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ARTICLE

Assessment of Human Papillomavirus in Lung Tumor Tissue

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Background Lung cancer kills more than 1 million people worldwide each year. Whereas several human papillomavirus (HPV)–associated cancers have been identified, the role of HPV in lung carcinogenesis remains controversial.

Methods We selected 450 lung cancer patients from an Italian population–based case–control study, the Environment and Genetics in Lung Cancer Etiology. These patients were selected from those with an adequate number of unstained tissue sections and included all those who had never smoked and a random sample of the remaining patients. We used real-time polymerase chain reaction (PCR) to test specimens from these patients for HPV DNA, specifically for E6 gene sequences from HPV16 and E7 gene sequences from HPV18. We also tested a subset of 92 specimens from all never-smokers and a random selection of smokers for additional HPV types by a PCR-based test for at least 54 mucosal HPV genotypes. DNA was extracted from ethanol- or formalin-fixed paraffin-embedded tumor tissue under strict PCR clean conditions. The prevalence of HPV in tumor tissue was investigated.

Results Specimens from 399 of 450 patients had adequate DNA for analysis. Most patients were current (220 patients or 48.9%) smokers, and 92 patients (20.4%) were women. When HPV16 and HPV18 type–specific primers were used, two specimens were positive for HPV16 at low copy number but were negative on additional type-specific HPV16 testing. Neither these specimens nor the others examined for a broad range of HPV types were positive for any HPV type.

Conclusions When DNA contamination was avoided and state-of-the-art highly sensitive HPV DNA detection assays were used, we found no evidence that HPV was associated with lung cancer in a representative Western population. Our results provide the strongest evidence to date to rule out a role for HPV in lung carcinogenesis in Western populations.

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More than 1 million people die of lung cancer each year (1). Although smoking is the major etiologic factor, most smokers will not develop lung cancer (2), indicating that additional cofactors are needed for lung carcinogenesis. Lung cancer is also a major cause of cancer deaths even among never-smokers (3).

Certain types of human papillomavirus (HPV) are firmly established as human carcinogens. These HPV types cause essentially all human cervical cancers (4,5) and are strongly implicated in the etiology of a substantial proportion of other anogenital cancers (6) and oropharyngeal cancers (7,8). HPV has been hypothesized to play a role in lung cancer pathogenesis, largely on the basis of more than 50 studies (9,10) in which HPV genomic DNA was detected in 0%–100% (median = 16.7%) of lung tumor tissues. However, these studies are not definitive because of power constraints, restricted study populations, use of nonspecific and/or insensitive laboratory approaches, and use of HPV detection techniques that are limited to regions of the viral genome that may be lost with viral integration into the cellular host DNA. Although geographic differences in the prevalence of HPV could potentially explain some variability in study results, interlaboratory variability and inadequate sample collection and handling leading to contamination may also contribute to the variability in reported results (10). Therefore, the role of HPV in lung carcinogenesis remains unclear.

Part of the reason why HPV is hypothesized to be associated with lung carcinogenesis is because the respiratory tract is in close proximity to the oropharynx, where HPV is known to cause a substantial proportion of cancers (7,8). HPV has been detected in respiratory papillomas, which are sometimes found in the bronchi and occasionally progress to malignancy, and also has been detected in respiratory tumors, as noted above (9,10). In addition, HPV can transform bronchial cells in vitro (11).

Furthermore, cervical cancer survivors and immunosuppressed populations are at increased risk for lung cancer and established HPV-related malignancies (12–14). Although smoking is an

CONTEXT AND CAVEATS

Prior knowledge

Human papillomavirus (HPV) is associated with cervical and oropharyngeal cancers. A role for HPV in lung cancer has been proposed, although previous reports of HPV in lung tumor tissue have been inconclusive.

Study design

Paraffin-embedded tissue specimens from 450 lung cancer patients who were smokers or never-smokers enrolled in the Environment and Genetics in Lung Cancer Etiology study were evaluated for HPV DNA by real-time polymerase chain reaction to determine the HPV prevalence in tissues from lung cancer patients in a representative Western population.

