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Abstract

Background: Although smoking and HPV infection are recognized as important risk factors for oropharyngeal cancer, how their joint exposure impacts on oropharyngeal cancer risk is unclear. Specifically, whether smoking confers any additional risk to HPV-positive oropharyngeal cancer is not understood.

Methods: Using HPV serology as a marker of HPV-related cancer, we examined the interaction between smoking and HPV16 in 459 oropharyngeal (and 1445 oral cavity and laryngeal) cancer patients and 3024 control participants from two large European multi-centre studies. Odds ratios and credible intervals [CrI], adjusted for potential confounders, were estimated using Bayesian logistic regression.

Results: Both smoking [odds ratio (OR [CrI]: 6.82 [4.52, 10.29]) and HPV seropositivity (OR [CrI]: 235.69 [99.95, 555.74]) were independently associated with oropharyngeal cancer. The joint association of smoking and HPV seropositivity was consistent with that expected on the additive scale (synergy index [CrI]: 1.32 [0.51, 3.45]), suggesting they act as independent risk factors for oropharyngeal cancer.

Conclusions: Smoking was consistently associated with increase in oropharyngeal cancer risk in models stratified by HPV16 seropositivity. In addition, we report that the prevalence of oropharyngeal cancer increases with smoking for both HPV16-positive and HPV16-negative persons. The impact of smoking on HPV16-positive oropharyngeal cancer highlights the continued need for smoking cessation programmes for primary prevention of head and neck cancer.

Keywords: Human papillomavirus, tobacco smoking, interaction, head and neck cancer risk, oropharynx cancer

Key Messages

- The incremental risk due to smoking in HPV16-positive oropharyngeal cancer (if any) remains unclear.
- Pooling two large HNC studies with HPV serology data, we examined the relationship between these risk factors.
- We demonstrated that smoking was consistently associated with increased risk of oropharyngeal cancer regardless of HPV status.
- These data demonstrate that tobacco exposure remains an important risk factor for oropharyngeal cancer irrespective of HPV status.

Introduction

It is estimated that nearly 600 000 new cancers of the oral cavity, pharynx and larynx are diagnosed each year worldwide, contributing to approximately 325 000 deaths each year. 1 Collectively referred to as head and neck cancers (HNC), most are squamous cell in origin and are grouped together due to aetiological similarities. 2 Nearly 33% of HNC are attributed to smoking alone, alcohol alone is estimated to cause nearly 4%, and the largest proportion of cases are attributed to the joint exposure to smoking and alcohol, nearly 35%. 3,4 Recently, infection by human

papillomavirus (HPV) has been associated with a subset of HNC arising at the base of tongue, the tonsils and the oropharynx. [5,6]

HPV-positive oropharyngeal carcinoma (OPC) patients tend to be more often never smokers compared with HPV-negative patients. [7,8] In addition, statistically non-significant associations between smoking and HPV-positive OPC have led to the speculation that smoking is not an important risk factor for HPV-positive OPC. 9 However, it is noteworthy that up to 30% of HPV-positive HNC occur among heavy smokers and alcohol drinkers. [8,10] Furthermore, previous analyses did not account for differing baseline risks by HPV status when interpreting the odds ratios (OR) for smoking. In addition, classification of HPV status has been an important challenge in such studies. We have previously demonstrated that circulating antibodies against HPV16 oncoprotein E6 constitute a highly specific marker of HPV16-related OPC, present in nearly 30% of OPC and less than 1% of controls, [11,12] whereas antibodies against HPV16 capsid protein L1 are regarded as markers of past exposure. [13,14] The rarity of OPC and the limited proportion of never smokers necessitate large pooled analysis to examine the relationships between these risk factors.

A significant proportion of OPC patients report a history of smoking at diagnosis (nearly 80% in the USA and European Union); [3,4,8,15] therefore we examined whether smoking increases the risk of HPV16-positive OPC. Further, we examined the impact of smoking on the prevalence of OPC among HPV-positive and -negative persons. We also examined the association between smoking, alcohol intake, HPV16 and the risk of non-oropharyngeal HNC.

