HPV infection in squamous cell carcinomas arising from different mucosal sites of the head and neck region. Is p16 immunohistochemistry a reliable surrogate marker?

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Background: Human papillomavirus 16 infection has been proven to be associated with oropharyngeal squamous cell carcinomas (SCCs) and is probably the main reason of the reported increase in the incidence. The role of high-risk (HR) HPV for carcinogenesis of other sites in the head and neck awaits confirmation. With the aim to evaluate the prevalence of HPV infection and the reliability of different diagnostic tools in SCCs of different sites, 109 consecutive untreated head and neck SCCs were enroled, and fresh tumour samples collected.

Methods: Human papillomavirus DNA was detected by Digene Hybrid Capture 2 (HC2). Human papillomavirus E6 and E7 mRNA were detected by NucliSENS EasyQ HPVv1. P16 expression was evaluated by immunohistochemistry.

Results: In all, 12.84% of cases were infected by HR genotypes and 1.84% by low-risk genotypes. Human papillomavirus 16 accounted for 87% of HR infections. The overall agreement between DNA and RNA detection is 99.1%. Although p16 expression clearly correlates with HPV infection ($P = 0.0051$), the inter-rater agreement is poor ($k = 0.27$). The oropharynx showed the highest HR HPV infection rate (47.6%) and was also the only site in which p16 immunohistochemistry revealed to be a fair, but not excellent, diagnostic assay ($\kappa = 0.61$).

Conclusion: The prognostic role of HR HPV infection in oropharyngeal oncology, with its potential clinical applications, underscores the need for a consensus on the most appropriate detection methods. The present results suggest that viral mRNA detection could be the standard for fresh samples, whereas DNA detection could be routinely used in formalin-fixed, paraffin-embedded samples.

More than 50 000 cases of head and neck cancer, mostly squamous cell carcinomas (head and neck SCCs (HNSCCs)), are estimated to have occurred in the United States in 2011, causing about 13 000 deaths (Siegel *et al*, 2011). Head and neck squamous cell carcinomas grossly accounts for 3.5% of all malignant tumours in the United States (Siegel *et al*, 2011), although in other parts of the world, such as India, Southeast Asia and Brazil, these tumours are much more prevalent (Shah and Lydiatt, 1995).

The best established risk factors are cigarette smoking and alcohol abuse (Shah and Patel, 2003; De Vita *et al*, 2008), although infection with high-risk (HR) HPV, whose role in carcinogenesis of the uterine cervix has been extensively studied (De Vita *et al*, 2008), is another emerging risk factor. The emergence of HR HPV infection underlies the marked increase in oropharyngeal SCC incidence (Hammarstedt *et al*, 2006; Chaturvedi *et al*, 2011), especially in the young (Sturgis and Cinciripini, 2007). Nevertheless, HNSCCs are an extremely heterogeneous group of tumours both from a molecular (Huang *et al*, 2002; Bosch *et al*, 2004) and a clinical point of view (Shah and Patel, 2003). The main clinical heterogeneity factor is the site of origin, which correlates with specific risk factors, symptoms, stage at diagnosis, tendency to local and distant metastasis, chemo- and radiosensitivity and ultimately prognosis (Shah and Patel, 2003; De Vita *et al*, 2008). In this context, the role of HR HPV in oropharyngeal carcinogenesis is established, as HPV infection predicts chemo-radiosensitivity and better prognosis (Gillison *et al*, 2000; Gillison, 2009; Ang *et al*, 2010); on the other hand, evidence of its significance in other head and neck sites is definitely lacking (Gillison *et al*, 2000).

