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Abstract

Background Human papillomavirus (HPV) is causally implicated in a subset of cancers of the upper aero-digestive tract (UADT).

Methods Associations between type-specific HPV antibodies were examined among 1496 UADT cancer case subjects and 1425 control subjects by estimating odds ratios (ORs) in logistic regression analyses adjusted for potential confounders. The agreement between serology and tumor markers of HPV infection, including presence of HPV DNA and p16 expression, were examined in a subset of tumors.

Results HPV16 L1 seropositivity was associated with increased risk of oral cavity and oropharyngeal cancer (OR = 1.94, 95% confidence interval [CI] = 1.03 to 3.65; OR = 8.60, 95% CI = 5.21 to 14.20, respectively). HPV16 E6 antibodies were present in 30.2% of oropharyngeal case subjects and only 0.8% of control subjects (OR = 132.0, 95% CI = 65.29 to 266.86). Combined seropositivity to HPV16 E6 and E7 was rare (n = 1 of 1425 control subjects). An agreement of 67% was observed between HPV16 E6 serology and the corresponding presence of an HPV-related cancer: four of six HPV DNA-positive/p16-overexpressing tumors were HPV16 E6 antibody positive. An HPV16 independent association was observed for HPV18 and oropharyngeal cancer

(OR = 8.14, 95% CI = 2.21 to 29.99 for HPV18 E6 seropositivity) and HPV6 and laryngeal cancer (OR = 3.25, 95% CI = 1.46 to 7.24 for HPV6 E7 seropositivity).
Conclusions These results confirm an important role for HPV16 infection in oropharyngeal cancer. HPV16 E6 antibodies are strongly associated with HPV16-related oropharyngeal cancers. Continuing efforts are needed to consider both HPV serology and p16 staining as biomarkers relevant to the etiology and natural history of HPV16-related oropharyngeal tumors. These results also support a marginal role for HPV18 in oropharyngeal cancer and HPV6 in laryngeal cancer.
Cancers of the upper aero-digestive tract (UADT), comprising the oral cavity, pharynx, larynx, and esophagus, contribute to more than a million new cancer cases each year worldwide. Annually, more than 700 000 people die of the disease (1). Tobacco and alcohol are known risk factors, and human papillomavirus (HPV) infection has been implicated in a subset of UADT cancers (2,3). The high-risk types, HPV16 and HPV18, are the commonly identified HPV types (>90% of HPV-positive tumors), consistent with observations in cervical and other ano-genital cancers (4–6). However, unlike in cervical cancer, not all HPV infections in UADT cancer are transcriptionally active (7).
The epidemiological, molecular, and mechanistic association of HPV16 and UADT cancer is strongest for the oropharynx (8–12). Even so, large variation in HPV16 DNA prevalence (8%–100%) is observed, possibly because of differences in study population, composition of tumor subsite, proportion of other known risk factors including smoking and alcohol, type of specimen assayed, and assay variability (5,13). Conversely, HPV18 appears to be rare in oropharyngeal cancers (5). Recently developed serological methods simultaneously detect type-specific antibodies to multiple HPV proteins, including early viral oncoproteins E6 and E7 that are considered markers of ongoing HPV-related malignancy. The studies published so far have consistently reported an association between presence of HPV16 antibodies and risk of oropharyngeal cancer (4,8,14–16). Even so, the usefulness of serological markers in identifying an HPV-related cancer is poorly understood. Further, the contribution of HPV infections to nonoropharyngeal sites and, particularly, of other mucosal HPV types remains unclear.
Using a large panel of markers of HPV infection in a large case–control study, we aimed to: 1) comprehensively evaluate the association between serological markers of oncogenic HPV infection and UADT cancer, 2) estimate how this varies by subsite, 3) examine the important serological associations in a subset of tumor biopsies, and 4) clarify the true proportion of HPV16-related UADT cancer by anatomic site.

Methods

Study Population

One thousand four hundred ninety-six case subjects and 1425 control subjects with an available plasma sample from the Alcohol-Related Cancers and Genetic Susceptibility in Europe (ARCAGE) study were included in this study. Details of the study have been described previously (17). Briefly, 2304 case subjects and 2227 control subjects were recruited from 10 European countries during the period from 2002 to 2005 using a standardized protocol in all centers (except France). Case subjects had histologically or cytologically confirmed primary cancers of the oral cavity (International Classification of Diseases for Oncology [ICD-O] C00.3–C00.9, C02.0–C06.9, 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), esophagus (ICD-O: C15), and nonspecified and overlapping sites (ICD-O: C02.8, C02.9, CO5.8, C05.9, C14.0, C14.8, C32.8). Cancer stage was ascertained based on the sixth edition of the staging atlas developed by the American Joint Committee on Cancer (AJCC). A comparable group of hospital- or population-based controls were recruited in each center and frequency matched for age, sex, and area of residence. All subjects underwent personal interviews to record lifestyle exposures; details are described elsewhere (17). Briefly, tobacco use was broadly categorized as ever or never smokers; ever smokers were defined as individuals who smoked any tobacco product at least once a week for a year. Ever drinkers were

those who reported ever consumption of any alcoholic beverage. The consumption of all types of alcoholic beverages were estimated, and the total frequency was expressed in terms of drinks of alcohol per day based on the definition that one drink equivalent was 14 grams, 18mL, or 0.49 ounces of alcohol (18). Informed consent was obtained from all participants in the study, and the study was approved by the ethical review boards at the participating centers and by the International Agency for Research on Cancer Ethical Review Committee.