Contribution

Two specimens were found to be HPV positive at low copy number but were negative on additional genotype-specific testing and on testing for a broad spectrum of HPV types. The study found no evidence to support an association between HPV and lung cancer.

Implications

HPV is not associated with lung carcinogenesis in lung cancer patients from a representative Western population.

Limitations

Multiple tissue specimens were used from each patient for analysis and were not necessarily adjacent to or from the same paraffin block. Also, the possible contamination of specimens during collection could not be determined. Because of geographic variances in the prevalence of HPV reported in lung tumors, further studies in non-Western populations should be completed.

From the Editors

established cofactor for cervical squamous cell carcinoma, it is not associated with an increased risk of cervical adenocarcinoma (15). However, cervical adenocarcinoma survivors have approximately twofold increased risk of lung cancer (16). In addition, HIV–AIDS patients, who have an increased risk of HPV and HPV-related malignancies (17), also have an increased risk of lung cancer that has not been completely accounted for by increased smoking (13). These epidemiological data are consistent with a hypothesis that HPV contributes to respiratory carcinogenesis in a smoking-independent manner.

A difficulty with the hypothesis that HPV is a lung carcinogen is the fact that HPV is not generally transmitted as an aerosol, making transmission to the lung difficult (18). However, the existence of rare recurrent respiratory papillomatosis demonstrates that HPV can reach the respiratory tract. In addition, recurrent respiratory papillomatosis shows a second peak in young adults aged approximately 20 years (19) that is associated with oral sex and number of lifetime sex partners (20), consistent with sexual transmission. The oral cavity, in which HPV infections are established largely through sexual transmission (21), may act as a reservoir for transmission of HPV to the respiratory tract, possibly through aspiration or mucosal transfer. The probability of such transmission may be exceedingly low, however, considering the rarity of recurrent respiratory papillomatosis.

The goal of our study was to complete a more definitive evaluation of HPV in lung cancer. We tested for the presence of HPV DNA with highly sensitive polymerase chain reaction (PCR) and took rigorous precautions against PCR contamination in tumor tissues from 450 lung cancer patients from the well-characterized population–based case–control study, Environment and Genetics in Lung Cancer Etiology (EAGLE).

Materials and Methods

Study Population

EAGLE is a large population-based case–control study with 2100 lung cancer patients that was designed to investigate the genetic and environmental determinants of lung cancer and smoking persistence (22). As previously described (22), individuals with newly diagnosed lung cancer were recruited from April 22, 2002, to February 28, 2005, from 13 hospitals in the Lombardy region of Italy (see Notes). Each participant provided written informed consent. The study was approved by the institutional review board of each participating hospital and university in Italy and by the National Cancer Institute, Bethesda, MD. Lung cancer was diagnosed by standard clinical criteria and confirmed by pathology reports from surgery, biopsy, or cytology samples (approximately 95% of patients) or through clinical history and imaging (approximately 5% of patients) (22). Tumor histology was classified by the 1999 World Health Organization Histological Typing of Lung and Pleural Tumors (23). The 13 Italian hospitals treat approximately 80% of all patients with newly diagnosed lung cancer in the area, which includes more than 1.3 million people aged 35–79 years from five cities and surrounding towns and villages. EAGLE had a participation rate of 86.6% among lung cancer patients. Thus, participants in the EAGLE study should be broadly representative of lung cancer patients in Western populations.

We decided to test 450 adult male and female lung cancer patients, including all 30 never-smokers and a random sample of 420 smokers who had an adequate number of unstained tissue slides and at least one slide available that was stained with hematoxylin and eosin. These stained slides were reviewed by H. Song to establish the presence and estimate proportion of tumor tissue in the sample, but these slides were not necessarily adjacent to the tissue that was tested for HPV DNA. The samples were formalin fixed for 399 patients and ethanol fixed for 51 patients.

