Human papillomavirus predicts the outcome following concomitant chemoradiotherapy in patients with head and neck squamous cell carcinomas

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Abstract. We investigated the prevalence of human papillomavirus (HPV) in a clinical series of 72 patients with head and neck squamous cell carcinoma (HNSCC) using a retrospective and prospective study design. The majority of patients were smokers and/or drinkers and were treated with concomitant chemoradiotherapy (CCR). Furthermore, we assessed the impact of HPV positivity on the response to CCR. Paraffin-embedded samples from HNSCC patients (n=72) were evaluated for the presence of HPV DNA using both GP5$^+$/GP6$^+$ consensus PCR and type-specific E6/E7 PCR to detect HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67 and 68. The type-specific E6/E7 PCR demonstrated that 20 out of 69 HNSCC patients (29%) presented with high-risk (HR) HPV types and that 5 of the 69 HNSCC patients (7%) presented with low-risk (LR) HPV types. Using the GP5$^+$/GP6$^+$ PCR, we observed that the rate of response was statistically lower in the HPV$^+$ group (P=0.02). Concerning patient outcomes in terms of recurrence and survival, we observed that the prognosis was poorer for HPV$^+$ patients. We showed for the first time that patients with HPV$^+$ HNSCC present with a worse prognosis after CCR. This observation highlights the need for prospective studies with large numbers of patients and a detailed history of tobacco and alcohol consumption before validating HPV as a marker of prognosis following CCR.

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Introduction

Head and neck squamous cell carcinomas (HNSCCs) remain a significant cause of morbidity worldwide, with as many as 466,831 and 168,368 cases diagnosed in 2008 among men and women, respectively (1-3). HNSCC patients with early clinical stage disease (stages I and II) have similar survival rates, with a 5-year survival rate between 70 and 90%, independent of the sublocation or the treatment (surgery vs. radiotherapy) (4). In contrast, HNSCC patients with advanced clinical stage disease (stages III and IV) display different survival rates depending on the histological type of the tumor and its sublocation (4,5). In this group, the combination of chemotherapy and radiotherapy allows for a better local-regional control rate of up to 65% (6,7). However, the obvious benefit of chemotherapy is associated with higher (grade III and IV) toxicity and mortality (8). It is, therefore, crucial to predict which patients will not benefit from concomitant chemoradiotherapy (CCR).

During the last 30 years, we have observed a clear increase in the incidence of carcinomas arising from the oral cavity and the oropharynx in the United States and in Europe, whereas the incidence of laryngeal carcinoma has been stable or has decreased slightly (9). This observation led us to propose that human papillomavirus (HPV) infection is a new risk factor for HNSCC in younger, non-smoking and non-drinking patients. In this subpopulation of HNSCC patients, HPV$^+$ tumors occur more frequently in the oropharynx than in other sites and appear to have a more favorable prognosis than HPV$^-$ carcinomas (10,11). The better prognosis of HPV$^+$ tumors was also reported for advanced oropharyngeal carcinomas treated by CCR (12-20). However, HPV-associated tumors have a different pathogenesis with less chromosomal aberrations than tumors caused by alcohol and tobacco abuse. In Belgium,

the situation is more complex since our HNSCC patients present with a higher incidence of HPV positivity associated with alcohol and tobacco abuse. In this context, we recently described that oral cavity HPV$^+$ carcinomas are associated with a worse prognosis that of HPV$^-$ carcinomas (21). The aim of the present study was to assess the impact of HPV positivity on the response to CCR in a series of 72 HNSCC patients.

Materials and methods

Histopathological and clinical data. Formalin-fixed, paraffin-embedded HNSCC specimens were obtained from 72 patients (57 males, 15 females) who underwent concomitant chemo-radiotherapy at the Saint-Pieter Hospital (Brussels) and Epicura (Baudour). The clinical data collected from this series of 72 HNSCC patients are described in Table I. This prospective and retrospective study was approved by the Institutional Review Board (AK/09-09-47/3805AD).

DNA extraction. The formalin-fixed, paraffin-embedded tissue samples (n=72) were sectioned (10x5 μm), de-paraffinized and digested with proteinase K by overnight incubation at 56˚C. DNA was purified using the QIAamp DNA Mini Kit (Qiagen, Benelux, Belgium) according to the manufacturer's recommended protocol.