Laboratory Methods

Serological Methods.

Plasma samples from 1496 case subjects and 1425 control subjects were tested for type-specific HPV antibodies using bead-based multiplex serology method, as described elsewhere (19). We report associations on 27 markers of mucosal HPV infection, including high-risk types—HPV16 (L1, E1, E2, E4, E6, E7), HPV18 (L1, E6, E7), HPV31 (L1, E6, E7), HPV33 (L1, E6, E7), HPV45 (L1, E6, E7), and HPV52 (L1, E6, E7)—and low-risk types—HPV6 (L1, E6, E7) and HPV11 (L1, E6, E7). Additionally, we tested antibodies to cutaneous HPV types (HPV1, HPV5, HPV8, and HPV38) and non-HPV-related antibodies (P53, P16, JCV, HHV, and EBV) as specificity controls. Serology data were generated as continuous measures of mean fluorescence intensity, which were dichotomized using cutoffs derived from earlier studies (20,21). Briefly, a bridging panel that included a reference set of sera from two previous studies of approximately 2000 Germans and 370 Korean students who tested negative for genital DNA of 25 HPV types and were self-declared as sexually naive was used to define seropositivity thresholds. Data normalization was performed using the ratio of predefined and extrapolated cutoffs, and seropositivity was set at mean plus three standard deviations.

Tumor Tissue Analyses.

One hundred fifty snap-frozen tumor tissues were identified that included 125 cases with a high a priori expectation for HPV infection based on serology (all HPV16 L1, E6, and E7 positives), tumor site (all cancers of the oropharynx and overlapping sites), and other characteristics (all women, young male never smokers) and 25 cases with a low a priori expectation, which included cancers of the oral cavity or larynx among male smokers. Based on pathological evaluation that aimed to confirm tumor histology and record cellular features, 30 tumors were excluded because of insufficient tumor tissue, absence of tumor tissue (only fibroconnective tissue, necrotic tissue, etc), unknown histology, or unknown tumor origin. The p16 expression was qualitatively evaluated using the CINtec Histology $P16^{INK4a}$ Kit (9511, mtmlabs, Heidelberg, Germany) following manufacturer's instructions. Expression was scored based on the percentage and intensity of nuclear or cytoplasmic staining. A combined score of four or greater was considered positive for $p16^{INK4a}$ overexpression (22). When this algorithm was subsequently compared with simpler p16 scoring methods used in other head and neck cancer studies (7,23,24), identical results were obtained. DNA extraction from biopsies was performed using the Qiagen BioRobot EZ1 (Qiagen, Hilden, Germany). HPV genotyping using the type-specific E7 polymerase chain reaction bead-based multiplex assay (TS-E7-MPG, IARC, Lyon, France) was performed to detect all high-risk HPV types (HPV16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68a, -68b, -73, and -82) and three low-risk HPV types (HPV6, -11, and -70) (25–27). Briefly, the reporter fluorescence was quantified using Luminex reader 200 (Luminex Corporation, Austin, TX), and cutoffs were computed by adding 5 to 1.1 multiplied by the median background value expressed as median fluorescence intensity.

Statistical Analysis

The association between serological markers of HPV infection and UADT cancer was examined by calculating the odds ratios (ORs) and corresponding 95% confidence intervals (CIs) using unconditional logistic regression models adjusted for age, sex, level of education (finished primary

school, finished secondary school, or university degree), pack years of tobacco smoking (never, <20, 20–39, 40–59, 60–79, and ≥80), number of alcoholic drinks consumed per day (never, <1, 1–2, 3–4, 5–6, ≥7), and country.

The HPV types included in this analysis fall into three main species: alpha7 (HPV18 and HPV45), alpha9 (HPV16, HPV31, HPV33, and HPV52), and apha10 (HPV6 and HPV11). Within these, it is possible that a positive infection from one type may result in seropositivity for another type because of cross-reactivity. To account for this, we performed antigen-specific sensitivity analysis. The association between HPV type-specific antibodies and UADT cancer were reexamined after exclusion of subjects who were seropositive for more than one homologous protein.

Multiple methods have been proposed to identify HPV-related head and neck cancers, including p16 immunohistochemistry, HPV DNA detection (by in situ hybridization or other polymerase chain reaction–based methods), and RNA expression of E6 and E7 genes (28–30). Although RNA expression is likely to represent the closest to a gold standard, it requires appropriate collection of fresh tumor tissue that preserves RNA integrity. Given that we did not collect RNA from tumor tissue, we adopted an alternate algorithm that included HPV DNA detection and p16 overexpression (7). Based on this algorithm, we classified tumors into HPV16 DNA-positive/p16-overexpressing (referred to as HPV-related), HPV16 DNA-positive/p16-negative, and HPV16 DNA-negative tumors. This algorithm was initially developed by comparing different methods for HPV detection among 48 oral and oropharyngeal cancers, and we are assuming it is also relevant for laryngeal cancers. We subsequently assessed the agreement between serological and tumor markers of HPV16 infection based on this algorithm. All statistical analyses were performed using STATA statistical software, version 11 (StataCorp, College Station, TX), and all reported *P* values are two sided. Statistical significance was set at *P* less than .05.