The increasing epidemiologic role of HPV and its value as a prognostic marker have raised great interest given their potential clinical implications in head and neck oncology, stimulating several studies in the past 10 years. Most of the acquired data on HPV-driven carcinogenesis comes from the uterine cervix model, although its validity in head and neck remains to be confirmed. For example, studies on the cervix model assigned the most significant role in the induction of malignant transformation to the *E6* and *E7* genes and their respective proteins (Munger *et al*, 1989; McDougall, 1994), showing that E7 interacts with and degrades RB, which then releases the transcription factor E2F from RB inhibition and upregulates p16^{INK4A}, providing the molecular basis for the typical p16 overexpression in cervical SCC (Dyson *et al*, 1989; Kiyono *et al*, 1998). Hence, detection of p16 overexpression by immunohistochemistry (IHC) has been proposed as a surrogate for HPV detection in the head and neck.

This study aims primarily to assess the prevalence of HPV infection in SCCs at different sites in the head and neck, and to determine the reliability of p16 immunohistochemistry as a diagnostic tool for HPV infection in head and neck oncology, in comparison with mRNA detection, which can be considered the gold standard.

MATERIALS AND METHODS

Patient characteristics. From March 2009 to October 2010, 109 patients affected by primary, previously untreated, HNSCC, arising from hypopharynx, oropharynx, oral cavity and larynx, were enroled in this study. Patients were evaluated by the multidisciplinary head and neck tumour board of the Policlinico Agostino Gemelli, Università Cattolica del Sacro Cuore (Rome, Italy) (Table 1).

Following squamous histology confirmation, completion of the diagnosis and staging according to the TNM classification (Edge *et al*, 2010) with a strict and careful clinicoradiological assessment of the site and subsite of origin (Table 1), and treatment planning, patients signed an informed consent for their participation in this study, which had been previously approved by the ethical committee.

HPV detection. Fresh tumour samples were obtained by biopsy and immediately stored as RNA at −80 °C until tested. Each sample was used for DNA and RNA detection. Nucleic acids'

Table 1. Descriptive statistics of the main variables concerning patients and tumour parameters		
Characteristic	**109 patients**	
Age at diagnosis		
Median	63	
Range	32–84	
Follow-up period (months)		
Median	13	
Range	3–32	
Pack-years of cigarette smoking		
Median	29	
Range	0–88	
Sex, no. (%)		
Male	88 (80.73%)	
Female	21 (19.27%)	
Site of origin, no. (%)	**Subsite, no. (%)**	
Hypopharynx	9 (8.26%)	Post-cricoid region 3 (2.75%)
		Pyriform sinus 6 (5.5%)
Larynx	46 (42.2%)	Glottic 18 (16.51%)
		Supraglottic 15 (13.76%)
		Transglottic 12 (11.01%)
		Subglottic 1 (0.92%)
Oral cavity	33 (30.28%)	Cheek 3 (2.75%)
		Floor of mouth 8 (7.34%)
		Hard palate 4 (3.67%)
		Mobile tongue 15 (13.76%)
		Retromolar trigone 3 (2.75%)
Oropharynx	21 (19.27%)	Base of tongue 6 (5.5%)
		Posterior wall 1 (0.92%)
		Soft palate 1 (0.92%)
		Tonsil 13 (11.93%)
Primary treatment, no. (%)		
Surgical	61 (57.55%)	
Radiochemotherapy	45 (42.45%)	
AJCC stage, no. (%)		
I	8 (7.34%)	
II	21 (19.27%)	
III	20 (18.35%)	
IV	IVa 53 (48.62%), IVb 5 (4.59%), IVc 2 (1.83%)	
cT classification, no.(%)		
T1	12 (11.01%)	
T2	32 (29.36%)	
T3	22 (20.18%)	
T4a	43 (39.45%)	
cN classification, no.(%)		
N0	61 (55.96%)	
N1	10 (9.17%)	
N2a	1 (0.92%)	
N2b	18 (16.51%)	
N2c	14 (12.84%)	
N3	5 (4.59%)	

Table 1. (Continued)

Characteristic	109 patients
Grading, no.(%)	
G1	3 (2.75%)
G2	63 (57.8%)
G3	43 (39.45%)
HPV DNA, no.(%)	
Negative	95 (87.16%)
High-risk HPV	12 (11.01%)
High and low risk	1 (0.92%)
Low risk	1 (0.92%)
High-risk HPV RNA, no.(%)	
Negative	95 (87.16%)
HPV16	12 (11.01%)
HPV18	1 (0.92%)
HPV45	1 (0.92%)
P16 immunostaining	
Positive	21 (24.77%)
Negative	88 (75.23%)