Detection of HPV by polymerase chain reaction (PCR) amplification. HPV detection was performed using PCR with GP5$^+$/GP6$^+$ primers (synthesized by Eurogentec, Liege, Belgium). The GP5$^+$/GP6$^+$ primers amplify a consensus region located within the L1 region of the HPV genome. The PCR amplification of the HPV-L1 DNA was performed in a 25-μl reaction mixture containing 2 μl of extracted DNA, 2.5 μl 1X PCR buffer, 0.025 U Taq DNA polymerase (Roche, Mannheim, Germany), 200 μM DNTPs and 0.5 pmol of each primer. The cycling conditions for the PCR were as follows: denaturation was performed at 94˚C for 1 min, annealing was performed at 55˚C for 1 min and 30 sec, and extension was performed at 72˚C for 2 min, for a total of 45 amplification cycles. The first cycle was preceded by a 7-min denaturation step at 94˚C, and the last cycle was followed by an additional 10-min extension step at 72˚C. Aliquots (10 μl) of each PCR product were electrophoresed through a 1.8% agarose gel and stained with ethidium bromide to visualize the amplified HPV-L1 DNA fragments.

Real-time quantitative PCR amplification of the HPV type-specific DNA. All DNA extracts were tested for the presence of 18 different HPV genotypes using TaqMan-based real-time quantitative PCR that targeted type-specific sequences of the following viral genes: 6 E6, 11 E6, 16 E7, 18 E7, 31 E6, 33 E6, 35 E6, 39 E7, 45 E7, 51 E6, 52 E7, 53 E6, 56 E7, 58 E6, 59 E7, 66 E6, 67 L1 and 68 E7 (22). For the various real-time quantitative PCR assays, the analytical sensitivity ranged from 1 to 100 copies and was calculated using standard curves generated with plasmids containing the entire genome of the different HPV types (23). Real-time quantitative PCR for the detection of β-globin was performed in each PCR assay to verify the quality of DNA in the samples and to measure the amount of input DNA (23,24). The following HPV types tested were considered high-risk (HR): 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59 and 66.

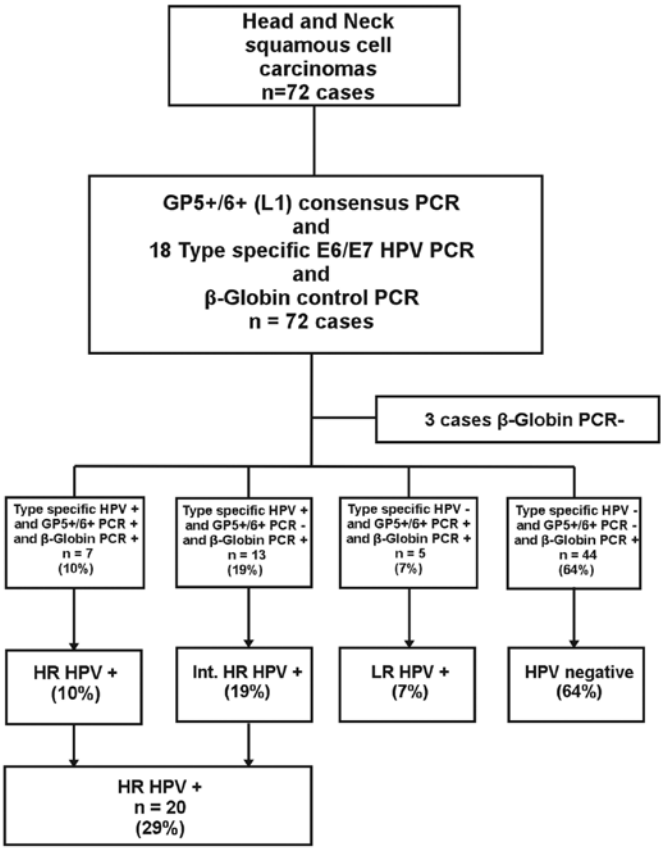

Figure 1. HPV PCR results from 72 HNSCC cases. β-globin could not be amplified in three samples; therefore, a total of 69 cases were analyzed using type-specific real-time PCR and GP5$^+$/GP6$^+$ consensus PCR. Among these patients, 29% tested positive for infection with one or several types of HR HPV, 7% tested positive for LR HPV and 64% were HPV-negative. HR, high risk; LR, low risk.