Results

Table 1 shows the characteristics of the study population. The analysis included 1496 case subjects and 1425 control subjects in the original study with an available plasma sample. The serology subset was comparable with the overall study on all the demographic and lifestyle factors (data not shown). Of the 1496 case subjects, 24% were diagnosed with oral cavity cancer, 22% were diagnosed with oropharyngeal cancer (123 were tonsillar), 35% were diagnosed with laryngeal/hypopharyngeal cancer, and 13% were diagnosed with esophageal cancer. Compared with control subjects, case subjects attained lower levels of education and were more often smokers. As expected, smoking and alcohol consumption were strong risk factors for UADT cancer. A clear dose–response relationship was observed between increasing pack years of smoking, number of alcoholic drinks consumed per day, and the risk of UADT cancer overall and for each subsite (data not shown). Cancer stage was ascertained for 81% of case subjects. The proportion of subjects missing stage information was not associated with any of the patient characteristics or exposures, including age, sex, smoking status, alcohol consumption, or the presence of HPV antibodies.

Table 1.
Demographic and lifestyle characteristics of the study group

	Serological series		Tumor subset
	Control subjects, No. (%)	Case subjects, No. (%)	Case subjects, No. (%)
Description	**(n = 1425)**	**(n = 1496)**	**(n = 120)**
Country			
Czech Republic	185 (13)	187 (12)	16 (13)
Germany	187 (13)	188 (13)	—
Greece	167 (12)	224 (15)	53 (44)
Italy	462 (32)	440 (29)	51 (43)
Ireland	16 (1)	33 (2)	—
Norway	168 (12)	162 (11)	—

	Serological series		Tumor subset
	Control subjects, No. (%)	Case subjects, No. (%)	Case subjects, No. (%)
Description	**(n = 1425)**	**(n = 1496)**	**(n = 120)**
United Kingdom	112 (8)	119 (8)	—
Spain	82 (6)	89 (6)	—
Croatia	46 (3)	54 (4)	—
Age group, years			
≤55	494 (35)	498 (32)	38 (32)
56–65	456 (32)	551 (37)	39 (33)
≥66	475 (33)	447 (30)	43 (36)
Sex			
Men	1059 (74)	1190 (80)	81 (68)
Women	366 (26)	306 (20)	39 (32)
Smoking status			
Never	516 (36)	172 (12)	32 (27)
Former	475 (33)	361 (24)	16 (13)
Current	434 (31)	963 (64)	72 (60)
Alcohol consumption*			
Never	172 (12)	89 (6)	8 (6)
Former	134 (9)	206 (14)	18 (15)
Current	1118 (79)	1201 (80)	94 (78)
Level of education attained*			
Finished primary	444 (31)	630 (42)	70 (58)
Finished secondary	834 (59)	798 (53)	40 (33)
University degree	147 (10)	66 (4)	10 (8)
Cancer site			
Oral cavity		366 (24)	42 (35)
Oropharynx		324 (22)	36 (30)
Hypopharynx/larynx		529 (35)	16 (13)
Esophagus		200 (13)	8 (7)
Overlapping†		77 (5)	18 (15)
Stage*			
I and II		530 (35)	48 (40)
III and IV		686 (46)	67 (56)

* Numbers do not add up to the total because of missing data: information on alcohol consumption was missing for one control subject, education level data was missing for two case subjects, and stage was missing for 280 case subjects.
† Includes cancers of overlapping topologies and nonspecified cancers of the head and neck.

HPV16 Antibodies and UADT Cancer

Table 2.
Human papillomavirus 16 (HPV16) antibodies and oropharyngeal cancer risk

	Control subjects (n = 1395)*	Oropharynx cancer	
HPV16 antibody	**Seropositive, No. (%)†**	**(n = 321)***	**OR (95% CI)‡**
L1	36 (2.6)	44 (13.7)	8.60 (5.21 to 14.20)
E1	31 (2.2)	69 (21.2)	22.63 (13.63 to 37.57)
E2	29 (2.1)	81 (25.2)	30.65 (18.56 to 50.64)
E4	85 (5.9)	41 (12.7)	2.59 (1.68 to 4.00)

HPV16 antibody	Control subjects (n = 1395)* Seropositive, No. (%)†	Oropharynx cancer (n = 321)*	OR (95% CI)‡
E6	11 (0.8)	97 (30.2)	132.0 (65.29 to 266.86)
E7	64 (4.6)	80 (24.9)	9.00 (6.06 to 13.36)

* Thirty control subjects and three oropharyngeal case subjects were missing data on smoking pack years or frequency of alcohol consumption.
† Represents seropositivity to the corresponding HPV16 antigen.
‡ Odds ratios (ORs) were adjusted for age, sex, level of education, smoking pack years, and number of alcoholic drinks consumed per day; corresponding seronegative group was considered as reference. CI = confidence interval.

Non-HPV16 Antibodies and UADT Cancer

Antibodies to high-risk types HPV18 (L1, E6, and E7), HPV31 (L1 and E7), HPV33 (L1, E6, and E7), HPV45 (L1, E6, and E7), and HPV52 (L1 and E7) and low-risk type HPV11 L1 were associated with oropharyngeal cancer (Table 3). To test for an HPV16-independent association, if any, we excluded all HPV16 L1, E6, and E7 seropositives. Although the HPV11 L1 association was consistent, only the associations between HPV18 L1 and E6 remained robust (OR = 2.37, 95% CI = 1.06 to 5.32; and OR = 8.14, 95% CI = 2.21 to 29.99, respectively). We found associations for HPV6 (L1 and E7) and UADT cancer that appeared to be driven by laryngeal cancer. An HPV16-independent effect was observed for HPV6 E7 and laryngeal cancer (OR = 3.25, 95% CI = 1.46 to 7.24, Supplementary Table 3, available online). No associations were observed between seropositivity to cutaneous HPV types and UADT cancer (data not shown).