Materials and Methods

Study sample

This analysis included two studies of HPV serology and HNC, the Alcohol-Related Cancers and Genetic Susceptibility in Europe (ARCAGE) study and the HNC case-control study nested within the European Prospective Investigation Into Cancer and Nutrition (EPIC) cohort. Briefly, the ARCAGE study was conducted during 2002–05 and included 1292 pathologically confirmed primary HNC and 1425 controls frequency-matched for age, sex and area of residence. [11,16] Ever smokers were defined as individuals who smoked any tobacco product at least once a week for 1 year, and ever drinkers were those who reported ever consuming any alcoholic beverage. 17 The EPIC cohort recruited 521 330 individuals during 1992 and 2000, of whom 385 747 participants contributed a blood sample. 18 This analysis included 612 incident HNC and 1599 controls. 12 Two controls (one in Denmark) were randomly selected for each cancer patient from appropriate risk sets consisting of all cohort participants alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the index case. Controls were matched on country, sex, date of blood collection (1 month, relaxed to 5 months for sets without available controls) and date of birth (1 year, relaxed to 5 years for sets without available participants). Ever smokers were individuals who reported ever smoking any tobacco product in their lifetime, and ever drinkers were individuals who reported ever consuming any alcoholic beverage. HNC included cancers arising at the oral cavity (International Classification of Diseases for Oncology (ICD-O) C00.3–C00.9, C02.0–C06.9, C14.0–C14.9, excluding C02.4, C02.8, C02.9, C05.1, C05.2, C05.8, C05.9), oropharynx (ICD-O: C01, C02.4, C05.1–C05.2, C09, C10), hypopharynx and larynx (ICD-O: C13, C32) and non-specified and overlapping sites (ICD-O: C02.8, C02.9, C05.8, C05.9, C32.8). Lymphomas were not included, and salivary gland cancers were omitted. This analysis included head and neck cancers of all histological subtypes, of which squamous cancers comprised the vast majority (~ 91%), and some other rarer non-squamous histologies (6%, in ARCAGE and 9% in EPIC). Informed consent was obtained from all participants in both the studies, and the studies were approved by the ethical review boards at the participating centres and the International Agency for Research on Cancer.

HPV serology

HPV antibodies were assayed using the bead-based multiplex serology method as described elsewhere. 19 Testing was performed blind to the case-control status of the participants. Mean fluorescence intensity (MFI) values were dichotomized by applying thresholds derived from a cross-sectional study among Korean students of mean plus 5 standard deviations (SD; for HPV16 E6) or the mean plus 3 SD excluding positive outliers (for HPV16 L1), 20 as described previously. 11,12

Statistical analysis

The overall associations between HPV16 (L1 and E6), smoking, alcohol intake and HNC risk were assessed by calculating odds ratios (ORs)and their corresponding 95% confidence intervals (CIs).These models included age, sex, smoking status (never, former, current), alcohol consumption (never, ever plus ethanol g/day at recruitment) and country as covariates. Since certain combinations of exposures were very rare (e.g. HPV16 E6-positive never smoking control subjects), Bayesian logistic regression models were used to calculate ORs and corresponding 95% credible intervals (CrI). These models use a prior distribution to shrink or penalize the regression coefficients, thus providing more stable estimates than maximum likelihood methods. Following Gelman *et al.* , 21 all regression inputs were centred, and continuous inputs were re-scaled to have a standard deviation of 0.5. All regression coefficients were then modelled with a weakly informative Cauchy prior distribution with mean 0 and scale 2.5, with the exception of the intercept, which was given a weaker Cauchy prior with scale 10. These models were fitted using the bayesglm function in the R package ARM. 21,22 In these analyses, former and current smokers were combined as ever smokers and given the few participants who reported never consumption of any alcoholic beverage; individuals who consumed 7 g or less of ethanol (equivalent of half a drink) per day were considered the reference. Since the results from ARCAGE and EPIC studies were similar, data were pooled in order to obtain more precise estimates. Interactions between smoking, alcohol intake and HPV16 were examined by the inclusion of an interaction term in the penalized regression models. Additive interactions were evaluated by estimating the synergy index (SI). 23 The prevalence of OPC by categories of smoking and HPV16 were calculated based on the ORs from the fitted models and assumed population prevalence of 0.003, based on the cumulative risk for pharyngeal cancer among men and women combined, in more developed regions of the world. 24 All statistical analyses were performed using Stata version 11.2 (StataCorp, College Station, TX, USA) and R version3.1.0. 25

Results

Study profile

A total of 1904 HNC patients included 459 OPC, 1445 non-oropharyngeal HNC and 3024 control subjects. Of these, 1292 HNC cases and 1425 controls were from the ARCAGE study and 612 HNC cases and 1599 controls from the EPIC study. OPC patients were similar to HNC patients, as they tended to be more often men, current smokers and current drinkers (Table 1).

