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No Causal Association Identified for Human Papillomavirus Infections in Lung Cancer

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Abstract

Human papillomavirus (HPV) infections have been implicated in lung carcinogenesis, but causal associations remain uncertain. We evaluated a potential causal role for HPV infections in lung cancer through an analysis involving serology, tumor DNA, RNA, and p16 protein expression. Association between type-specific HPV antibodies and risk of lung cancer was examined among 3,083 cases and 4,328 controls in two case–control studies (retrospective) and one nested case–control study (prospective design). Three hundred and thirty-four available tumors were subjected to pathologic evaluation and subsequent HPV genotyping following stringent conditions to detect all high-risk and two low-risk HPV types. All HPV DNA-positive tumors were further tested for the expression of p16 protein and type-specific HPV mRNA. On the basis of the consistency of the results, although HPV11 and HPV31 E6 antibodies were associated with lung cancer risk in the retrospective study, no association was observed in the prospective design. Presence of type-specific antibodies correlated poorly with the presence of the corresponding HPV DNA in the tumor. Although nearly 10% of the lung tumors were positive for any HPV DNA (7% for HPV16 DNA), none expressed the viral oncogenes. No association was observed between HPV antibodies or DNA and lung cancer survival. In conclusion, we found no supportive evidence for the hypothesized causal association between HPV infections and lung cancer. *Cancer Res; 74(13); 3525–34. ©2014 AACR.*

Introduction

Lung cancer accounts for over a million deaths worldwide each year (1). Although smoking is the most important risk factor, lung cancer also occurs in never smokers with particularly high rates reported among women in south Asia (2, 3). Among other known risk factors, a role for human papillomavirus (HPV) infections has been postulated (4).

HPV infections cause almost all cervical cancers, nearly half of anogenital cancers, and a substantial proportion of oropharyngeal cancers (5). Evidence for the association between HPV

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and lung cancer comes from more than 50 case studies that have examined the presence of HPV DNA in lung tumor tissues. A recent meta-analysis estimated that nearly 7% [95% confidence interval (CI), 4.7–10.6] of lung cancer is HPV16 associated and almost 6% (95% CI, 3.5–9.0) is HPV18 associated (6). Interpreting these findings is difficult due to the variation in HPV prevalence, both across and within geographic regions. A more recent study did not observe any HPV DNA in nearly 400 lung tumors examined (7). A recent Taiwanese study, however, associated HPV DNA presence with improved lung cancer survival. This study, based on nearly 200 cases, focused on lung adenocarcinoma and oversampled women (8).

That HPV can reach the lung is well established through the occurrence of respiratory papillomas (9). This rare benign condition, caused by HPV6 and HPV11, occasionally transforms to frank malignancies. Known risk factors for HPV transmission such as lifetime number of sexual partners and oral-genital sexual contact are also associated with adult recurrent respiratory papillomatosis (10, 11). One of the challenges in the hypothesis of HPV as a lung carcinogen lies in establishing the functional consequence of the viral presence in the tumor. Though experimental studies have demonstrated that HPV oncoproteins E6 and E7 can cooperate with tobacco smoke in transforming lung epithelial cells *in vitro*, and can promote tumor angiogenesis both *in vitro* and *in vivo* (12, 13), limited direct evidence exists. The few studies on the expression of HPV oncogenes in lung tumors have been inconclusive (14–16). These limitations led the International Agency for Research on Cancer (IARC; Lyon, France) Monograph expert group to conclude that there was limited evidence for the carcinogenicity of HPV to the lung. The expert group also argued that the exclusively case-only design, limited sample size (typically <100 cases), assay limitations, and lack of comprehensive characterization using multiple methods were important limitations in the interpretation of the association between HPV infection and lung cancer (4).

To conclusively evaluate the causal role of HPV infection in lung cancer, we have (i) examined the association between type-specific HPV antibodies and lung cancer risk in multiple series of lung cancer cases and controls; (ii) tested lung tumors for the presence of HPV genomes of all high-risk types and two low-risk types using rigorous precautions against potential contamination; (iii) assessed the expression of p16 protein; and (iv) examined the transcriptional state of the HPV oncogene E6.