HPV DNA Testing

All 450 patients were tested for HPV16 and HPV18 DNA because these are the most common types found in cervical cancer (24) and account for the overwhelming majority of noncervical HPV–associated cancers (25). Type-specific PCR for HPV16 and HPV18 was conducted in a laboratory at the Ohio State University, as described below. For a subset of 100 patients including all never-smokers and a random selection of smokers, a second tissue specimen was sent to DDL Diagnostic Laboratory (Voorburg, the Netherlands) for broad-spectrum HPV typing.

Type-Specific PCR for HPV16 and HPV18. All specimen receipt, processing, and pre-amplification analysis procedures were performed in ultraviolet light–irradiated laminar flow hoods in a

laboratory with positive-pressure ventilation. All equipment and reagents were specific to the pre-amplification analysis laboratory with strict one-way transport of specimens from this laboratory to a physically separate postamplification analysis laboratory. Additional protocols to prevent specimen contamination included sample processing in small batches, change of gloves after DNA extraction of each sample, exclusive use of barrier pipette tips, weekly cleaning of all laboratory surfaces and equipment with 70% ethanol, and soaking of all racks and PCR plate holders in 10% bleach for a minimum of 10 minutes between each experiment. Additionally, one negative control per seven experimental samples was included in each column of each 96-well PCR plate.

DNA was extracted from 450 formalin- or ethanol-fixed paraffin-embedded tissue samples by means of paraffin removal with octane, proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation. Briefly, after octane exposure, the sample was washed with 100% ethanol, resuspended in 200 μL of digestion buffer (50 mM Tris–HCl at pH 8.5, 1 mM EDTA, and proteinase K at 20 μg/mL), and incubated for 48 hours at 55°C. If tissue remained, 10 μL of proteinase K (400 μg/mL) was added, and digestion was continued for an additional 24–48 hours. DNA was extracted in phenol–chloroform by use of Qiagen MaXtract tubes (Qiagen, Germantown, MD) and ethanol precipitated in the presence of glycogen (0.02 mg/mL). Pellets were washed once in 70% ethanol, dried at 37°C in a dry incubator, and resuspended in 50 μL of diethylene pyrocarbonate-treated water. DNA quantity and purity (calculated by use of the ratio of the absorbance at 260 nm to that at 280 nm [260/280 ratio]) were measured with the Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc, Wilmington, DE).

An estimate of the number of cells analyzed in each PCR was made by custom TaqMan real-time PCR assays targeting a single-copy human gene on chromosome 7, human endogenous retrovirus 3 (*ERV3*) (26). In a pilot study of 19 specimens, DNA integrity was evaluated through amplification of ERV3 sequences with primers generating an amplicon of 58, 135, or 354 base pairs (bp) in size (Supplementary Table 1, available online). Primer and probe sequences for the 58-bp amplicon were as follows: forward primer, 5′-GATAATTTCACACTAACCGCC-3′; reverse primer, 5′-AGATGCTCTGACTTGATGGT-3′; and the probe sequence, 5′-56-FAM-CTCTTCCCTCGAACCTGC-3BHQ1-3′ (where 56-FAM is 5′6-carboxy-fluorescein and 3BHQ1 is 3-Black Hole Quencher-1). Primer and probe sequences for the 135-bp amplicon were as follows: forward primer, 5′-CATGGGAAGCAAG GGAACTA-3′; reverse primer, 5′-CCCAGCGAGCAATACAG AATTT-3′; and probe, 5′-56-FAM-TCTTCCCTCGAACCTG CACCATCAAGTCA-3BHQ1-3′. Primer and probe sequences for the 354-bp amplicon were as follows: forward primer, 5′-CATGGGAAGCAAGGGAACTAATG-3′; reverse primer, 5′-GCCCACAGATCCAGTAGAGG-3; and probe were the same as for the 135-bp amplicon above. Reaction conditions were as previously published (26). Briefly, purified genomic DNA (2 μL) from lung cancer specimens was analyzed. A standard curve was generated in duplicate from a fivefold dilution series (from 150 000 to 1.92 cells) of a diploid human cell line, CCD-18LU (catalog number CCL-205, ATCC [American Type Culture Collection], Manassas, VA). Results were reported as the number of human diploid genome equivalents of purified genomic DNA from tumor samples that were evaluated for HPV16 and HPV18 DNA by real-time PCR. All samples were tested once, and positive samples were retested in duplicate. Although DNA was degraded because of fixation, the mean yield was 54.5 ng/mL. This subset analysis indicated that DNA integrity was sufficient to support amplification of targets with amplicon size between approximately 58 and 135 bp. The 58-bp ERV3 fragment was amplified for the 431 remaining specimens. DNA specimens with at least 100 evaluable cell equivalents and a 260/280 ratio between 1.5 and 2.2 (27) were considered to have adequate DNA quantity and quality for further analysis.