Results

HPV status in our clinical series of HNSCC patients treated by CCR. Of the 72 cases, three were excluded from further analysis since β-globin could not be amplified (Fig. 1). Ultimately, 69 β-globin PCR-positive specimens were typed by quantitative real-time PCR using primers for 18 different HPV types. We identified 20 (29%) patients whose tumors tested positive for the following HR HPV types: 16 (15 cases), 59 (2 cases), 53 (2 cases), 58 (1 case), 66 (1 case) and 67 (1 case). One patient was infected with multiple types of HR HPV (HPV 59 E7 and HPV 33, 52, 58, 67 L1). Among the 20 patients with HR HPV$^+$ tumors, 7 tumors were both GP5$^+$/GP6$^+$ positive (L1 detection) and type-specific HPV positive (HR HPV$^+$ group). However, 13 tumors were GP5$^+$/GP6$^+$ negative and type-specific HPV positive, corresponding to an integrated HPV$^+$ group (int. HR HPV$^+$). In the HR HPV-negative group (n=49), 5 patients tested positive for HPV using the GP5$^+$/GP6$^+$ consensus primers and were considered to be infected with low-risk (LR) HPV types. Forty-four tumors (64%) were negative for both GP5$^+$/GP6$^+$ and type-specific HPV upon PCR analysis (Fig. 1).

Table I. Clinical data of the HNSCC patients.

Variable	Patients (n=72)
Gender	
Male	57
Female	15
Age (years)	
Mean	57.9
Range	42-83
Localization	
Oral cavity	19
Oropharynx	29
Hypopharynx	12
Larynx	12
Grade (differentiation)	
Well	29
Moderate	15
Poor	13
In situ	2
Not recorded	13
TNM stage	
T1N2	4
T1N3	1
T2N0	1
T2N1	3
T2N2	5
T3N0	8
T3N1	9
T3N2	10
T4N0	5
T4N1	6
T4N2	19
T4N3	1
TNM stage I-IV	
I	0
II	1
III	20
IV	51
Risk factors	
Tobacco	
Smoker	54
Non-smoker	12
Former smoker	6
Alcohol	
Drinker	55
Non-drinker	7
Former drinker	10
Treatment	
Cisplatin 100 mg/m^2 (Day 1-21-42)	
Two doses	12
Three doses	23
Cisplatin 40 mg/m^2 (weekly)	7
Carboplatin (weekly)	7
Cisplatin 100 mg/m^2 (1 cycle) and carboplatin 40 mg/m^2 (weekly)	2
Erbitux	18
Erbitux (2 cycles) and cisplatin 40 mg/m^2 (weekly)	1
Carboplatin + 5FU	1
Cisplatin 100 mg/m^2 (2 cycles) and erbitux (1 cycle)	1
Radiotherapy (n=70)	
70 Gy	66
>70 Gy	1
<70 Gy	3
Responders	
Yes	38
No	34
Lymph node dissection	
Yes	10
Positive node	3
Negative node	7
No	62
Recurrence	
Local	10
Nodal	5
Distant metastases	4
Local + distant metastases	1
Nodal + distant metastases	1
Follow-up[a]	
Range (months)	1-106
Mean (months)	30

[a]Forty-six deaths, including 35 caused by the HNSCC and 11 deaths that were unrelated to HNSCC.

Correlation between HPV detection and clinical data. The HPV$^+$ group was composed of more men (n=20, 77%) than women (n=6, 23%). The age ranged from 43 to 78 years, and most patients had stage IV disease (4 had stage III and 22 had stage IV). There was a clear predominance of smokers (n=19, 73%) or former smokers (n=6, 23%) and drinkers (n=23, 88%) or former drinkers (n=1, 4%) compared to patients who did not consume tobacco (n=1, 4%) or alcohol (n=2, 8%) (Table I). However, no statistical correlation was found between the HPV status and the following clinical data: gender, smoking status, alcohol status, sublocation, differentiation, T and N stage.