Abbreviations: AJCC = American Joint Committee on Cancer; cN = clinical N; cT = clinical T; HPV = human papillomavirus.

extraction from tissues was performed with the NucliSens easyMAG platform (BioMérieux, Rome, Italy), according to the manufacturer's instructions. Human papillomavirus DNA detection was performed with the Digene Hybrid Capture 2 (HC2) assay (Qiagen Inc., Valencia, CA, USA), which allows the detection of 18 HPV genotypes, and can differentiate between HR HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59 and 68) and low-risk (LR) HPV (types 6, 11, 42, 43 and 44), but does not allow the identification of specific genotypes.

As for RNA, samples were analysed for HPV E6 and E7 mRNA by NucliSENS EasyQ HPV v.1 test (BioMérieux, Rome, Italy), based on the NASBA technique. It specifically detects the E6/E7 mRNA of five HR HPV types (HPV16/18/31/33/45); the other HR and LR genotypes are not detectable with this assay. Briefly, three premixes were made by the reconstitution of reagent spheres in the reagent diluent, followed by the addition of primer/molecular beacon mixes (either U1A/HPV16, HPV18/31 or HPV33/45). A measure of $10\,\mu l$ of premix were distributed to each well, followed by $5\,\mu l$ sample, and incubated for 4 min at $65\,^{\circ}\text{C}$ and 2 min at $41\,^{\circ}\text{C}$. The reaction was started by the addition of enzymes and measured in real time using the EasyQ analyser at $41\,^{\circ}\text{C}$. Data analysis was performed using the EasyQ Director software.

P16 immunohistochemistry. Formalin-fixed, paraffin-embedded (FFPE) tumour specimens were evaluated for p16 expression with a monoclonal antibody (clone E6H4; CINtec p16INK4a Histology Kit; Mtm-Laboratories, Heidelberg, Germany).Tissue sections were cut at 2 to $4\,\mu m$ and deparaffinised. Cervical cancer sections known to be HPV positive were used as a positive control and omission of primary antibody was used as a negative control. After antigen unmasking for 20 ± 1 min at 95 to $99\,^{\circ}\text{C}$ in Tris buffer, pH 9.0, slides were allowed to cool down in the solution for 20 ± 1 min to room temperature. Endogenous peroxidase blocking was performed in 3% hydrogen peroxide for 5 ± 1 min. Slides were then stained with the primary antibody (p16ink4a ready to use for 40 ± 1 min at room temperature) and then with the visualisation reagent provided with the kit for 30 ± 1 min at room temperature.

After development with substrate–chromogen solution (DAB; Mtm-Laboratories, Heidelberg, Germany) for 10 ± 1 min, p16-stained slides were finally counterstained with haematoxylin (Dako) (Negri *et al*, 2008). Positive p16 expression was defined as a strong and diffuse nuclear and cytoplasmic staining in 70% or more of the tumour cells. Immunohistochemical evaluation was carried out by three histopathologists in independent readings (VGV, GFZ and GR). Each reader was 'blinded' to the results obtained for HPV infection and to the results obtained by the other histopathologists. Cases that varied among readers were re-evaluated to determine a consensus.

Statistical analysis. Statistical analysis was performed using the JMP software, release 7.0.1, from the SAS Institute (Cary, NC, USA).

Correlation between p16 IHC and HPV status itself was evaluated by χ^2.

The agreement among detection methods was quantified by calculating κ, which is a measure of agreement and takes on the value zero if there is no more agreement between test and outcome than can be expected on the basis of chance. It is considered that κ values <0.4 represent poor agreement, values between 0.4 and 0.75 fair to good agreement and values >0.75 excellent agreement.