Type-specific TaqMan real-time PCR targeted to the oncogenic E6 region of the genome for HPV16 (amplicon size = 201 bp) and the E7 region of the genome for HPV18 (amplicon size = 137 bp) were performed in a 96-well plate format as previously described (28). To account for DNA degradation in formalin-fixed and paraffin-embedded tissues, all samples were also evaluated by means of a TaqMan real-time PCR assay designed to amplify an 81-bp amplicon in the E6 region of the genome for HPV16. Primers and probes were manufactured at IDT Technologies (Coralville, IA) and sequences were: forward, 5′-GAGAAC TGCAATGTTTCAGGACC-3′; reverse, 5′-TGTATAGTTGT TTGCAGCTCTGTGC-3′; and probe, 5′-56-FAMCAGGAGC GACCCAGAAAGTTACCACAGTT-3BHQ1-3′. Standard curves were generated in duplicate by use of a fivefold dilution series from 2.5×10^6 copies of plasmid vector containing full-length viral genome (pGEM HPV16) in a background of human placental DNA (5 ng/μL). Reactions that were performed in an Applied Biosystems 7300 PCR system contained 2X TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), 0.1 μmol of probe, 0.2 μmol of each primer, and 2 μL of purified tumor DNA. Amplification conditions included a 12-minute incubation at 95°C, followed by 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The cycle threshold (CT) of unknown samples was determined from an equation derived from a linear regression through the log CT of the standard curve according to the manufacturer's recommendations. For positive samples, HPV viral load was normalized to ERV3 copy number by real-time PCR, as a measure of viral copy number per cell analyzed. Samples with any detectable HPV16 or HPV18 DNA were tested in duplicate, with a mean viral load of greater than one viral copy per sample classified as real-time PCR positive. A mean viral load of greater than or equal to one viral copy per cell was considered indicative of a clonal viral–tumor relationship.

Broad-Spectrum HPV Typing. Specimens from all 30 never-smokers and 70 randomly sampled current and former smokers were selected to test for broad-spectrum HPV typing at DDL Diagnostic Laboratory. HPV typing used PCR with short PCR fragment (SPF_{10}) primers, which targets the HPV L1 gene, and a mixture of conservative probes that recognize at least 54 mucosal HPV genotypes. We used the DNA enzyme immunoassay (DEIA) to determine HPV DNA positivity by detecting amplimers, synthesized by biotinylated PCR primers, through hybridization to a mixture of HPV-specific probes, as described previously (29). Stringent PCR contamination precautions that were similar to the type-specific testing for HPV16 and HPV18 conducted at the

Ohio State University were taken. All pre-amplification procedures were performed in ultraviolet light–irradiated laminar flow hoods that were separate from a postamplification analysis laboratory. During DNA isolation, PCR amplification, and post-PCR analyses, specific negative (water blank) and positive (HPV DNA extracted from the HPV18-positive HeLa cell line) controls were included. We monitored potential contamination of samples with negative controls and the sensitivity of the procedure with low-concentration positive controls. All reagents had been prepared in a separate reagent laboratory and subjected to strict quality control to confirm efficacy and the absence of contaminants.