Table 3.
Human pappilomavirus (HPV) type-specific antibodies and oropharyngeal cancer risk

	Control subjects (n = 1395)*	Oropharynx cancer		Control subjects (n = 1288)	Oropharynx cancer	
	Seropositive, No. (%)†	(n = 321)*	OR (95% CI)‡	Seropositive, No. (%)†	(n = 198)	OR (95% CI)‡
HPV antibody	Unstratified analyses			Excluding HPV16 L1, E6, and E7 positives		
Antibodies to mucosal high-risk HPV types						
HPV18						
L1	50 (3.6)	21 (6.5)	2.32 (1.33 to 4.04)	32 (2.3)	9 (4.5)	2.37 (1.06 to 5.32)
E6	7 (0.5)	11 (3.4)	8.16 (2.81 to 23.66)	6 (0.4)	7 (3.5)	8.14 (2.21 to 29.99)
E7	5 (0.4)	8 (2.5)	9.31 (2.75 to 31.50)	3 (0.2)	2 (1.0)	4.81 (0.65 to 35.45)
HPV31						
L1	51 (3.7)	16 (5.0)	1.90 (1.03 to 3.51)	38 (2.7)	3 (1.5)	0.92 (0.27 to 3.12)
E6	17 (1.2)	7 (2.2)	1.71 (0.66 to 4.41)	16 (1.1)	2 (1.0)	0.67 (0.14 to 3.28)
E7	14 (1.0)	53 (16.5)	32.33 (16.93 to 61.74)	10 (0.7)	1 (0.5)	0.77 (0.09 to 6.57)
HPV33						
L1	37 (2.7)	19 (5.9)	2.73 (1.46 to 5.09)	26 (1.9)	8 (4.0)	1.97 (0.78 to 4.96)
E6	7 (0.5)	14 (4.4)	12.95 (4.90 to 34.28)	4 (0.3)	—	—
E7	21 (1.5)	62 (19.3)	26.48 (15.18 to 46.20)	15 (1.1)	2 (1.0)	0.62 (0.11 to 3.56)
HPV45						
L1	41 (2.9)	15 (4.7)	1.89 (0.99 to 3.61)	29 (2.1)	5 (2.5)	1.19 (0.42 to 3.40)
E6	11 (0.8)	7 (2.2)	3.50 (1.28 to 9.57)	10 (0.7)	2 (1.0)	1.79 (0.32 to 9.86)
E7	10 (0.7)	6 (1.9)	4.29 (1.47 to 12.50)	10 (0.7)	2 (1.0)	2.55 (0.53 to 12.27)

	Control subjects (n = 1395)*	Oropharynx cancer		Control subjects (n = 1288)	Oropharynx cancer	
	Seropositive, No. (%)†	(n = 321)*	OR (95% CI)‡	Seropositive, No. (%)†	(n = 198)	OR (95% CI)‡
HPV antibody	**Unstratified analyses**			**Excluding HPV16 L1, E6, and E7 positives**		
HPV52						
L1	33 (2.4)	15 (4.7)	2.81 (1.43 to 5.50)	20 (1.4)	4 (2.0)	1.94 (0.61 to 6.20)
E6	11 (0.8)	4 (1.2)	1.48 (0.41 to 5.31)	10 (0.7)	—	—
E7	25 (1.8)	28 (8.7)	7.79 (4.27 to 14.19)	20 (1.4)	4 (2.0)	2.11 (0.65 to 6.82)
Antibodies to mucosal low-risk HPV types						
HPV6						
L1	223 (16.0)	65 (20.2)	1.17 (0.84 to 1.63)	187 (13.4)	46 (23.2)	1.53 (1.02 to 2.30)
E6	10 (0.7)	1 (0.3)	0.36 (0.04 to 2.95)	9 (0.6)	—	—
E7	18 (1.3)	5 (1.6)	1.10 (0.38 to 3.22)	16 (1.1)	3 (1.5)	1.35 (0.34 to 5.39)
HPV11						
L1	72 (5.2)	30 (9.3)	1.85 (1.15 to 2.98)	22 (1.6)	2 (1.0)	1.83 (0.96 to 3.48)
E6	22 (1.6)	2 (0.6)	0.36 (0.08 to 1.71)	11 (0.8)	1 (0.5)	0.53 (0.10 to 2.86)
E7	12 (0.9)	4 (1.2)	1.33 (0.37 to 4.83)	198 (14.2)	25 (12.6)	0.29 (0.02 to 3.40)

* Thirty control subjects and three oropharyngeal case subjects were missing data on smoking pack years or frequency of alcohol consumption.
† Represents corresponding HPV type-specific seropositivity.
‡ Odds ratios (ORs) were adjusted for age, sex, level of education, smoking pack years, and number of alcoholic drinks consumed per day; corresponding seronegative group was considered the reference. CI = confidence interval.

Sensitivity Analysis

The observed results were insensitive to varying definitions of seropositivity, either as continuous data or after doubling of calculated thresholds (data not shown). This could be because of the high HPV16 antibody titers among oropharyngeal case subjects. The antibody titers for the majority of the HPV16 E6 seropositive control subjects (n = 11) and nonoropharyngeal case subjects (n = 21), on the other hand, were just above the cutoff. For example, upon doubling of the seropositivity threshold for HPV16 E6, although there was a marginal decrease in the proportion of seropositive control subjects (from 0.8% to 0.5%), the proportion of positive oropharyngeal cancer did not vary (30.2% to 29.6%). Consistently, the effect estimate remained robust (OR = 190.9, 95% CI = 82.85 to 440.0). Further, the important associations, including those of HPV16, HPV18, and HPV6, did not change substantially upon exclusion of phylogenetically related, homologous, and potentially cross-reacting proteins (Supplementary Table 4, available online).