Materials and Methods

Study population

This analysis included three large lung cancer studies; the Central Europe and the L2 study (retrospective design), and the European Prospective Investigation into Cancer and Nutrition (EPIC) study (prospective design). The Central Europe study was conducted during 1998–2002 across six central European countries. Information on demographics, lifestyle factors, and occupational exposures were recorded upon personal interview using standardized questionnaires (17). The present analysis included 2,035 controls and 1,286 case subjects with an available plasma sample. The L2 study was conducted in Moscow during 2007 and 2010, and similar to the L1 study, lifestyle and exposure data were recorded on personal interview. The present analysis included 348 cases and 694 controls who donated a blood sample. Briefly, both the retrospective studies recruited histologically or cytologically confirmed incident lung cancer cases. Cases were hospitalized subjects (recruited mostly within 3 months of diagnosis), identified through active search of diagnostic departments of the participating hospitals who reported to have resided in the area of recruitment for at least a year before diagnosis. Corresponding hospital-based controls, none of whom reported a previous cancer history or any tobacco-related disease, were frequency matched to cases based on age, gender, and area of residence. Both retrospective studies defined smokers as individuals who smoked at least 100 cigarettes in their lifetime. Details of the ongoing EPIC study are published elsewhere (18). Briefly lifestyle, dietary, and anthropometric data were recorded from 519,978 subjects ages 25 to 70 years during 1992–2000 from 23 centers across 10 countries in Europe. Smokers were defined as those who responded to have ever smoked a cigarette or cigar or pipe in their lifetime. Follow-up and identification of incident cancer were based on record linkage and active follow-up through study participants and their next of kin. In this analysis, we excluded all prevalent cancers (other than nonmelanoma skin cancers), secondary lung cancer cases, and subjects lost to follow-up. HPV serology data were available among a similar group of cancer-free cohort members as a part of a parallel head and neck cancer study (19). Even though controls in the nested case–control study were matched to head and neck cancer cases 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), they were processed and analyzed at the same time as the lung cases and under identical conditions. A total of 1,449 lung cancer cases and 1,599 controls were available from the EPIC study. All three studies were approved by the IARC Ethics Committee and local ethical review boards of the participating centers.

HPV serology

The three high-risk (HPV16, HPV18, and HPV31) and two low-risk (HPV6 and HPV11) types tested in this study were selected on the basis of preexisting evidence of their association with benign conditions and frank malignancies of the respiratory epithelium. In addition, antibodies against cellular proteins, non-HPV viral proteins, and cutaneous HPV-types were tested as specificity controls. In total, plasma samples from 4,328 controls and 3,083 lung cancer cases were tested using the bead-based multiplex serology method described elsewhere (20). Serology data were dichotomized on the basis of study-specific cutoffs derived using a set of known reference sera (21, 22). Thresholds derived for the prospective study, which were higher in general, were applied to the retrospective studies to allow for comparisons. Serology testing was performed blind to case–control status.

Tumor analyses

Tissue processing. Tumor tissues were available only for the retrospective studies. Of the 334 tumors available, 290 tumors passed pathologic evaluation performed in-blind to serology results. The reasons for pathologic exclusion included tissue size (too small to allow for complete testing, $n = 10$), lack of confirmation of lung origin of the tumor ($n = 6$), insufficient tumor cell content ($n = 15$), presence of nontumor lung tissue such as reactive lymph nodes ($n = 7$), fibroconnective tissue ($n = 2$), necrotic tissue ($n = 2$), or inflammation ($n = 2$). To avoid potential contamination, tumors were processed in small batches with frequent change of gloves and cleaning of the worktable using DNA Away (Molecular BioProducts). Furthermore, a negative control for tumor sectioning included processing one blank block every 10 tumor blocks.

HPV genotyping. DNA was extracted from snap-frozen ($n = 139$) tumor tissues using the Qiagen BioRobot EZ1 with the EZ1 DNA tissue kit according to the manufacturer's instructions (Qiagen), while crude DNA extraction method was used for formalin-fixed paraffin-embedded (FFPE) tumor tissues ($n = 151$). Briefly, FFPE tissues were incubated in proteinase K and buffer G2 (Qiagen) at 56°C until the tissue was completely lysed and crude cell extract containing nucleic acids was used for HPV genotyping. To ensure DNA quality, β-actin gene primers were included as positive control; all 290 tumors were β-globin positive. One extraction control was included every 10 tumors to monitor potential cross-contamination. In addition, negative controls for the PCR assay (1 per 95 samples) and luminex beads (1 per batch) were also included. HPV genotyping was performed under strict pre- and post-PCR separation conditions using the Type-Specific E7 PCR-MPG Assay (TS-E7-MPG) that detects 19 high-risk (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a, 68b, 70, 73, 82) and two low-risk types (HPV6, 11; ref. 23). Results were dichotomized by applying cutoffs calculated as the median background value plus five SDs (24). We adopted a stringent definition to call a HPV DNA-positive tumor based on two rounds of HPV genotyping. In the first genotyping round, all cases that passed the independent pathologic evaluation were tested ($n = 290$). Subsequently, to confirm the initial findings and reduce potential false positivity, all tumors that tested HPV DNA positive in the first genotyping round were subjected to a second round of tissue preparation and genotyping. In addition, 16 HPV DNA negatives were included as additional controls. Only cases positive for the same HPV-type in both rounds were considered true HPV DNA positives.

Expression of p16 protein. The expression of p16 protein was qualitatively evaluated among all cases subjected to the second genotyping round. These included tumors that tested positive for any HPV DNA at least once and an additional 16 HPV DNA negatives. Immunohistochemical staining was performed using the CINtec Histology $p16^{INK4a}$ Kit (9511, mtm laboratories) following manufacturer's instructions. Expression was scored using a composite score based on the percentage of stained cells and the intensity of the nucleic or cytoplasmic staining. A score of 4 or lesser was considered low to moderate expression of p16 protein.