RESULTS

Patient and tumour characteristics are shown in Table 1. None of the patients who accepted to participate in this study was lost at follow-up.

The most frequent site of origin of the SCC was the larynx (42%), followed by oral cavity (30%) and oropharynx (19%), whereas the most frequent subsite was the glottis. A marked prevalence of advanced stages was observed (stages III and IV together account for $>73\%$ of the cases in our series). In particular, $>40\%$ of our patients had clinically positive lymph nodes at diagnosis. These results are in agreement with the epidemiology of HNSCC in Italy (Bray *et al*, 2002; Ferlay *et al*, 2010).

DNA of LR HPV genotypes was found only in 2 out of 109 cases (1.8%), whereas DNA of HR genotypes was detected in 13 cases (12%), with one case of HR and LR co-infection. Human papillomavirus mRNA detection demonstrated that 12 out of 14 HR HPV cases were due to HPV 16. Interestingly, the two remaining HR HPV referred to tumours localising outside the oropharynx (in the oral cavity and glottic larynx, respectively; Table 2), and were identified as HPV18 and 45. In any case, HPV16 was by far the most frequent genotype detected and the only one detected in the tonsil, base of the tongue and supraglottic larynx, which were in turn the most frequently positive subsites. In fact, most HR-positive cases were in the oropharynx (10 out of 14), in which the overall prevalence of HR HPV infection was 47.6%; conversely, HR HPV infection rate did not reach 5% in the present series (4/88) when all other head and neck sites were pooled together. Positivity to p16 immunostaining was markedly more frequent in the whole series (25%) and, in contrast to HPV infection, it was commonly recorded outside the oropharynx (19/88: 21.6%).

Many authors (Braakhuis *et al*, 2004; Shi *et al*, 2009; Schlecht *et al*, 2011) consider mRNA detection performed on fresh samples to be the most reliable diagnostic test for HR HPV infection in the cervix and also in the head and neck; for this reason, we decided to define mRNA detection as the standard when assessing the value of the other detection methods by the estimation of κ. The overall agreement between the HPV genomic DNA detection results and the mRNA detection was close to 100% ($\kappa = 0.96$), being absolute in all sites except the glottic larynx, where the Digene test missed an unexpected positivity for the mRNA of HPV45, associated with

Table 2. Results of the different detection methods for HPV infection in our series[a]

Site of origin, no.	HPV status (high-risk HPV RNA), no. (%)	High-risk HPV DNA, no. (κ)	P16 over-expression (κ)	Subsite of origin, no.	HPV RNA positivity (genotype), %	HPV DNA positivity (high vs low risk), %	P16 IHC positivity, %
Hypopharynx, 9	0	0	1	Post-cricoid region, 3	0	0	0
				Pyriform sinus, 6	0	0	1, 17%
Larynx, 46	3 (6.5%)	2 (0.79)	8 (0.1)	Glottic, 18	1 (HPV45), 5%	0	4, 22%
				Supraglottic, 15	2 (HPV16), 13.3%	2 (high risk), 13.3%	4, 27%
				Transglottic, 12	0	0	0
				Subglottic, 1	0	0	0
Oral cavity, 33	1 (3%)	1 (1)	10 ($-$0.058)	Cheek, 3	0	0	0
				Floor of mouth, 8	1 (HPV18), 12.5%	1(low + high risk), 12.5%	0
				Hard palate, 4	0	0	2, 50%
				Mobile tongue, 15	0	1 (low risk), 6%	8, 53%
				Retromolar trigone, 3	0	0	0
Oropharynx, 21	10 (47.6%)	10 (1)	8 (0.61)	Base of tongue, 6	3 (HPV16), 50%	3 (high risk), 50%	2, 33%
				Posterior wall, 1	0	0	0
				Soft palate, 1	0	0	0
				Tonsil, 13	7 (HPV16), 54%	7 (high risk), 54%	6, 46%

Abbreviations: HPV = human papillomavirus.
[a]In the left part of the table the agreement (κ) of Digene (HPV DNA) and p16 IHC with the reference method (RNA), by site of origin is shown. In the right part, the results of the different detection methods are shown in detail for each subsite, evidencing that HPV16 is by far the most frequent genotype (12 out of 14 high-risk cases), and the only one in the oropharynx, and that most positive cases (10 out of 14) arose from the tonsil and from the base of the tongue. The only high-risk case missed by Digene was in the glottic larynx and was positive for HPV45 RNA.