Tumor Tissue Analysis

Because HPV16 antibodies formed the principal associations observed in this study and the results appeared to be driven by the oropharyngeal cancer, we prioritized a subset of 120 tumors for HPV genotyping and p16 expression to examine the agreement between HPV serology and cellular markers of HPV infection in the corresponding tumor. These included all HPV16 seropositives and all available oropharyngeal tumors. We identified 47 tumors that were positive for any HPV DNA (39.2%). Of these, 44 were positive for HPV16 (93.6%), two were positive for HPV31 (4.2%), six were positive for HPV33 (12.8%), two were positive for HPV35 positive (4.2%), and one was positive for low-risk HPV66 (2.1%) (Figure 2). Eight multiple infections involving HPV16 were identified, two involving HPV31 and six involving HPV33. Three non-HPV16-positive tumors were identified (two for HPV35 and one for HPV66). The largest proportion of HPV16 positives

were observed for oropharynx, followed by oral cavity, larynx, esophagus, and overlapping topologies (43%, 32%, 11%, 7%, and 7%, respectively; data not shown). An algorithm for detecting HPV-related head and neck cancer has been proposed that includes an initial test for p16 overexpression followed by HPV DNA detection. Only cases positive at both stages were judged to have an HPV-related tumor (7). In all, nine tumors overexpressed the p16 protein: seven were cancers of the oropharynx, one was a cancer of the larynx, and one was a cancer of the esophagus. Of the nine p16-positive tumors, six were concurrently positive for HPV16 DNA, indicative of an HPV-related tumor; all six were oropharyngeal cancers. Thirty-seven tumors were negative for p16 but positive for HPV DNA. Based on serology, ten of the 120 tumors tested were HPV16 E6 positive (all oropharyngeal case subjects), of which four were positive for HPV16 DNA and overexpressed p16 protein, three were HPV16 DNA-positive/p16-negative tumors, and three were negative for both HPV16 DNA and p16 (Figure 3). We observed a 67% agreement between HPV16 E6 serology and tumor measures of HPV infection, although it is important to mention that these results were based on small numbers. Seropositivity was observed in four of six HPV16 DNA-positive/p16-positive tumors (Figure 3; Supplementary Table 5, available online). Based on serology, 30.2% of oropharyngeal cancers were positive for antibodies to HPV16 E6. Based on HPV16 DNA presence and p16 overexpression, the HPV16-related fraction was 17% at the oropharynx; none of the cancers at other sites of UADT appeared to be HPV related (Table 4).

Table 4.
Human papillomavirus 16 (HPV16)–related upper aero-digestive tract (UADT) cancer fraction based on serology and tumor analyses

	HPV16 related	
	E6 serology*	Tumor markers†
Cancer site	Positive/total, No. (%)	Positive/total, No. (%)
UADT cancer	119/1496 (8.0)	6/120 (5)
Oropharynx	98/324 (30.2)	6/36 (17)
Oral cavity	4/366 (1.1)	0/42
Larynx‡	8/529 (1.5)	0/16
Esophagus	5/200 (2.6)	0/8

* Proportion of case subjects in each category positive for HPV16 E6 antibodies.
† Indicates HPV16 DNA-positive and p16-overexpressing tumors by total tumors in each category.
‡ Includes larynx and hypopharynx case subjects.

Discussion

In this large case–control study, we examined the associations between 27 serological markers of mucosal HPV infection and the risk of UADT cancer. Among the various HPV types assessed, strong associations were observed between HPV16 antibodies and oropharyngeal cancer. The agreement between HPV16 E6 seropositivity and tumor markers of HPV infection was 67% in this series (four of six HPV DNA-positive/p16-overexpressing tumors were HPV16 E6 antibody positive). Additionally, we found associations for HPV18 antibodies and oropharyngeal cancer and HPV6 and laryngeal cancer.

HPV16 L1 antibodies are considered markers of previous exposure (31,32). In interpreting the association between L1 antibodies and cancer, it is important to note that capsid seropositivity represents a mixed group of current and past infections in a subset of individuals who seroconvert. It is interesting that even so, such antibodies are consistently associated with oropharyngeal cancer (4,8,15,16). It can be argued that the presence of capsid antibodies may reflect systemic exposure from any mucosal HPV infection. This would appear unlikely given we observed consistent associations across sex and included first primary cancers of the UADT. Higher risk estimates

among never smokers support the notion that HPV16 E6 antibody-positive and smoking-related cancers of the UADT follow distinct etiologies (9). HPV16 E6 and E7 antibodies are regarded markers of current HPV-related malignancy, and low E6 and E7 antibody prevalence in the general control population in previous studies support this view (4,8,15,16). Consistently, HPV16 E6 seroprevalence was rare (0.8%) in this study, whereas HPV16 E7 was more common (4.6%), possibly because of assay limitations. HPV16 E6-seropositive UADT case subjects were more likely to be men, never smokers, and light drinkers (data not shown), consistent with the previously described risk profile (9). Contrarily, antibodies to E1, E2, and E4 are less well understood. E1 and E2 proteins are expressed in episomal viral infection and often disrupted during viral integration into the host genome (33). In this study, such antibodies were associated with oropharyngeal cancer risk. These results confirm findings from a previous study involving 40 oropharynx cancers and 50 cancer-free control subjects (34). This analysis included both squamous and nonsquamous histologies. Because these cancers are likely to vary based on etiology, stratified analyses were performed. Although the non-HPV16 associations were principally driven by squamous cell cancers, presence of HPV16 antibodies were associated with increased risk of both squamous and nonsquamous oropharyngeal cancer (Supplementary Table 6, available online). It is important to note that the number of nonsquamous oropharyngeal cancer case subjects were limited (n = 22). Although the presence of an undiagnosed current or prior cervical neoplasia among the female oropharyngeal case subjects cannot be ruled out, this is unlikely to explain the strong associations observed in this study. Future studies that determine HPV status in cervico-vaginal samples are warranted.