Expression of HPV oncogenes. Two assays for the detection of HPV-E6 mRNA were performed that differed in the HPV types tested and their sensitivity. The two assays were performed in independent laboratories and blind to HPV DNA status. Because HPV16 has been reported as the most important noncervical oncogenic HPV type, we performed a dedicated assay to detect the most abundant splice variant (E6*I; ref. 25). This assay included all 19 HPV16 DNA-positive tumors alongside seven HPV16 DNA negatives. The second assay utilized a recently developed method to detect HPV type-specific transcripts of the E6 gene (26). This assay was customized to detect transcripts of all HPV types identified in this study (details provided upon request). In the second assay, 34 tumors were tested in total that included 28 HPV DNA positives (14 single HPV16 infections, nine single infections of non-HPV16 type, five multiple infections including HPV16) and six HPV DNA negatives, which also included all 26 tumors from the first assay.

Statistical analysis

The association between HPV antibodies and the risk of lung cancer was examined by calculating the ORs and corresponding 95% CI. Unconditional logistic regression models were adjusted for age, sex, smoking status (never, former, or current), and country. Further stratified analyses were performed by sex and smoking status (ever, never) to examine any potential effect modification. We used χ^2 tests to examine the heterogeneity in OR by study design. The correlation between HPV DNA presence and seropositivity to the corresponding type-specific antibodies, and expression of p16 as a surrogate marker was examined by linear regression, coefficients of correlation and P values at 95% CI are reported. Because HPV positivity has been associated with improved survival in some nongenital cancers such as the oropharynx (19), we tested the association between HPV markers and all-cause mortality among patients diagnosed with lung cancer. Multivariate Cox regression models were used using years since diagnosis as the time variable. HRs were adjusted for age at lung cancer diagnosis, sex, country, and smoking status. Statistical significance was set at $P < 0.05$, and all reported P values are two sided. All statistical analyses were performed using STATA statistical software, version 11 (StataCorp).

Results

The retrospective Central Europe study contributed the largest number of participants ($n = 3{,}321$), followed by the EPIC nested case–control study ($n = 3{,}048$). Lung cancer cases were more often men and compared with controls, were more likely to complete lower levels of education, and ever smoke (Table 1). A schematic representation of the tests performed in the retrospective studies is represented in Fig. 1.

HPV serology and lung cancer associations

Retrospective studies. Combined results of the retrospective studies are presented (Table 2). Because early antibodies are considered markers of HPV-related malignancy, they are

Table 1. Description of study group

	Retrospective studies				Prospective study	
	Central Europe study		L2 study		EPIC study	
Description	**Controls (%)**	**Cases (%)**	**Controls (%)**	**Cases (%)**	**Controls (%)**	**Cases (%)**
Study design	Retrospective case–control				Nested case–control	
Recruitment period	1998–2002		2007–2010		1992–2000	
Sample size	2,035	1,286	694	348	1,599	1,449
Demographic and lifestyle factors						
Age, y						
Mean (SD)	59.6 (9.7)	60.4 (8.5)	53.0 (14.6)	62.1 (9.5)	56.7 (7.8)	58.9 (7.6)
Median	61	60	56	62	57	59
Sex[a]						
Men	1,463 (72)	981 (76)	500 (72)	252 (72)	1,105 (69)	819 (57)
Women	572 (28)	305 (24)	194 (28)	96 (28)	494 (31)	630 (43)
Level of education[a]						
Primary school	505 (25)	218 (17)	42 (6)	27 (8)	643 (40)	748 (52)
Secondary school	1,389 (68)	992 (77)	237 (34)	116 (33)	604 (38)	511 (35)
University or higher	138 (7)	71 (6)	414 (60)	203 (59)	302 (19)	149 (10)
Smoking status[a]						
Never smokers	708 (35)	98 (8)	339 (49)	116 (33)	631 (39)	206 (14)
Former smokers	515 (25)	244 (19)	123 (18)	115 (33)	553 (35)	345 (24)
Current smokers	811 (40)	943 (73)	232 (33)	117 (33)	385 (24)	882 (61)
Histology[a]						
Adenocarcinoma	—	269 (21)	—	106 (31)	—	467 (32)
Squamous	—	550 (43)	—	135 (39)	—	303 (21)
Small	—	182 (14)	—	12 (3)	—	187 (13)
Large	—	24 (2)	—	0	—	63 (4)
Other	—	261 (20)	—	40 (11)	—	122 (9)
Unknown	—	0	—	55 (16)	—	85 (6)
Tumor subset and markers of HPV infection						
Pathology evaluation	—	180 (14)	—	—	—	154 (44)
HPV serology[b]	2,035 (100)	1,286 (100)	694 (100)	347 (100)	1,599 (100)	1,449 (100)
HPV genotyping[c]	—	139 (11)	—	151 (44)	—	—
p16 expression[d]	—	12 (1)	—	52 (15)	—	—
HPV mRNA analysis[e]	—	—	—	34 (7)	—	—