Main effects of smoking, alcohol consumption and HPV antibodies in OPC

HPV16 E6-positivity was observed in 0.7% of 3024 controls and 31.6% of 459 OPC subjects, and was strongly associated with OPC risk [OR (95% CI): 147.31 (83.07, 361.24)]. The odds of OPC was higher among current smokers compared with never smokers [OR (95% CI): 5.34 (3.89, 7.33)].

Consumption of ≥ 28 g of ethanol per day (two or more drinks per day) was also associated with increased risk of OPC [OR (95% CI): 2.43 (1.77, 3.33)] (Figure I).

Combined effects of smoking and HPV16 in OPC

To understand the combined effects of smoking and HPV16 in OPC, we examined the relative odds of OPC given smoking and HPV16 status (Table 2). Compared with HPV16 E6-negative never smokers, ever smoking was associated with 6.82 times increased OPC risk (95% CrI: 4.52, 10.29), whereas HPV16 E6-positivity alone was associated with 235.69 times increased odds (95% CrI: 99.95, 555.74). HPV16 E6-positive ever smokers had 355.82 timeshigher OPC risk (95% CrI: 177.0, 715.30) compared with individuals negative for both risk factors. HPV16 E6-positivity and smoking appeared to interact on a less than multiplicative scale (OR [95% CrI] for the interaction term: 0.22 [0.08, 0.62]). The SI for the interaction was calculated to be 1.32 [95% CrI: 0.51, 3.45], suggesting that the risks associated with HPV16 E6 and smoking might be additive. Interestingly, similar results were observed when HPV16 L1-positivity was examined (OR [95% CrI] for the multiplicative interaction term: 0.23 [0.13, 0.43], SI [95% CrI]: 0.75 [0.51, 1.12]) (Table 2). These results remained unchanged when analyses were restricted to squamous cell cancers of the oropharynx (Supplementary table V , available as Supplementary data at *IJE* online). In models stratified by HPV status (Supplementary table I , model 1, available as Supplementary data at *IJE* online), we observed that OPC risk increased with the number of cigarettes smoked per day (CPD) among HPV16 L1-negatives (OR [95% CrI] for > 15 CPD: 8.71 [6.01, 12.64]), as well as HPV16 E6-negatives (OR [95% CrI] for > 15 CPD: 10.69 [7.06, 16.20]). Among HPV16-positives, we observed no additional increase in OPC risk with smoking dose (Supplementary Table I , model 1). The limited proportion of HPV16-positive controls in these comparisons is noteworthy; whereas 2.5% of HPV16 L1-positive controls reported smoking > 15 CPD, this proportion was down to only 0.2% among HPV16 E6-positives (Supplementary Table I , model 2). Our previous analysis of predictors of HPV16 E6-positivity among cancer-free individuals suggested that the possibility of an underlying undiagnosed cancer cannot be ruled out in such subjects. 26 Therefore, we excluded all HPV16 E6-positive controls under the assumption that they could be false positives or harbour other undiagnosed HPV-related cancer. We then compared HPV16 E6-positive cases with HPV16 E6-negative controls, to clarify whether smoking might confer any further risk in HPV16-positive OPC (Table 3). Results indicate that the OPC risk among HPV16-positive persons increased with smoking. Compared with never smokers, both former smokers (OR [95% CrI]: 1.49 [0.95,2.36]) and current smokers (OR [95% CrI]: 1.86 [1.17,2.96]) were at increased OPC risk. However, we observed a modest trend toward increased OPC risk with smoking dose (OR [95% CrI] for ≤ 15 CPD: 1.61 [1.03, 2.54] and OR [95% CrI] for > 15 CPD: 1.77 [1.11, 2.84]).

Relatedly, the relationship between smoking and the risk of HPV-positivity remains poorly understood. It remains unclear whether smoking may enhance HPV exposure and/or infection. Using HPV seropositivity as the endpoint, Kelsey *et al.* have recently demonstrated that younger smokers are more likely to be seropositive for HPV16 L1 than older smokers. 27 We sought to understand whether any age-dependent association existed between smoking and HPV seropositivity in this study. Briefly, in analysis restricted to controls, age was divided into tertiles as young (< 54), intermediate (54–62) and old (> 62 years of age) subjects. We then examined the association between these age categories and HPV16 seropositivity by smoking status. Overall, age did not appear to be strongly associated with HPV seropositivity, either HPV16 L1 (OR [95% CI]: 0.78 [0.56, 1.09]) or HPV16 E6 (OR [95% CI]: 0.67 [0.22, 2.02]), albeit based on small numbers. Current smoking, on the other hand, modestly reduced the risk of HPV16 L1 seropositivity (OR [95% CI]: 0.79 [0.62, 1.00]) but not HPV16 E6 (OR [95% CI]: 1.00 [0.67, 1.51]) (Supplementary Table VI , available as Supplementary data at *IJE* online). We found no evidence for an interaction between age and smoking towards HPV16 seropositivity (Supplementary Table VII , available as