HNSCC in only a few reports (Lindel et al, 2001). On the other hand, even though the correlation between p16 IHC and HPV status itself was clear ($P = 0.0051$ at χ^2), when p16 was evaluated as a diagnostic test, the agreement rate was poor ($\kappa = 0.27$) with a 0.23 error rate. The oropharynx, where the higher rate of HR HPV infection was observed (47.6%), was also the only site in which the agreement rate of p16 IHC ($\kappa = 0.61$) was fair (Table 2).

DISCUSSION

The increasing epidemiologic role of HPV and its value as prognostic marker in head and neck oncology has stimulated a growing number of studies in the past 10 years; nevertheless, some critical issues, such as the real prevalence of HR HPV infection in sites outside the oropharynx and the methods of choice to diagnose the infection itself in HNSCC, have not yet been definitely defined.

The results of this study confirm the relevance of HPV infection as a risk factor for oropharyngeal SCC (Gillison et al, 2000; Gillison, 2009). However, the prevalence of HPV infection in SCC of other head and neck sites was found definitely lower than what was reported in recent studies (Sethi et al, 2011; Heath et al, 2012). Hence, the role of HPV as a risk factor for non-oropharyngeal sites remains to be fully evaluated, appearing certainly less relevant. The subsite with the highest prevalence (13.3%) outside the oropharynx was the supraglottic larynx, whose marginal region is contiguous with the oropharynx. This may explain the HR HPV infection rate reported in some studies on laryngeal SCCs (Almadori et al, 2001, 2002). Notably, our total (13%) and oropharyngeal (47.6%) positivity rates are very close to those from a recent study evaluating the overall genetic pattern of HNSCCs (Stransky et al, 2011) in a different population from a different geographic area (North America).

Human papillomavirus 16 mRNA was detected in about 86% of all HPV-positive samples, and in 100% of the positive samples from the oropharynx and the supraglottic larynx (Table 2). This finding confirms also in the Italian population that only HPV16 can be classified at present as cancer causing in oropharyngeal

tissues (Bouvard et al, 2009). Notably, the other HPV genotypes were detected in non-oropharyngeal subsites: in the floor of the mouth (HPV18) and the glottic larynx (HPV45). However, an effective and conclusive evaluation of the putative association of specific HPV genotypes with different sites in head and neck requires a wider study with more homogeneous series according to the site of origin of the SCCs.

Concern has been expressed that the hypersensitivity of viral genomic DNA detection methods (Braakhuis et al, 2004; Kreimer et al, 2005) would increase the risk of identifying contaminant HPV, leading to a high number of false positives. In this study, viral genomic DNA detection shows 100% specificity (no false-positive cases) when compared with the mRNA detection test. In addition, only the HPV45-positive case (sensitivity: 93%) was not detected by the genomic DNA technique used, which may therefore be considered a perfect surrogate of HPV16 mRNA detection in HNSCC samples. Such reliability of genomic DNA HPV-detecting techniques in HNSCCs seems confirmed also by the recent influential paper of Stransky and co-workers (2011), performing an accurate mutational profiling.