[a]Other category includes mixed and unclassified histologies. Numbers do not add up due to missing values; 222 cases were missing histology data in the prospective EPIC study.
[b]Population characteristic of the type-specific HPV serology subset is the same as described above.
[c]HPV genotyping performed using the E7 MPG assay among all cases that passed pathology evaluation.
[d]On the basis of immunostaining among a subset of tumors that included all HPV positives.
[e]Performed using two independent assays.

expected to be rare. Consistent with this notion, the prevalence of antibodies to early proteins (E6 and E7) was lower among controls compared with cases. When were grouped based on their carcinogenic potential into any high risk, any low risk, or any HPV categories, seropositivity to most early and late antibodies was associated with increased risk of lung cancer. Furthermore, some type-specific antibodies were consistently associated with lung cancer, including HPV16-L1, HPV16-E7, HPV18-L1, HPV18-E7, HPV31-E6, HPV6-L1, HPV11-L1, -E6, and -E7. We observed directional consistency in the risk estimates of capsid and early protein antibodies by HPV type (Table 2). Irrespective of whether study-specific thresholds, higher cutoff of the two studies, or twice the highest threshold were applied, the association between type-specific antibodies and the lung cancer risk remained consistent (Supplementary Table S1). We observed no effect modification by smoking status, except for HPV31-E7 where a stronger association was observed among never smokers (OR = 6.59; 95% CI, 2.59–16.77; Supplementary Table S2).

Prospective study. None of the 15 candidate antibody markers tested was significantly associated with lung cancer risk in the prospective design. Importantly, the lack of

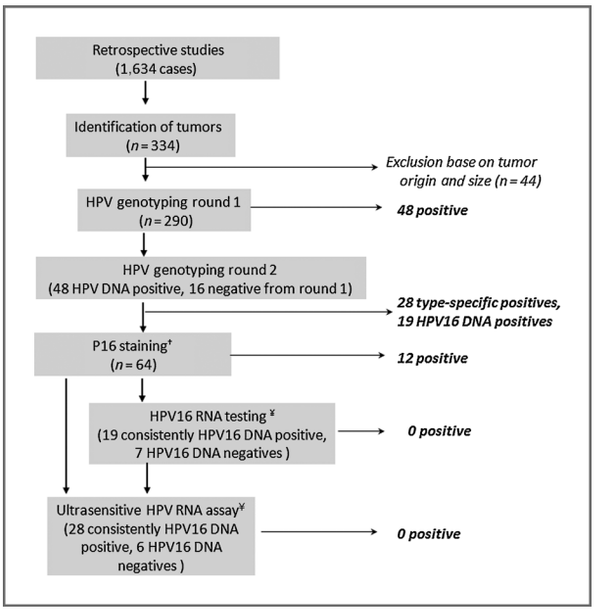


Figure 1. Overall schematic for HPV testing in lung cancers. †, same cases as for HPV genotyping round 2 were included for p16 testing. Positives indicate p16-expressing tumors. Of the 12 p16 positives, two were HPV16 DNA positive, two HPV11 positive, one HPV16, and 31 DNA positive and one HPV11 and HPV16 positive, and six HPV DNA negatives. ¥, included all 19 HPV16 DNA-positive tumors and randomly selected HPV 16 DNA negatives. ‡, included all 28 HPV DNA positives and six HPV DNA negatives

association between HPV16 E6 and HPV18 E6 antibodies and lung cancer risk was also replicated (Table 2).

Tumor markers of HPV infection

Performance of quality indicators. Tumor testing was accompanied by inclusion of negative and positive controls at each step of the multiple assays performed. These included negative controls for (i) sectioning where blank blocks were processed 1 in every 10 tumor blocks, (ii) DNA extraction that included 1 tube per batch containing buffer alone, (iii) PCR that consisted of PCR buffers and primers only, 1 per 96 samples, and (iv) luminex that included testing of 1 sample containing luminex beads alone. Together, these comprised 42 controls in the first genotyping round. Of these, two (of 12) DNA extraction controls were HPV DNA positive and two (of 22) sectioning controls were HPV DNA positive, indicating potential contamination in the respective sample processing steps. In the second genotyping round that involved repetition of all the sample preparation steps, none of the 34 controls incorporated were positive for any HPV DNA. In addition, all tumor samples were positive for the β-globin gene and ubiquitin expression as positive controls for DNA and RNA quality, respectively.

On the basis of the previously described definition of a HPV DNA-positive tumor, we identified 28 positives (9.7%), 19 of which were positive for HPV16 DNA (6.6%) and 9 were positive for any non-HPV16 type (3.1%). Non-HPV16 infections included single infections of HPV11 ($n = 6$), HPV51 ($n = 1$), and HPV58 ($n = 2$). Twenty-three of 28 HPV DNA-positive tumors were positive for a single HPV type ($n = 23$), all multiple infections were double infections involving HPV16 (Table 3). In general, the presence of HPV DNA correlated poorly with the presence of corresponding type-specific antibodies (Table 4). Of the 19 HPV16 DNA-positive tumors, none were positive for HPV16 E6 antibodies, whereas two HPV16 DNA negatives were HPV16 E6 seropositive. Similarly, none of the seven HPV11 DNA-positive tumors were HPV11 E6 seropositive, whereas two HPV11 DNA-negative tumors were HPV11 E6 seropositive. Sixty-four tumors were tested for p16 protein expression, 12 expressed low to medium levels (19%). Of these, four (1.4%) were also positive for HPV16 DNA. Among the 52 tumors negative of p16 expression, 21 were HPV DNA positive (14 HPV16 DNA positive). Expression of p16 protein was also observed among eight HPV16 DNA-negative tumors (2.8%), reflecting background p16 expression (Table 4).