Correlation between HPV detection and of response rate to CCR. When analyzing the impact of HPV positivity on the rate of response and non-response to CCR, we tested the potential correlation using the two tests (GP5$^+$/GP6$^+$ PCR and qPCR) separately and also in combination. We investigated whether one of these two tests or their combination could predict the response to CCR. Interestingly, using the PCR GP5$^+$/GP6$^+$ as a tool for HPV detection, the rate of response to CCR

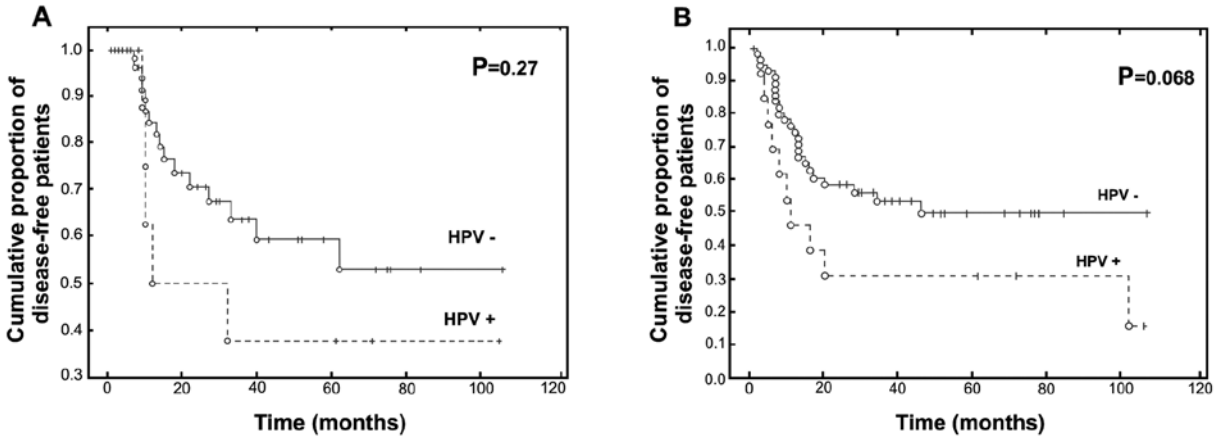

Figure 2. These graphs show, respectively, the recurrence rate (A) and the survival rate (B) of patients with HPV$^+$ and HPV$^-$ carcinomas. Both analyses reveal a less favorable prognosis for the HPV$^+$ subgroup, with a recurrence rate of 38 vs. 27% for HPV$^-$ patients (P=0.27) (A) and a survival rate at 5 years of 23 vs. 57% for the HPV$^-$ population (P=0.068) (B). The percentages shown in the two curves do not represent the total number of patients since the follow-up of several patients was shorter than the first event of recurrence or death. Standard survival time analyses were performed using Kaplan-Meier curves, the Gehan-Wilcoxon text and the log-rank test.

Table II. Studies that found a positive correlation between HPV and response to chemoradiotherapy in HNSCCs.

Authors (ref.)	No. of patients	HPV prevalence (%)	Anatomical site	Smokers (n)	Drinkers (n)	Detection methods
Kumar *et al* (12)	42	64	Oropharynx	34	Not listed	qPCR
Chung *et al* (17)	46	50	Oropharynx	Not listed	Not listed	PCR *in situ* hybridization
Nichols *et al* (18)	44	61	Oropharynx	Not listed	Not listed	*In situ* hybridization
Fallai *et al* (20)	22	23	Oropharynx	Not listed	Not listed	qPCR
de Jong *et al* (19)	75	49	Pharynx Oral cavity	Not listed	Not listed	Genetic signature
Rischin *et al* (13)	172	65	Oropharynx	111	Not listed	PCR *in situ* hybridization
Hong *et al* (15)	35	24	Head and neck squamous cell carcinomas	Not listed	Not listed	qPCR
Lill *et al* (14)	29	38	Head and neck squamous cell carcinomas	Not listed	Not listed	PCR *in situ* hybridization

was statistically lower, with 23% of responders in the HPV$^+$ group against 59% in the HPV$^-$ group (P=0.02, Fisher's exact test). There was no statistical correlation between the type of chemotherapy administered and the number of responders according to HPV status determined through consensus PCR or qPCR. Using the qPCR for HPV detection, no statistical correlation was observed, and the rate of response was lower in the HPV$^+$ group (50%) than in the HPV$^-$ group (57%).