Several studies have examined the association between HPV16 and esophageal cancer; however the results have been inconsistent (35,36). We observed inconsistent associations based on HPV16 serology (data not shown). Even though three tumors were HPV16 DNA positive, p16 overexpression was not observed, possibly indicating inactive infections. Hence, we conclude that although HPV16 infections are common (37.5%), any contribution to esophageal squamous carcinoma is unlikely. The observed site-specific differences in the association of HPV16 markers could reflect differences in viral load, viral states (episomal or integrated), site-specific immune differences, or true noncausal associations. Even though HPV16 DNA was observed in nonoropharyngeal sites of UADT, none of the tumors overexpressed p16 protein. Further, among HPV16 DNA-positive tumors, the proportion of HPV16E seropositivity (E1, E2, E4, E6, and E7) was lower for nonoropharyngeal cancers than for cancers of the oropharynx, although the proportion of L1 seropositivity did not differ, indicating that. although infection rates at heterogeneous UADT sites are likely to be similar, HPV16-related cancer rates are lower for nonoropharyngeal sites. These conclusions are supported by serological associations for HPV16 that appear to principally driven by oropharyngeal cancer.

Among other mucosal HPV antibodies examined, we observed strong associations for HPV18, particularly L1 and E6 antibodies, and oropharyngeal cancer risk that were robust upon several sensitivity analyses. Because HPV18 DNA was not identified and the seropositive oropharyngeal cancer fraction was small (7%), we conclude that the contribution of HPV18 to oropharyngeal cancer is likely to be marginal. We did not observe an HPV16-independent association for HPV31, HPV33, HPV45, and HPV52 antibodies and oropharyngeal cancer. We did not identify any HPV45 or HPV52 DNA. Given the low seroprevalence of these markers (1% for HPV45 E6, and E7 and 2% for HPV52), the contribution of these types to oropharyngeal cancer, at least in Europe, is likely to be small. It is possible that these types may be more important in other populations with higher infection prevalence, such as observed for HPV52 among Asian women (31). Serological analysis indicated a sevenfold increased risk of UADT cancer with HPV31 E7, albeit in the presence of HPV16. This is supported by tumor analyses where we found that both of the HPV31 DNA-positive tumors were concomitantly HPV16 positive. Even so, the correlation between HPV31 E7 serology and HPV31 positive tumor was moderate (50%; data not shown). Similarly, all six HPV33 DNA-positive tumors were concurrently positive for HPV16 as indicated by serology. Again, the type-

specific correlation of HPV33 E6 and E7 antibodies and HPV33 DNA was poor (17% and 29%, respectively). Given that serology supports a role of 5% of HPV31 and almost 6% of HPV33 in oropharyngeal cancer, the contribution of these types warrants further investigation. The causal association, if any, will be difficult to disentangle given the concurrence with HPV16. Low-risk mucosal HPV types such as HPV6 and HPV11 are associated with benign laryngeal papillomas, a rare disease that occasionally undergoes malignant transformation (37). In this study, HPV6 L1 and E7 were associated with laryngeal cancer independent of HPV16 serology. The proportion of HPV6-related cancers (E6 laryngeal seropositivity = 3.5%), although small, is consistent with previous estimates, indicating larger focused studies with detailed tumor analyses will be required to clarify the rare causal contribution of HPV6 to laryngeal cancer. It is important to mention that the tumor subset was prioritized to examine the serological associations of HPV16 and oropharyngeal cancer and the results for non-HPV16 types and nonoropharyngeal cancer sites should be interpreted with caution.

In this study, nearly 94% of all HPV-positive tumors were positive for HPV16 DNA, consistent with earlier reports (4,10,38,39). Other types found were HPV31, HVP33, HPV35, and HPV66, together contributing a total of 23%, higher than previous estimates (5,13). Presence of HPV16 DNA, although necessary, is not sufficient to establish causality because it includes a subset of transient infections. In this study, we found 37 HPV DNA-positive/p16-negative tumors, indicating that the majority of HPV16 infections (84%) may be inactive. Interestingly, more than 60% of these were smokers (all oropharyngeal case subjects), suggestive of a smoking-related etiology. Viral transcription and the production of viral oncoprotein E7 leads to the upregulation of p16 by the retinoblastoma pathway (33). A previous study found 100% sensitivity for p16 as a surrogate marker to identify HPV16-related cancers of the head and neck (7). It can be argued that HPV-independent mechanisms could result in the upregulation of p16. We found three p16-positive and HPV16 DNA-negative tumors (2.5%), lower than the previous estimates (13). It is also plausible that infection by non-HPV16 types could upregulate p16. In this study, we did not observe p16 overexpression among any of the HPV16-negative tumors, although three of the six HPV16-related tumors were concomitantly positive for HPV31 or HPV33. Although HPV16 DNA-positive/p16-negative tumors have been described as HPV-unrelated tumors, the true etiological involvement of HPV remains to be demonstrated. Future studies testing multiple markers of HPV activity will be required to address this. Based on serology, we found that nearly 30% of oropharyngeal cancers were HPV16 E6 positive. These results are highly consistent with the recent report on the global burden of cancer attributable to infections, where the estimated HPV-related oropharyngeal cancer fraction in Europe was between 17% and 39% (40). HPV16 E6 serology identified 67% of HPV-related cancers (four of six HPV16 DNA-positive/p16-overexpressing tumors), higher than previously published, possibly because of higher seropositivity thresholds (7). Inclusion of HPV16 E2 or E4 markers further increased the detection from 67% to 83%, warranting further investigation on the panel of serological markers that can accurately identify HPV-driven cancers. In light of the high specificity of HPV16 E6 (0.1% among controls), the strong oropharyngeal-specific association observed in this study, and the promising sensitivity to identify HPV-related oropharyngeal cancer (though based on small numbers; 4 of 6 HPV16 DNA-positive/p16-overexpressing tumors of 120 tumors tested), and based on additional data from other groups indicating improved prognosis among HPV16 E6 antibody-positive case subjects (41,42), further studies are needed to evaluate HPV16 serology as a biomarker for HPV16-related oropharyngeal cancer.