In determining the causal association between HPV infection and lung cancer, we considered the expression of HPV E6 mRNA as the gold standard. On the basis of the HPV16-specific assay, we found no evidence for the expression of HPV16 E6 mRNA in the 26 tumors examined, 19 of which were HPV16 DNA positive (Table 4). Further validation using the ultrasensitive Luminex-based assay capable of detecting E6 mRNA of additional high- and low-risk HPV types confirmed the absence of not only HPV16 transcripts, but also HPV31, HPV33, HPV56, HPV58, and HPV11 (data not shown).

HPV infection and lung cancer survival. In the analysis of all-cause mortality, 221 cases with serology data were available in the L2 study and 114 deaths were recorded (87 from lung cancer). One hundred and twenty-three cases were available in the tumor analysis and 62 deaths were recorded. The longest follow-up time was 6.1 years and the median follow-up time was 3.62 years. In the EPIC study, a total of 1,423 cases were available and the maximum follow-up time was 14.7 years during which 1,203 deaths occurred. We observed no association between the presence of any HPV antibodies or DNA and overall mortality. Individually, neither HPV16 or HPV11 antibodies, nor HPV16 or HPV11 DNA were associated with all-cause mortality following lung cancer (Table 5). Further adjustment for stage at diagnosis did not change the results (data not shown). Furthermore, none of the markers of HPV infection were associated with cause-specific mortality due to lung cancer (data not shown).

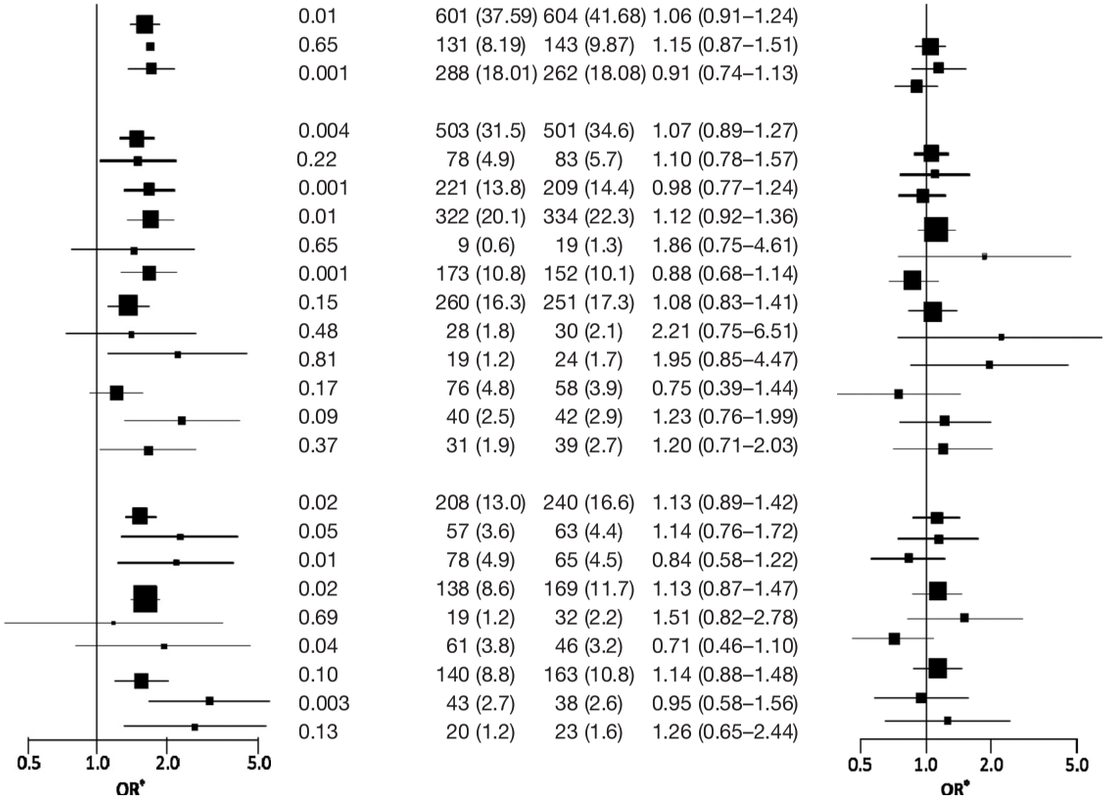
Discussion

In this study, we found no evidence for a causal association between HPV infection and lung cancer. On the basis of a stringent definition of HPV DNA positivity, we observed that 9.7% of lung cancers in Europe were positive for any HPV type, 6.6% for HPV16 and 2.4% for HPV11. Though 1.4% (4 of 290) of the lung tumors was positive for HPV16 DNA and p16 protein expression combined, no transcriptional activity of the HPV16 E6 gene was observed, indicating inactive infections.