Supplementary data at *IJE* online). Similarly, when stratified by smoking, age was not associated with risk of HPV16 L1 seropositivity in former (OR [95% CI]: 0.91 [0.56, 1.48]) or current smokers (OR [95% CI]: 1.36 [0.79, 2.36]) (Supplementary Table VIII , available as Supplementary data at *IJE* online).

OPC prevalence by smoking and HPV16 status

In order to provide a simple appreciation of the joint importance of HPV infection and tobacco smoking, we estimated the prevalence of OPC given smoking and HPV16 E6 status, assuming an overall OPC population prevalence of 0.003. OPC prevalence among HPV16 E6-negative never smokers was negligible (0.05%; 95% CrI: 0.03%, 0.07%), which increased to 0.13% (95% CrI: 0.08%, 0.20%) in former smokers, and further increased to 0.54% (95% CrI: 0.36%, 0.80%) in current smokers. Similarly, among HPV16-positive participants, the baseline prevalence in never smokers was 8.94% (95% CrI: 4.20%, 18.01%), 11.58% (95% CrI: 5.38%, 23.19%) in former smokers, and further increased to 17.68% (95% CrI: 7.29%, 36.95%) in current smokers (Figure 2).

Non-oropharyngeal HNC risk factors

For non-oropharyngeal HNC, current smoking (OR (95% CI): 6.82 (5.51, 8.44)), alcohol consumption of > 28 g per day (OR (95% CI): 2.31 (1.87, 2.85)) and HPV16 E6-positivity were associated with increased risk for non-oropharyngeal HNC (OR (95%CI): 2.57 (1.24, 5.33)), whereas HPV16 L1-positivity was not (OR (95% CI): 1.05 (0.82, 1.35)) (Supplementary Figure I , available as Supplementary data at *IJE* online). No interaction was observed between HPV16 and smoking for non-oropharyngeal HNC (Supplementary Table II , available as Supplementary data at *IJE* online). Alcohol consumption and HPV16 antibody status did not appear to interact to affect risk of either oropharyngeal or non-oropharyngeal HNC (Supplementary Tables III and Supplementary Data , available as Supplementary data at *IJE* online). Study-specific associations between smoking, alcohol intake, HPV16 and OPC risk and non-oropharyngeal HNC risk are presented in Supplementary Figures II-V , (available as Supplementary data at *IJE* online).

Discussion

This study supports the notion that smoking and alcohol are important risk factors for all HNC subsites, and HPV16 infection is relevant at the oropharynx. We demonstrate that smoking increases the risk of OPC, irrespective of HPV16 status. Importantly, OPC remains higher in smokers compared with never smokers, among both HPV16-positive and -negative persons.

The manner in which two (or more) distinct risk factors interact to influence disease risk can be tested by examining the joint effects. Biologically, components (or exposures) that lie within the same causal pathway are thought to interact, implying that disease will not occur (or will occur at the population baseline rates) in the absence of any one of the exposures. Statistically, the combined effect may be quantified on the multiplicative or additive scale. Most risk factors interact in greater than a multiplicative scale. For example, whereas smoking and alcohol consumption are each associated with substantially increased risks of HNC, smokers who also consume alcohol experience risk far greater risk than the product of the two. [3,4] Such a 'supermultiplicative' risk is often interpreted to indicate that the risk factors potentiate the carcinogenic effects of each other. Truly additive effects, where the combined risk is similar to the sum of individual risks, are rare. Such additive interactions are thought to indicate independence of risk factors. In this study we observe that the joint effect of HPV16 (L1 and E6 antibody status) and smoking are consistent with

that expected on the additive scale. This result suggests that HPV16 and smoking are independent risk factors for OPC, a conclusion supported by previous studies. [7,9,28–30] However, that the association of smoking with risk of OPC in HPV16-positives has not been statistically significant has been interpreted as the absence of risk due to smoking, and has led to the speculation that presence of HPV16 could protect against the adverse effects of smoking. [9] In this study, we clearly demonstrate that the OPC risk increases with smoking even in the context of HPV16-positive OPC. The consistency of these observations when considering HPV16 L1- or HPV16 E6-positivity as markers of HPV16 status further strengthens our conclusions. These results would also seem to suggest that smoking may induce molecular changes in HPV16-positive OPC. This hypothesis is supported by results from the Radiation Therapy Oncology Group (RTOG) trial where OPC patients were stratified into three distinct risk-of-death groups; HPV-positive never smokers experienced the best prognosis and HPV-negative smokers the worst, and the combined presence of smoking and HPV constituted an 'intermediate' risk group. [31]