Correlation between HPV infection and prognosis in HNSCC patients. Based on the GP5$^+$/GP6$^+$ PCR detection, we observed that the HPV$^+$ group exhibited a worse prognosis in terms of survival. In fact, the recurrence rate was higher in patients with HPV$^+$ carcinomas, at 38%, while it reached only 27% among patients with HPV$^-$ carcinomas (log-rank test, P=0.27) (Fig. 2A). However, the treatment did not influence the recurrence rate; there was no significant difference between patients who received platin or erbitux. Therefore, the disease-free survival rate at 5 years was 57% for patients with HPV$^-$ carcinoma vs. 23% for patients with HPV$^+$ carcinoma (Gehan-Wilcoxon test, P=0.068) (Fig. 2B).

The percentages shown in the two curves do not represent the total number of patients. The follow-up of several patients was shorter than the first event of recurrence or death.

Discussion

In the early 2000s, with the advent of CCR, we observed a clear increase in the 5-year disease-free survival of HNSCC stage IV patients, from 45 to 66% (25). However, CCR was also associated with significant morbidity and mortality (notably, a higher incidence of dysphonia and dysphagia) (26). Therefore, clinicians are

searching for new reliable prognostic markers of CCR response and are considering the growing interest in HPV infection in the biology of HNSCC. We decided to investigate whether a correlation exists between HPV positivity and the response to CCR in a series of 72 HNSCC patients with a history of tobacco and alcohol use (in more than 90% of our population).

In our study, the prevalence of HPV positivity reached 36%, with 29% of samples containing HR HPV DNA and 7% of samples containing LR HPV DNA. Moreover, we observed that the rate of response was statistically lower in the HPV$^+$ group. Several studies have reported that HPV DNA detection was closely correlated to a more favorable prognosis in HNSCC patients treated with CCR (Table II) (12-20).

We recently revealed, in a large clinical series of 162 oral cavity carcinoma patients, that HPV$^+$ tumors were significantly associated with a poorer prognosis (27). The association between HPV positivity and poor prognosis was also previously reported in two Swedish studies in which oral HPV infection was associated with a dramatically increased risk of oral squamous cell carcinoma (OSCC) development (28,29). Clayman *et al* also showed that HPV$^+$ carcinomas were significantly correlated with a decreased survival rate (30). In fact, our results could be explained by the fact that our series was mainly composed of smokers and/or drinkers (Table I), which is contrary to the majority of studies describing a favorable prognosis for patients with HPV$^+$ tumors (Table II). It should also be emphasized that HPV$^+$ tumors related to tobacco and alcohol consumption constitute a distinct biological and clinical entity from HPV$^+$ tumors without classical risk factors, which are associated with a better outcome. In this regard, trends in smoking behavior in Europe present some significant differences (31). A greater decline in smoking habits was observed among Norwegian, Finnish and Dutch populations, highlighting that individuals in Northern European countries are less exposed to classical risk factors than those residing in Southern European countries (31). Therefore, all studies investigating the HPV status in HNSCC need to be interpreted with caution since many are small clinical series without information on the alcohol consumption and smoking status of their patients. Moreover, our clinical series was composed of patients with an extremely long-term follow-up (ranging from 0 to 106 months), which is a crucial point for assessing the prognostic implications of HPV infection.

A persistent HPV infection that can lead to the development of epithelial cancer requires immune tolerance. Thus, HPV has also developed several mechanisms to avoid detection by the host immune defense system, such as downregulation of INF-α and toll-like receptor 9, production of TGF-β and maintenance of low viremia (viral protein synthesis is confined to keratinocytes without an increase in cell death) (32-34). In the absence of cell lysis, there is little or no release of the pro-inflammatory cytokines that are crucial for the activation and migration of dendritic cells (32,35). There are limited data describing the interaction between the host immune system during HPV infection in the context of HNSCC, which means that the role of innate and adaptive immunity in this context is largely unknown. As mentioned previously, in several studies, HPV$^+$ HNSCC was associated with an unfavorable outcome. From these results, some authors supported the hypothesis that immunosuppression favors HPV infection and that a failing immune response may be negative in terms of prognosis for HPV$^+$ HNSCC. In fact, Tung *et al* reported the presence of HPV-16 or HPV-18 and the Epstein Barr virus in 80% of nasopharyngeal carcinoma samples (36). Another study showed that herpes simplex virus-2 infection was associated with an increased risk of HPV infection (37). In 2004, Kreimer *et al* demonstrated that tonsillar HPV infection was strongly associated with HIV co-infection and immunosuppression (38). More recently, we studied different markers of the immune system in a large series of 110 HNSCC cases (36 HPV$^+$ cases vs. 74 HPV$^-$ cases) to study the involvement of HPV infection in the alterations of the immune system in a population of smokers and drinkers. We observed a significant decrease in the number of natural killer cells and dendritic cells in HPV$^+$ samples compared to HPV$^-$ samples (unpublished data).