Serologic methods that detect antibodies to HPV proteins provide an alternative method to test the association between type-specific HPV infections and cancer. Although capsid

Table 2. Antibodies to type-specific HPV proteins and the risk of lung cancer

	Retrospective studies[a]					Prospective study				
	Seropositive					Seropositive				
HPV antibody	Controls (n = 2,729)	Cases (n = 1,634)	OR[b] (95% CI)		Study heterogeneity	Controls (n = 1,599)	Cases (n = 1,449)	OR[b] (95% CI)	
	N (%)	*N* (%)		Any mucosal HPV types		*N* (%)	*N* (%)		
Any HPV LI	991 (36.3)	791 (48.4)	1.62 (1.42–1.85)		0.01	601 (37.59)	604 (41.68)	1.06 (0.91–1.24)	
Any HPV E6	101 (3.7)	100 (6.1)	1.69 (1.25–2.30)		0.65	131 (8.19)	143 (9.87)	1.15 (0.87–1.51)	
Any HPV E7	189 (6.9)	194 (11.8)	1.72 (1.38–2.16)		0.001	288 (18.01)	262 (18.08)	0.91 (0.74–1.13)	
High-risk mucosal HPV types									
Any HPV LI	502 (18.4)	404 (24.7)	1.50 (1.28–1.76)		0.004	503 (31.5)	501 (34.6)	1.07 (0.89–1.27)	
Any HPV E6	73 (2.7)	71 (4.4)	1.51 (1.05–2.16)		0.22	78 (4.9)	83 (5.7)	1.10 (0.78–1.57)	
Any HPV E7	168 (6.2)	171 (10.5)	1.69 (1.33–2.14)		0.001	221 (13.8)	209 (14.4)	0.98 (0.77–1.24)	
HPV16 LI	205 (7.5)	185 (11.4)	1.71 (1.36–2.14)		0.01	322 (20.1)	334 (22.3)	1.12 (0.92–1.36)	
HPV16 E6	26 (0.9)	23 (1.4)	1.44 (0.78–2.65)		0.65	9 (0.6)	19 (1.3)	1.86 (0.75–4.61)	
HPV16 E7	116 (4.3)	124 (7.6)	1.68 (1.27–2.22)		0.001	173 (10.8)	152 (10.1)	0.88 (0.68–1.14)	
HPV18 LI	293 (10.8)	233 (14.3)	1.37 (1.13–1.67)		0.15	260 (16.3)	251 (17.3)	1.08 (0.83–1.41)	
HPV18 E6	23 (0.8)	22 (1.4)	1.41 (0.74–2.66)		0.48	28 (1.8)	30 (2.1)	2.21 (0.75–6.51)	
HPV18 E7	18 (0.7)	19 (1.2)	2.24 (1.12–4.48)		0.81	19 (1.2)	24 (1.7)	1.95 (0.85–4.47)	
HPV31 LI	174 (6.4)	114 (7.0)	1.22 (0.94–1.58)		0.17	76 (4.8)	58 (3.9)	0.75 (0.39–1.44)	
HPV31 E6	24 (0.9)	33 (2.0)	2.33 (1.32–4.12)		0.09	40 (2.5)	42 (2.9)	1.23 (0.76–1.99)	
HPV31 E7	40 (1.5)	39 (2.4)	1.65 (1.03–2.67)		0.37	31 (1.9)	39 (2.7)	1.20 (0.71–2.03)	
Low-risk mucosal HPV types									
Any HPV LI	682 (25.0)	568 (34.8)	1.55 (1.34–1.78)		0.02	208 (13.0)	240 (16.6)	1.13 (0.89–1.42)	
Any HPV E6	29 (1.1)	31 (1.9)	2.26 (1.30–3.91)		0.05	57 (3.6)	63 (4.4)	1.14 (0.76–1.72)	
Any HPV E7	27 (1.0)	34 (2.1)	2.17 (1.25–3.78)		0.01	78 (4.9)	65 (4.5)	0.84 (0.58–1.22)	
HPV6 LI	634 (23.3)	553 (33.9)	1.62 (1.40–1.88)		0.02	138 (8.6)	169 (11.7)	1.13 (0.87–1.47)	
HPV6 E6	9 (0.3)	6 (0.4)	1.18 (0.40–3.49)		0.69	19 (1.2)	32 (2.2)	1.51 (0.82–2.78)	
HPV6 E7	11 (0.4)	13 (0.8)	1.93 (0.82–4.58)		0.04	61 (3.8)	46 (3.2)	0.71 (0.46–1.10)	
HPV11 LI	155 (5.7)	122 (7.5)	1.56 (1.20–2.03)		0.10	140 (8.8)	163 (10.8)	1.14 (0.88–1.48)	
HPV11 E6	22 (0.8)	30 (1.8)	3.06 (1.68–5.58)		0.003	43 (2.7)	38 (2.6)	0.95 (0.58–1.56)	
HPV11 E7	16 (0.6)	23 (1.4)	2.67 (1.32–5.39)		0.13	20 (1.2)	23 (1.6)	1.26 (0.65–2.44)	



NOTE: *N* represents the number of cases.