DNA amplification by PCR with SPF_{10} primers was followed by a reverse hybridization line-probe assay ($LiPA_{25}$, version 1; Labo Bio-Medical Products, Rijswijk, the Netherlands) for genotyping of 25 HPV types in SPF_{10}- and DEIA-positive samples. These primers produce very short PCR fragments of approximately 65 bp, making them ideal for amplification in formalin-fixed specimens (29–31). Eight specimens did not appear to have tumors in the tissue specimen received for testing. DNA was extracted for 92 specimens (from 65 ever-smokers and 27 never-smokers) with identifiable tumor in the tissue specimen. For each specimen, half of the tissue section was removed from the slide with a swab after confirming the presence of tumor cells in that part of the section. This part of the tissue was transferred to a microtube (Sarstedt, Etten-Leur, the Netherlands) and digested with proteinase K buffer (45 mM Tris–HCl at pH 8, 0.9 mM EDTA, 0.45% Tween 20, and proteinase K at 1 mg/mL). Proteinase K solution (100 μL) was added to each microtube and incubated for 16–24 hours at 56°C. Proteinase K solution was inactivated by incubating the tubes at 95°C for 10 minutes. Resulting DNA preparations were stored at −20°C. During this process, negative and positive DNA isolation controls were included. In these samples, DNA quality and quantity were adequate as measured by real-time PCR for the human β-actin gene in the same DNA specimens as used for the SPF_{10} PCR. For one patient (a current smoker) where additional investigation was warranted due to weak HPV16 positivity in the sample tested at the Ohio State University, type-specific PCR for HPV16 was performed with a 92-bp amplicon as previously described (32).

Statistical Analysis

Our goal in determining sample size was to be able to rule out a true HPV prevalence greater than 1.0%. Using cii in Stata (StataCorp LP, College Station, TX), we determined that zero HPV positives among 450 lung cancer patients would generate a one-sided 97.5% confidence interval (CI) of 0% to 0.82% and that one observed HPV-positive patient among 450 lung cancer patients would provide a two-sided 95% confidence interval of 0.0056% to 1.2%; zero positives among 400 lung cancer patients would provide a one-sided 97.5% confidence interval of 0% to 0.92%, and one observed HPV-positive patient would provide a two-sided 95% confidence interval of 0.0063% to 1.4%. The chance of finding at least one positive when the true prevalence is 1.0% or more was 98.9% with a sample size of 450 patients; thus, finding no positives with a sensitive assay rules out even a low prevalence of infection with high confidence.

In addition to the sample size calculations described above, we used the cii command in Stata (StataCorp LP), version 9.0, to estimate HPV prevalence and corresponding confidence intervals.

Results

Among the 450 lung cancer patients, 246 (54.7%) had adenocarcinoma and 137 (30.4%) had squamous cell carcinoma (Table 1). Most patients had a history of tobacco smoking: 220 patients (48.9%) were current smokers, 198 patients (44.0%) were former smokers, and 30 patients (6.7%) were never-smokers. The median age at diagnosis of lung cancer was 67.6 years (range = 35.4–79.9 years), and the median body mass index was 25.3 kg/m^2 (range = 15.9–60.8 kg/m^2). Among smokers, the median duration of smoking was 45 years (range = 1–70 years), the median average smoking intensity was one pack per day (range = 0–3 packs per day), the median pack-years was 43.8 (range = 0.1–192 pack-years), and the median age at initiation of smoking was 16 years of age (range = 6–45 years). Ninety-two patients (20.4%) were women. The majority of patients had less than a high school education (72.5% or 319 patients), drank alcohol (84.3% or 365 patients), were married or cohabitating (82.2% or 356 patients), had abnormal respiratory

Table 1. Characteristics of 450 lung cancer patients from the population-based case–control Environment and Genetics in Lung cancer Etiology study, including histology, smoking status, and human papillomavirus (HPV) status