In conclusion, we showed for the first time, in a clinical series of 72 HNSCC patients, that the rate of response to CCR was statistically lower in the HPV$^+$ group. Notably, the association between HPV positivity and an unfavorable prognosis was discovered in a population of smokers and drinkers with HNSCC. Our study also highlights the need for prospective, controlled studies with larger numbers of patients, a detailed history of tobacco and alcohol use among patients, and homogeneous treatments and anatomical sites in order to confirm the impact of HPV infection in HNSCCs treated with CCR.

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References

1. World Health Organization: International Agency for Research on Cancer. Globocan, 2008. Available from: http://globocan.iarc.fr/.
2. Grandis JR, Pietenpol JA, Greenberger JS, Pelroy RA and Mohla S: Head and neck cancer: meeting summary and research opportunities. Cancer Res 64: 8126-8129, 2004.
3. Shah JP and Patel SG: Head and Neck Surgery and Oncology. 3rd edition. Mosby, New York, pp232-236, 352, 2003.
4. Forastiere AA and Trotti A: Radiotherapy and concurrent chemotherapy: a strategy that improves locoregional control and survival in oropharyngeal cancer. J Natl Cancer Inst 91: 2065-2066, 1999.
5. Denis F, Garaud P, Bardet E, *et al*: Final results of the 94-01 French Head and Neck Oncology and Radiotherapy Group randomized trial comparing radiotherapy alone with concomitant radiochemotherapy in advanced-stage oropharynx carcinoma. J Clin Oncol 22: 69-76, 2004.
6. Stenson KM, Kunnavakkam R, Cohen EE, *et al*: Chemoradiation for patients with advanced oral cavity cancer. Laryngoscope 120: 93-99, 2010.
7. Milano MT, Vokes EE, Kao J, *et al*: Intensity-modulated radiation therapy in advanced head and neck patients treated with intensive chemoradiotherapy: preliminary experience and future directions. Int J Oncol 28: 1141-1151, 2006.
8. Hu M, Ampil F, Clark C, Sonavane K, Caldito G and Nathan CA: Comorbid predictors of poor response to chemoradiotherapy for laryngeal squamous cell carcinoma. Laryngoscope 122: 565-571, 2012.
9. Sturgis EM and Cinciripini PM: Trends in head and neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers? Cancer 110: 1429-1435, 2007.