[a]Central Europe and L2 studies were combined; thresholds from the prospective study were used to define HPV seropositivity.

[b]ORs were adjusted for age, sex, smoking status (never, former, and current), and country; corresponding HPV-negative group was considered the reference category.

Table 3. HPV DNA positives identified by genotyping

	HPV genotyping	
HPV DNA status	**Round 1[a]**	**Round 2[b]**
	***N* (%)[c]**	***N* (%)**
Negative	242 (83)	37
Positive	48 (17)	28 (9.7)
Single infections	34 (11.7)	23 (7.9)
HPV16	24 (12)	14 (4.8)
HPV31	1 (0.3)	—
HPV33	3 (1.0)	—
HPV51	—	1 (0.3)
HPV58	2 (0.6)	2 (0.7)
HPV11	4 (1.4)	6 (2.1)
Multiple infections[d]	14 (4.8)	5 (1.7)
HPV16	11 (3.8)	5 (1.7)
HPV31	5 (1.7)	2 (0.7)
HPV33	1 (0.3)	1 (0.3)
HPV51	1 (0.3)	—
HPV56	1 (0.3)	1 (0.3)
HPV11	7 (2.4)	1 (0.3)

[a]All 290 tumors that passed pathology were included in the first round of HPV genotyping.
[b]Confirmation round included 48 HPV DNA-positive tumors and 16 HPV DNA-negative tumors were included in the confirmation stage.
[c]Percentage positivity is based on the entire series of 290 tumors tested.
[d]Multiple HPV infections do not add up to total because certain HPV types appear more than once.

antibody positivity (L1) indicates recent HPV infection, early antibodies (E6 and E7) are considered markers of an ongoing HPV-related malignancy (27, 28). Presence of HPV16 E6 antibodies in particular, are thought to be cancer-specific markers owing to their rarity among controls (<1% seropositivity in nearly 6,500 controls based on studies of head and neck cancer; refs. 29–32). Conversely, HPV16 E6 seems to be a specific marker for HPV16-positive oropharyngeal cancer (19). We applied HPV serology as a test in the retrospective and prospective designs to address independent questions. In the retrospective studies, serologic testing was applied as an initial screen to examine the potential associations between type-specific HPV antibodies and lung cancer and also determine whether HPV serology could predict the presence of the corresponding HPV DNA in the tumor. The prospective analysis was conducted to examine the temporality of the association and to determine whether antibody markers may be detectable before clinically apparent disease, as we recently observed for oropharyngeal cancer (19). In the interpretation of the serologic associations, we considered: (i) the consistency of antibody prevalence across the studies, (ii) directional consistency within a study between capsid and early antibodies by HPV type, (iii) potential effect modification by smoking status,

Table 4. Concordance between serology and tumor markers of HPV infection

	Serology[a]						Tumor markers					
	Capsid serology		E6 serology		E7 serology		P16INK4a*			HPV16 RNA†		
HPV DNA Status	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative ($n = 52$)**	**Positive ($n = 12$)**		**Negative ($n = 26$)**	**Positive ($n = 0$)**	
HPV16	$\rho = 0.09$; $P = 0.83$		$\rho = -0.07$; $P = 0.71$		$\rho = -0.04$; $P = 0.43$		$\rho = 0.06$; $P = 0.66$					
Negative	233	38	269	2	241	30	38	8		7	—	
Positive	16	3	19	0	18	1	14	4		19	—	
HPV11	$\rho = -0.03$; $P = 0.35$		$\rho = -0.02$; $P = 0.82$		$\rho = -0.02$; $P = 0.69$		$\rho = 0.17$; $P = 0.09$					
Negative	251	32	281	2	277	6	48	9		18	—	
Positive	7	0	7	0	7	0	4	3		2	—	

[a]Represents data from 290 cases with overlapping HPV DNA and serology data. ρ represents correlation coefficient and *P* the associated *P* value.
[b]Sixty-four tumors were included in this analysis and reflect cases with any level of p16 expression.
[c]Represents data from 26 cases included for HPV16 E6*I assay. Similar results were observed for the type-specific assay that included 34 tumors.

