classification of tumors with the help of biomarkers may aid the clinician to avoid over- or under-treatment of patients. HPV-positive patients may gain from deintensification of treatment as they respond better and/or have a better prognosis.[23,24] However, it is important not to detect false-positive HPV in OPSCC patients.

In conclusion, OPSCC of patients in northern Spain between 1990 and 2009 showed a very low, but increasing prevalence of HPV, going from 1.3% in the time period 1990–1999 to 6.1% in 2000–2009.

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