Our study has several limitations. First, the reported significance levels were not adjusted for multiple testing. However, even under the assumption of complete independence, important associations (particularly HPV16, HPV18, and HPV6) were robust. Second, reverse causality is a concern in the interpretation of these results given that blood samples were drawn at diagnosis. However, our results are concordant with the prospective case–control study that found twofold increased head and neck cancer risk with HPV16 capsid seropositivity (43). Third, we restricted our analyses to the alpha papillomavirus family; although this could potentially underestimate the role

of HPV in UADT cancer, literature indicates that these constitute known carcinogenic types. Our study has several strengths. The study was large enough to allow examination of site-specific associations of HPV infection. The results of the sero-epidemiologic study were examined in a tumor subset that reflected an agreement of 67% between serology and presence of a HPV16-related tumor (four of six HPV DNA-positive/p16-overexpressing tumors were HPV16 E6 antibody positive). Additionally, our results were robust upon various sensitivity analyses.

In conclusion, the majority of the HPV16 infections in the UADT appear to be inactive. HPV16 E6 antibodies are promising markers to identify HPV16 DNA-positive/p16-overexpressing tumors, indicating that at least 30% of oropharyngeal cancers are HPV16 related. Larger focused studies will be required to clarify the appropriate algorithm to accurately identify HPV-related UADT cancers. Given the increasing proportion of oropharyngeal cancers in Europe, the value of HPV16 E6 serology warrants further investigation. It will be important to determine when in the course of a malignancy antibodies to HPV16 E6 develop. Do such antibodies precede a clinically diagnosable disease? Large cohort studies will be required to address some of these questions.

Funding

The ARCAGE study was supported by the grant from European Commission's 5th Framework Program (contract QLK1-2001-00182). This project was partly funded by the Health General Directorate of the French Social Affairs and Health Ministry. 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).

Notes

None of the funding agencies participated at any stage in the designing of the study, collection, analysis, interpretation of the data; drafting of the manuscript and the decision to submit the manuscript for publication.

We would like to thank all the patients and their families for their participation. We are also grateful to the support of many clinicians and staff of the hospitals, interviewers, data managers, pathology departments, and primary care clinics. Gary J. Macfarlane and Tatiana V. Macfarlane partly worked on ARCAGE study while at the University of Manchester. We acknowledge the help of Professor Martin Tickle in study conducted in the Manchester center and Professor Phil Sloan who in addition coordinated sample collection and processing for all the UK centers.

References

1. Ferlay J , Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer . 2010;127(12):2893–2917.
2. Bouvard V, Baan R, Straif K, WHO International Agency for Research on Cancer Monograph Working Group. Lancet Oncol . 2009;10(4);321–322 .
3. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer . 2011;11(1):9–22.
4. Herrero R, Castellsague X, Pawlita M, et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. J Natl Cancer Inst . 2003;95(23):1772–1783.
5. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiology, Biomarkers & Prevention . 2005;14(2):467–475.
6. Roden R, Wu TC. How will HPV vaccines affect cervical cancer? Nat Rev Cancer . 2006;6(10):753–763.
7. Smeets SJ , Hesselink AT, Speel EJ, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer . 2007;121(11):2465–2472.
8. D'Souza G, Kreimer AR, Viscidi R, et al. Case–control study of human papillomavirus and oropharyngeal cancer. N Engl J Med . 2007;356(19):1944–1956.
9. Gillison ML, D'Souza G, Westra W, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst . 2008;100(6):407–420.
10. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal associa - tion between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst . 2000;92(9):709–720.
11. Jung AC, Briolat J, Millon R, et al. Biological and clinical relevance of tran - scriptionally active human papillomavirus (HPV) infection in oropharynx squamous cell carcinoma. Int J Cancer . 2010;126(8):1882–1894.
12. Smith EM, Ritchie JM, Summersgill KF, et al. Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. Int J Cancer . 2004;108(5):766–772.
13. Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. Lancet Oncol . 2010;11(8):781–789.
14. Zumbach K, Hoffmann M, Kahn T, et al. Antibodies against oncoproteins E6 and E7 of human papillomavirus types 16 and 18 in patients with head- and-neck squamous-cell carcinoma. Int J Cancer . 2000;85(6):815–818.
15. Smith EM, Ritchie JM, Pawlita M, et al. Human papillomavi - rus seropositivity and risks of head and neck cancer. Int J Cancer . 2007;120(4):825–832.
16. Ribeiro KB, Levi JE, Pawlita M, et al. Low human papillomavirus preva - lence in head and neck cancer: results from two large case-control studies in high-incidence regions. Int J Epidemiol . 2011;40(2):489–502.
17. Lagiou P , Georgila C, Minaki P, et al. Alcohol-related cancers and genetic susceptibility in Europe: the ARCAGE project: study samples and data col - lection. Eur J Cancer Prev . 2009;18(1):76–84.
18. Lee YC, Marron M, Benhamou S, et al. Active and involuntary tobacco smoking and upper aerodigestive tract cancer risks in a multicenter case– control study. Cancer Epidemiol Biomarkers Prev . 2009;18(12):3353–3361.
19. W aterboer T, Sehr P, Michael KM, et al. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. Clin Chem . 2005;51(10):1845–1853.