10. Dahlstrand H, Nasman A, Romanitan M, Lindquist D, Ramqvist T and Dalianis T: Human papillomavirus accounts both for increased incidence and better prognosis in tonsillar cancer. Anticancer Res 28: 1133-1138, 2008.
11. Reimers N, Kasper HU, Weissenborn SJ, *et al*: Combined analysis of HPV-DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. Int J Cancer 120: 1731-1738, 2007.
12. Kumar B, Cordell KG, Lee JS, *et al*: Response to therapy and outcomes in oropharyngeal cancer are associated with biomarkers including human papillomavirus, epidermal growth factor receptor, gender, and smoking. Int J Radiat Oncol Biol Phys 69: 109-111, 2007.
13. Rischin D, Young RJ, Fisher R, *et al*: Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol 28: 4142-4148, 2010.
14. Lill C, Kornek G, Bachtiary B, *et al*: Survival of patients with HPV-positive oropharyngeal cancer after radiochemotherapy is significantly enhanced. Wien Klin Wochenschr 123: 215-221, 2011.
15. Hong AM, Dobbins TA, Lee CS, *et al*: Human papillomavirus predicts outcome in oropharyngeal cancer in patients treated primarily with surgery or radiation therapy. Br J Cancer 103: 1510-1517, 2010.
16. Sedaghat AR, Zhang Z, Begum S, *et al*: Prognostic significance of human papillomavirus in oropharyngeal squamous cell carcinomas. Laryngoscope 119: 1542-1549, 2009.
17. Chung YL, Lee MY, Horng CF, *et al*: Use of combined molecular biomarkers for prediction of clinical outcomes in locally advanced tonsillar cancers treated with chemoradiotherapy alone. Head Neck 31: 9-20, 2009.
18. Nichols AC, Faquin WC, Westra WH, *et al*: HPV-16 infection predicts treatment outcome in oropharyngeal squamous cell carcinoma. Otolaryngol Head Neck Surg 140: 228-234, 2009.
19. de Jong MC, Pramana J, Knegjens JL, *et al*: HPV and high-risk gene expression profiles predict response to chemoradiotherapy in head and neck cancer, independent of clinical factors. Radiother Oncol 95: 365-370, 2010.
20. Fallai C, Perrone F, Licitra L, *et al*: Oropharyngeal squamous cell carcinoma treated with radiotherapy or radiochemotherapy: prognostic role of TP53 and HPV status. Int J Radiat Oncol Biol Phys 75: 1053-1059, 2009.
21. Pim D, Collins M and Banks L: Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. Oncogene 7: 27-32, 1992.
22. Depuydt CE, Benoy IH, Bailleul EJ, Vandepitte J, Vereecken AJ and Bogers JJ: Improved endocervical sampling and HPV viral load detection by Cervex-Brush Combi. Cytopathology 17: 374-381, 2006.
23. Depuydt CE, Boulet GA, Horvath CA, Benoy IH, Vereecken AJ and Bogers JJ: Comparison of MY09/11 consensus PCR and type-specific PCRs in the detection of oncogenic HPV types. J Cell Mol Med 11: 881-891, 2007.
24. Arbyn M, Benoy I, Simoens C, Bogers J, Beutels P and Depuydt C: Prevaccination distribution of human papillomavirus types in women attending at cervical cancer screening in Belgium. Cancer Epidemiol Biomarkers Prev 18: 321-330, 2009.
25. Filleul O, Preillon J, Crompot E, Lechien J and Saussez S: Incidence of head and neck cancers in Belgium: comparison with worldwide and French data. Bulletin du Cancer 98: 1173-1183, 2011.
26. Saussez S: Cancer of the upper aero-digestive tract: elevated incidence in Belgium, new risk factors and therapeutic perspectives. Bull Mem Acad R Med Belg 165: 453-461, 2010.
27. Duray A, Descamps G, Decaestecker C, *et al*: Human papillomavirus DNA strongly correlates with a poorer prognosis in oral cavity carcinoma. Laryngoscope 122: 1558-1565, 2012.
28. Rosenquist K, Wennerberg J, Annertz K, *et al*: Recurrence in patients with oral and oropharyngeal squamous cell carcinoma: human papillomavirus and other risk factors. Acta Otolaryngol 127: 980-987, 2007.
29. Hansson BG, Rosenquist K, Antonsson A, *et al*: Strong association between infection with human papillomavirus and oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. Acta Otolaryngol 125: 1337-1344, 2005.
30. Clayman GL, Stewart MG, Weber RS, el-Naggar AK and Grimm EA: Human papillomavirus in laryngeal and hypopharyngeal carcinomas. Relationship to survival. Arch Otolaryngol 120: 743-748, 1994.
31. Giskes K, Kunst AE, Benach J, *et al*: Trends in smoking behavior between 1985 and 2000 in nine European countries by education. J Epidemiol Community Health 59: 395-401, 2005.
32. Stanley MA: Immune responses to human papillomaviruses. Indian J Med Res 130: 266-276, 2009.
33. Hasan UA, Bates E, Takeshita F, *et al*: TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. J Immunol 178: 3186-3197, 2007.
34. Lepique AP, Daghastanli KR, Cuccovia IM and Villa LL: HPV16 tumor associated macrophages suppress antitumor T cell responses. Clin Cancer Res 15: 4391-4400, 2009.
35. Stanley MA: Immune responses to human papillomavirus. Vaccine 24: 16-22, 2006.
36. Tung YC, Lin KH, Chu PY, Hsu CC and Kuo WR: Detection of human papillomavirus and Epstein Barr virus DNA in nasopharyngeal carcinoma by polymerase chain reaction. Kaohsiung J Med Sci 15: 256-262, 1999.
37. Moscicki AB, Hills N, Shiboski S, *et al*: Risks for incident human papillomavirus infection and low-grade squamous intraepithelial lesion development in young females. JAMA 285: 2995-3002, 2001.
38. Kreimer AR, Alberg AJ, Daniel R, *et al*: Oral human papillomavirus infection in adults is associated with sexual behavior and HIV serostatus. J Infect Dis 189: 686-698, 2004.