Some authors (Smeets et al, 2007; Marur et al, 2010; El-Naggar and Westra, 2011; Harris et al, 2011) expressed concerns on the use of p16 IHC as a surrogate marker for the HPV infection in the head and neck. However, several groups, probably assuming that data acquired from the uterine cervix could be easily translated in other models, have considered for a long time p16 expression as equivalent to HPV infection in all head and neck sites, even those outside the oropharynx (Lassen et al, 2011), where the prevalence of proved HR HPV infection was markedly lower (Gillison et al, 2000; Stransky et al, 2011). These assumptions may have detracted from a thorough evaluation of diagnostic tools for HPV in head and neck oncology and may have led to an overestimation of the HPV infection relevance in head and neck carcinogenesis (Kaminagakura et al, 2011; Lassen et al, 2011). A recent study (Schlecht et al, 2011) compared p16 IHC with in situ hybridisation and concluded that the former is a better surrogate for HPV mRNA detection, despite the reported sensitivity of 69% in the oropharynx and only 52% in the head and neck as a whole. The overall low specificity of p16 IHC for the diagnosis of HR HPV

infection in the present series is striking and is certainly very different to what was observed in the cervix, in which virtually all the SCCs are associated with HR HPV and p16 overexpression. In the head and neck, where causative factors, clinical behaviours and molecular profiles, are definitely more heterogeneous (Braakhuis *et al*, 2004; Agrawal *et al*, 2011; Stransky *et al*, 2011), p16 overexpression may derive from different mechanisms than HR HPV infection (El-Naggar and Westra, 2011), especially in mobile tongue SCC (53% of positivity without any demonstration of HR HPV infection in the present series). The present results undermine the usefulness of p16 as a diagnostic surrogate for HPV infection in sites outside the oropharynx. In the oropharynx, p16 overexpression is associated with HPV infection, and agreement can be considered fair ($\kappa = 0.61$) but not excellent. A recent work demonstrated a low diagnostic value for p16 immunohistochemistry in oropharyngeal SCCs arising outside tonsils and base of tongue (Marklund *et al*, 2012). In our opinion, even in the oropharynx, there is no reason to prefer a surrogate, such as p16 IHC, to more reliable and direct diagnostic methods based on HPV nucleic acid detection, which are objective, as they do not require the reading of a trained histopathologist, and are becoming less expensive and widely available in the microbiology departments all over the world. Another option for the diagnosis of HPV infection in FFPE samples could be the algorithm proposed by Smeets *et al* (2007)), involving both p16 immunohistochemistry and GP5 + / 6 + PCR.

One of the main issues of head and neck oncology is that, despite the multiplicity of clinical prognosticators, the only consistent clinical predictors for disease control and disease-specific survival of SCCs are T and, to a greater extent, N (Spector *et al*, 2001; Franchin *et al*, 2003; Licitra *et al*, 2003; Shah and Patel, 2003), and the stratification of patients remains often inadequate. This is probably due, in spite of the histological homogeneity (most head and neck malignancies are SCC), to the extreme biological heterogeneity, and contributes to a lack of consistency in treatment planning. The molecular characterisation is an attempt to define homogeneous groups of patients for prognosis as well as for responsiveness to treatments (Almadori *et al*, 2005). The growing body of evidence about the prognostic role of HR HPV infection in oropharyngeal oncology (Gillison *et al*, 2000; Kumar *et al*, 2008; Worden *et al*, 2008; Gillison, 2009; Ang *et al*, 2010), which is associated with a better prognosis and an increased radio-sensitivity, suggests that it may be a useful molecular marker to be integrated in the clinical management of oropharyngeal SCCs. Nevertheless, the inconsistency of detection methods for the most studied molecular markers in head and neck oncology, and for HPV infection in particular, is probably one of the main obstacles to their employment in the clinic (Almadori *et al*, 2008; Bussu *et al*, 2012). For this reason, a consensus on the methods to be used for HR HPV detection in HNSCC is mandatory (Braakhuis *et al*, 2009), and this study provides useful indications. In fact, mRNA detection, considered the gold standard for the diagnosis of HPV infection (Braakhuis *et al*, 2004; Shi *et al*, 2009; Schlecht *et al*, 2011), could be performed when fresh tumour samples are available; on the other hand, genomic DNA-based methods, alone or combined with other assays in a diagnostic algorithm (Smeets *et al*, 2007), if the high specificity of new genomic DNA detecting techniques will be confirmed and validated, could be used on common FFPE samples.

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