	Smoking status, No. (%)				HPV DNA status by PCR* testing, No. (% HPV positive)	
Lung cancer histology	Never	Former	Current	Unknown	Type-specific HPV16 and HPV18†	Broad-spectrum HPV typing‡
Adenocarcinoma (n = 246)	25 (83.3)	103 (52.0)	117 (53.2)	1 (50.0)	221 (0.0)	63 (0.0)
Squamous cell (n = 137)	2 (6.7)	68 (34.3)	66 (30.0)	1 (50.0)	123 (0.0)	27 (0.0)
Large cell (n = 30)	1 (3.3)	10 (5.1)	19 (8.6)	0 (0.0)	24 (0.0)	0 (0.0)
Small cell (n = 13)	0 (0.0)	5 (2.5)	8 (3.6)	0 (0.0)	11 (0.0)	0 (0.0)
Other (n = 24)§	2 (6.7)	12 (6.1)	10 (4.5)	0 (0.0)	20 (0.0)	2 (0.0)

* PCR = polymerase chain reaction.

† Results from 399 specimens with adequate DNA of 450 selected for testing.

‡ Results from 92 specimens with tumor present in the sample of 100 selected for testing.

§ Includes mixed type (adenosquamous, n = 10), synchronous (two different lung cancers, n = 6), non-small cells (n = 2), and other (including poorly differentiated, epithelial, neuroendocrine, mucoepidermoid, non-small cell, pleomorphic, sarcomatoid, n = 6).

function (51.7% or 186 patients), and had stage IIB disease or less (71.3% or 321 patients) (Table 2).

The median number of cells evaluable for the HPV16 and HPV18 PCR as measured by ERV3 real-time PCR was 3680 cells (range = 1.9–399 750 cells). One specimen was negative for ERV3. The median 260/280 ratio was 1.63 (range = 1.23–1.94). Combining these data, 399 (88.7%) of 450 specimens had adequate DNA quantity and quality for analysis (ie, 100 or more cell equivalents and a 260/280 ratio between 1.5 and 2.2). On review of the 450 slides that were stained with hematoxylin and eosin, 412 specimens contained tumor cells. Of those tumor-positive specimens, 73.8% (n = 304 specimens) contained greater than 60% tumor. We included all specimens with adequate DNA (n = 399 specimens) in the prevalence estimate.

Table 2. Descriptive characteristics of 450 lung cancer patients from the population-based case–control Environment and Genetics in Lung cancer Etiology study*

Characteristic	No. of patients (% of total)
Age, y	
<60	93 (20.7)
60–69	181 (40.2)
≥70	176 (39.1)
Sex	
Male	358 (79.6)
Female	92 (20.4)
Area of Italy	
Brescia	72 (16.0)
Milano	268 (59.6)
Monza	54 (12.0)
Pavia	22 (4.9)
Varese	34 (7.6)
Education†	
None	27 (6.1)
Elementary school	161 (36.6)
Middle school	131 (29.8)
High school (teaching training, job training, college training)	105 (23.9)
University degree (bachelor, master, PhD)	16 (3.6)
Alcohol consumption†	
No	68 (15.7)
Yes	365 (84.3)
Marital status†	
Married or cohabitating	356 (82.2)
Neither married nor cohabitating	77 (17.8)
Spirometry-based respiratory function†	
Normal	174 (48.3)
Mild COPD	51 (14.2)
Moderate COPD	68 (18.9)
Severe and very severe COPD	17 (4.7)
Potentially restrictive disease	50 (13.9)
Stage	
IA	84 (18.7)
IB	126 (28.0)
IIA	14 (3.1)
IIB	97 (21.6)
IIIA	58 (12.9)
IIIB	43 (9.6)
IV	28 (6.2)

* COPD = chronic obstructive pulmonary disease.

† Numbers do not sum to total because of missing values.