Unlike the recently published report, [27] we found no evidence for an interaction between age and smoking in the risk of HPV seropositivity. This largely implies that age is not associated with HPV seropositivity regardless of smoking status. Conversely, the association between smoking and HPV seropositivity, for either L1 or E6, does not appear to vary with age in this study. In other words, smoking did not appear to alter either exposure or infection to HPV16 (L1 and E6, respectively) differentially by age in this study.

Further, our data emphasize that it remains critical to interpret relative risks in the context of the dramatically differing baseline risks between HPV16-positive and -negative people. We estimated the absolute difference in OPC prevalence between never and current smokers to be 0.5% in HPV16 E6-negative individual and 8.8% in HPV16 E6-positives, re-emphasizing the importance of smoking regardless of HPV16 status. These data suggest that smoking remains an important risk factor for OPC, at least in Europe where almost a quarter of the patients are both HPV16 positive and report smoking history at diagnosis. In this study, we did not observe a strong interaction between alcohol consumption and HPV16. This is perhaps unsurprising given that alcohol appears to affect HNC risk at higher doses and primarily in the presence of tobacco smoking. [3,4]

To the best of our knowledge, this constitutes the largest study examining the joint effects of smoking and HPV16 infection in OPC. It is important to note that antibody status is a systemic marker, and might therefore reflect infection in any location in the body rather than an oropharyngeal infection specifically. However, we and others have previously demonstrated HPV16 E6 to be a highly specific [11,12,28–31] and likely sensitive marker for HPV16-related OPC. [11,12] Since this uncertainty applies to both cases and controls, it is not expected to lead to biased comparisons. Further, self-reported data on alcohol and tobacco use were used and thus may contribute to some measurement error, especially in the retrospective case-control study.

In summary, these results indicate that smoking and HPV16 follow independent pathways towards OPC. The impact of smoking on HPV16-positive OPC may have important implications for treatment, survival and recurrence of OPC and re-emphasizes the continuing need for tobacco cessation programmes.

Funding

The ARCAGE study was supported by a grant from the European Commission's 5th Framework Program (contract QLK1-2001-00182). The EPIC study has been supported by the Europe Against Cancer Program of the European Commission (SANCO); Deutsche Krebshilfe, Deutsches Krebsforschungszentrum, German Federal Ministry of Education and Research; Danish Cancer

Society; Health Research Fund (FIS) of the Spanish Ministry of Health, Spanish Regional Governments of Andalucia, Asturias, Basque Country, Murcia and Navarra; Catalan Institute of Oncology, Spain; the ISCIII of the Spanish Ministry of Health (RETICC DR06/0020); Cancer Research UK; Medical Research Council, UK; Greek Ministry of Health; Stavros Niarchos Foundation; Hellenic Health Foundation; Italian Association for Research on Cancer (AIRC); Italian National Research Council, Fondazione-IstitutoBanco Napoli, Italy; Associazione Italiana per la Ricerca sul Cancro-AIRC Milan; Compagnia di San Paolo; Dutch Ministry of Public Health, Welfare and Sports; World Cancer Research Fund; Swedish Cancer Society; Swedish Scientific Council; Regional Government of Vasterbotten, Sweden; NordForsk (Centre of excellence programme HELGA), Norway; French League against Cancer (LNCC), France; National Institute for Health and Medical Research (INSERM), France; Mutuelle Generale de l'Education Nationale (MGEN), France; 3M Co, France; Gustave Roussy Institute (IGR), France; and General Councils of France. The serology testing of EPIC prospective study was supported by the National Cancer Institute Intramural Research Program. The work done in this study was supported in part by a grant from the European Commission's 7th Framework Program (contract FP7-HEALTH-2011–282562) and partly by the Health General Directorate of the French Social Affairs and Health Ministry.

Conflict of interest: None declared.

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