20. Clifford GM, Shin HR, Oh JK, et al. Serologic response to oncogenic human papillomavirus types in male and female university students in Busan, South Korea. Cancer Epidemiol Biomarkers Prev . 2007;16(9):1874–1879.
21. Michael KM, Waterboer T, Sehr P, et al. Seroprevalence of 34 human papillomavirus types in the German general population. PLoS Pathog . 2008;4(6):e1000091.
22. Koo CL, Kok LF, Lee MY, et al. Scoring mechanisms of p16INK4a immu - nohistochemistry based on either independent nucleic stain or mixed cytoplasmic with nucleic expression can significantly signal to distinguish between endocervical and endometrial adenocarcinomas in a tissue micro - array study. J Transl Med . 2009;7(1):25.
23. Singhi AD, Westra WH. Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. Cancer . 2010;116(9):2166–2173.
24. W einberger PM, Yu Z, Haffty BG, et al. Molecular classification identifies a subset of human papillomavirus—associated oropharyngeal cancers with favorable prognosis. J Clin Oncol . 2006;24(5):736–747.
25. Gheit T, Landi S, Gemignani F, et al. Development of a sensitive and specific assay combining multiplex PCR and DNA microarray primer extension to detect high-risk mucosal human papillomavirus types. J Clin Microbiol . 2006;44(6):2025–2031.
26. Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T. Bead-based multiplex genotyping of human papillomaviruses. J Clin Microbiol . 2006;44(2):504–512.
27. Schmitt M, Dondog B, Waterboer T, Pawlita M, Tommasino M, Gheit T. Abundance of multiple high-risk human papillomavirus (HPV) infections found in cervical cells analyzed by use of an ultrasensitive HPV genotyping assay. J Clin Microbiol . 2010;48(1):143–149.
28. Pannone G, Santoro A, Papagerakis S, Lo Muzio L, De Rosa G, Bufo P. The role of human papillomavirus in the pathogenesis of head & neck squamous cell carcinoma: an overview. Infect Agent Cancer . 2011;6:4.
29. Robinson M, Sloan P, Shaw R. Refining the diagnosis of oropharyngeal squamous cell carcinoma using human papillomavirus testing. Oral Oncol . 2010;46(7):492–496.
30. Schache AG, Liloglou T, Risk JM, et al. Evaluation of human papil - loma virus diagnostic testing in oropharyngeal squamous cell carcinoma: sensitivity, specificity, and prognostic discrimination. Clin Cancer Res . 2011;17(19):6262–6271.
31. Clifford GM, Gallus S, Herrero R, et al. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: a pooled analy - sis. Lancet . 2005;366(9490):991–998.
32. Dillner J. The serological response to papillomaviruses. Semin Cancer Biol . 1999;9(6):423–430.
33. Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer . 2010;10(8):550–560.
34. Anderson KS, Wong J, D'Souza G, et al. Serum antibodies to the HPV16 proteome as biomarkers for head and neck cancer. Br J Cancer . 2011;104(12):1896–1905.
35. Sitas F , Egger S, Urban MI, et al. InterSCOPE study: associations between esophageal squamous cell carcinoma and human papillomavirus serologi - cal markers. J Natl Cancer Inst . 2012;104(2):147–158. 36. Syrjanen KJ . HPV infections and oesophageal cancer. J Clin Pathol . 2002;55(10):721–728.
37. Herrero R. Chapter 7: Human papillomavirus and cancer of the upper aerodigestive tract. J Natl Cancer Inst Monogr . 2003(31):47–51.
38. Schwartz SM, Daling JR, Doody DR, et al. Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection. J Natl Cancer Inst . 1998;90(21):1626–1636
39. Smith EM, Hoffman HT, Summersgill KS, Kirchner HL, Turek LP, Haugen TH. Human papillomavirus and risk of oral cancer. Laryngoscope . 1998;108(7):1098–1103.
40. de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attrib - utable to infections in 2008: a review and synthetic analysis. Lancet Oncol . 2012;13(6):607–615.
41. Rotnaglova E, Tachezy R, Salakova M, et al. HPV involvement in tonsil - lar cancer: prognostic significance and clinically relevant markers. Int J Cancer . 2011;129(1):101–110.
42. Smith EM, Pawlita M, Rubenstein LM, Haugen TH, Hamsikova E, Turek LP. Risk factors and survival by HPV-16 E6 and E7 antibody sta - tus in human papillomavirus positive head and neck cancer. Int J Cancer . 2010;127(1):111–117.
43. Mork J , Lie AK, Glattre E, et al. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N EnglJ Med . 2001;344(15):1125–1131