Among the 399 specimens with adequate DNA, one female former smoker with adenocarcinoma was positive for HPV16 on initial testing. Given the very low viral load in this tumor (average = 0.4 per 1000 cell equivalents across three tests of the same sample), we tested another tumor tissue specimen from this patient. We found no evidence of HPV16 or HPV18 DNA in this second specimen. The hematoxylin–eosin slide from this specimen consisted of greater than 80% cancer cells, indicating there was adequate tumor available to detect any HPV DNA if it were present. This patient was diagnosed with stage IB lung cancer at 72 years of age and had smoked for 48 years at an average intensity of 0.75 packs per day (36 pack-years).

PCR analysis targeting an 81-bp amplicon in the HPV16 E6 region confirmed the prior positive test (average of two viral copies per 1000 cell equivalents) in the first sample, and the second tumor tissue specimen from this patient was again negative. Another patient who was initially negative for HPV16 tested positive with this assay but at low copy number (131 per 1000 cell equivalents). This second patient was a male with squamous cell carcinoma diagnosed at 64 years of age who had smoked for 43 years at an average intensity of one pack per day (43 pack-years). The hematoxylin–eosin slide from this patient also contained greater than 80% cancer cells.

In the subset of 92 specimens tested with the broad-spectrum assay SPF_{10}-DEIA-$LiPA_{25}$ (including those from the two patients who tested positive with low viral load, as described above), no specimens were positive for HPV16 DNA or for any HPV type. DDL Diagnostic Laboratory conducted type-specific testing for HPV16 as a follow-up test for the male patient identified as being HPV positive by PCR analysis targeting an 81-bp amplicon in the HPV16 E6 region. The specimen tested negative for HPV DNA with this type-specific test. These results indicate that the prevalence of HPV DNA is 0.0% (Table 1) with an upper 97.5% confidence interval of 0.92% for the 399 specimens with adequate DNA, including 370 ever-smokers (upper 97.5% confidence bound = 0.99%) and 27 never-smokers (upper 97.5% confidence bound = 12.8%).

Discussion

In this study, which is to our knowledge the largest study of HPV in lung tumor tissue from a Western country, we found no evidence that HPV is associated with lung carcinogenesis. Extensive laboratory efforts to avoid DNA contamination and state-of-the-art, highly sensitive HPV DNA detection assays were performed. The two tumors that tested positive had a very low viral load of less than one copy of HPV16 per cell, despite being composed of greater than 80% tumor cells. One of these patients tested negative in a separate tissue specimen, and the other was negative on additional type-specific HPV16 testing in a separate sample. None of the subset of 92 patients was positive for any other HPV type by the very sensitive SPF_{10}-DEIA-$LiPA_{25}$ testing method. HPV-related carcinogenesis at other anatomical sites indicates that HPV should be present in every tumor cell if it truly contributed to the development of that tumor (25). Thus, whereas low-level HPV positivity in one tissue sample and complete lack of HPV in a separate sample may be possible, such tumor heterogeneity is unlikely

to reflect a truly causal association. On the basis of these results, the prevalence of HPV in lung tumor tissue from this population was essentially 0%.

A limitation of this study was that the tissue specimens were not necessarily adjacent or from the same block, although as described above, lack of uniform HPV results throughout the tumor would suggest that HPV was not associated with tumorigenesis. Because tissue was collected for diverse purposes, it was not possible to address contamination in specimen collection. However, all laboratory assays were performed under stringent precautions to avoid contamination. We also used viral load to assess whether detected HPV DNA was present at a meaningful level, as described above. Although DNA degradation may occur in paraffin-embedded tissues, DNA quality was confirmed by satisfactory 260/280 ratio and real-time PCR for the human β–actin gene and/or ERV-3 in 399 of the 450 specimens. These results indicate that the contamination precautions used during PCR analysis were largely sufficient to avoid contamination and that the DNA from the paraffin-embedded tissues was adequate for HPV DNA detection.