Table 5. Association between the HPV markers and survival from lung cancer

	Retrospective studies[a]			Prospective study[b]		
HPV status	**Events/*N***	**HR (95% CI)[c]**	***P***	**Events/*N***	**HR (95% CI)[c]**	***P***
Any HPV status						
L1 seronegative	84/160	1		853/1,000	1.0	
L1 seropositive	30/61	0.90 (0.58–1.41)	0.65	340/407	0.96 (0.84–1.08)	0.49
E6 seronegative	114/211	1		1,147/1,351	1.0	
E6 seropositive	0/10	NA		46/56	1.01 (0.75–1.35)	0.96
E7 seronegative	97/188	1		1,047/1,237	1.0	
E7 seropositive	17/33	0.98 (0.59–1.66)	0.12	146/170	1.06 (0.89–1.26)	0.54
DNA negative	47/99	1		NA		
DNA positive	15/24	1.67 (0.93–2.99)	0.09			
HPV16 status						
L1 seronegative	95/182	1.0		915/1,077	1.0	
L1 seropositive	19/39	0.89 (0.53–1.50)	0.67	278/330	0.98 (0.85–1.12)	0.74
E6 seronegative	114/219	1.0		1,179/1,388	1.0	
E6 seropositive	0/2	NA		14/19	0.71 (0.42–1.20)	0.20
E7 seronegative	100/192	1.0		1,064/1,256	1.0	
E7 seropositive	14/29	0.91 (0.52–1.60)	0.74	129/151	1.05 (0.87–1.26)	0.61
DNA negative	50/105	1.0		NA		
DNA positive	12/18	1.74 (0.92–3.29)	0.09			
HPV11 status						
L1 seronegative	96/183	1.0		1,062/1,246	1.0	
L1 seropositive	18/38	0.89 (0.54–1.49)	0.66	131/161	0.94 (0.79–1.13)	0.53
E6 seronegative	114/212	1.0		1,160/1,369	1.0	
E6 seropositive	0/9	NA		33/38	1.23 (0.87–1.74)	0.25
E7 seronegative	110/215	1.0		1,172/1,384	1.0	
E7 seropositive	4/6	1.39 (0.51–3.77)	0.52	21/23	1.24 (0.81–1.91)	0.33
DNA negative	59/116			NA		
DNA positive	3/7	0.92 (0.29–2.94)	0.89			

NOTE: *N* represents total number of cases available in the analysis for each category.

[a]In the retrospective study, a total of 221 cases were available (114 deaths recorded) for serologic analysis and 123 cases (62 deaths) in the tumor DNA analysis.

[b]In the prospective study, a total of 1,407 cases were available (16 excluded because of missing smoking status) and 1,193 deaths were recorded. The model additionally included country of recruitment as a covariate.

[c]HR adjusted for age at diagnosis, sex, country, and smoking status.

and (iv) sensitivity of the results to seropositivity thresholds. In this study, the association between type-specific HPV antibodies and lung cancer seemed to differ by study design. It remains possible that the apparent difference by study design could be attributed to false findings or cancer-specific immune changes in the retrospective cases. However, it is important to note that consistently no association was found for HPV16 E6 seropositivity, considered a cancer-specific marker and also the HPV type with the strongest *a priori* hypothesis. It is important to mention that the observations in the retrospective studies may be interpreted independently given the strengths of comprehensive HPV evaluation. Here, although nearly 7% of lung tumors were consistently HPV16 DNA positive, no expression of HPV16 E6 mRNA was observed, with two different methodologies applied. Furthermore, based on the criteria previously outlined, though some HPV antibodies (HPV11 and HPV31 E6) seemed to be consistently associated with increased risk of lung cancer, no correlation with tumor HPV DNA presence was observed. Furthermore, no functional consequence of viral presence could be established.

This study raises several questions about the published associations between HPV and lung cancer. First, despite extensive efforts taken to avoid contamination and following a stringent definition for HPV DNA positivity, we observed that 28 lung tumors consistently tested positive for the same type (of the 48 initial positives, nearly 58%). The high proportion of false positivity may explain the large variation in HPV DNA prevalence reported within the same country, such as the observed range of 0% to 78% in Japan (33, 34) and 12% to 55% in China (35, 36). Second, some of the regional difference in HPV prevalence has been attributed to varying proportions of nonsmoking women and lung histologies (37). In this study, we observed that the proportion of HPV16 DNA positivity was comparable between smoking men (7%) and nonsmoking

women (7.5%) as well as across different lung cancer histologies (Supplementary Table S3). Third, this study demonstrates that although nearly 10% of lung tumors may be consistently HPV DNA positive, none expressed viral oncoproteins, emphasizing that HPV DNA (and/or p16) is insufficient to establish a causal role for HPV infection in lung carcinogenesis. Our conclusions are supported by the only study reported to date that included a large series of lung tumors and took adequate precautions to avoid potential contamination (7). On the basis of this evidence, we suggest that future publications reporting HPV DNA prevalence in lung cancer should: (i) include and describe precautions taken to avoid potential contamination and (ii) address the transcriptional state of the HPV genome.

In conclusion, using multiple markers, extensive precautions against potential contamination, and state-of-the-art HPV detection methods, we demonstrate that several high- and low-risk HPV types are present in the lung tissue and can be detected with varying level of evidence, but are unlikely to contribute causally to lung carcinogenesis. These data do not support a causal role for HPV infections in lung cancer.

Disclosure of Potential Conflicts of Interest

M. Pawlita has commercial research grant from Roche and Qiagen. No potential conflicts of interest were disclosed by the other authors.

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