A particular strength of this study was that two independent laboratories extracted DNA (at the Ohio State University using phenol–chloroform extraction and at DDL Diagnostic Laboratories using crude extraction methods, as described in "Materials and Methods") from different tissue specimens from the same patient and conducted separate PCR assays for HPV DNA. All 450 specimens were tested for the E6 and E7 oncogenes of HPV16 and HPV18, the two types most strongly associated with cancer outside the cervix (25), which circumvented concerns about false-negative results because of loss of the L1 gene through integration. A substantial proportion (92 specimens from 450 lung cancer patients) was tested for a broad range of HPV types with the L1-based SPF_{10}-DEIA-$LiPA_{25}$ system. The SPF_{10}-DEIA-$LiPA_{25}$ system is the gold standard for HPV DNA testing in paraffin-embedded tissue because of its short PCR product (31) and ensured that specimens were tested for all carcinogenic and many noncarcinogenic HPV types.

Geographic differences in the prevalence of HPV in lung tumor tissue may be associated with variation in smoking habits, sexual behaviors, or other factors related to environmental exposures, culture, topography, or genetics (10). Asian studies typically report higher PCR-based HPV prevalences of HPV in lung tumors than European studies, with a summary meta-estimate of 11.6% (95% CI = 9.5% to 14.2%) for HPV16 and 8.8% (95% CI = 6.0% to 12.8%) for HPV18 in lung tumor tissues from Asia compared with 3.5% (95% CI = 2.3% to 5.3%) for HPV16 and 3.6% (95% CI = 2.3% to 5.7%) for HPV18 in lung tumor tissues from Europe (10). The largest PCR-based Asian studies found HPV DNA in 42.0% (n = 92 specimens, 95% CI = 35.6% to 48.6%) of 219 paraffin-embedded lung tumor tissues (33,34) and 44.1% (n = 138 specimens, 95% CI = 38.5% to 49.8%) of 313 fresh-frozen lung tumor tissues (10,35). Similar to our study, the largest previous European PCR-based study from France found a very low prevalence of HPV DNA in 218 fresh-frozen lung tumor tissues (1.8% prevalence, 95% CI = 0.7% to 4.8%, n = 4 positive samples) (10,36). Part of this geographic discrepancy may be because of differences in smoking habits. For example, Asian women typically do not smoke. A study of lung cancer in Taiwan found that nonsmoking female lung cancer patients were more likely to have HPV-positive lung tumor tissue than male lung cancer patients, who were more likely to smoke (37). Environmental tobacco exposure is unlikely to account entirely for the increased risk in never-smokers. Non–smoking-related factors must therefore contribute to lung cancer among never-smokers. Given the high upper confidence limit of our prevalence estimate in never-smokers (12.8%), further study of HPV in lung tumor tissue from never-smokers may be warranted.

Nevertheless, the prevalence of HPV can vary markedly within the same country. For example, the PCR-based prevalence of HPV in lung tumor tissues from Japan ranges from 0.0% to 78.3% (10). Although we found essentially no HPV in lung tumor tissues from Italy, others have reported 12.8%–21.1% HPV DNA positivity in Italian lung cancer patients (10). Such disparities within the same geographic region emphasize the importance of taking precautions to avoid PCR contamination and prevent false-positive results (38) when using sensitive HPV DNA detection assays to avoid false-negative results. Our study used such precautions in two independent laboratories, verified DNA quality before HPV detection, and used multiple sensitive methods for HPV DNA detection. Although a few previous Asian and European studies have found 0% prevalence of HPV in lung tumor tissues, these studies included less than 100 patients. With nearly 400 patients evaluated, we had a sufficient sample size to detect any true prevalence of HPV greater than 1%. Our study was twice as large as the next largest study in Europe (n = 218 specimens).

In conclusion, using multiple state-of-the-art methods to evaluate the presence of HPV DNA in resected lung cancer tumors from a representative Western study population, we found no evidence that HPV is associated with the development of lung cancer. Although we detected essentially no HPV in specimens from the EAGLE study, evaluation in a larger population of never-smokers, in which the attributable risk of non–smoking-related risk factors is necessarily higher, may be informative. Differences in smoking habits could potentially account for the higher prevalence of HPV DNA detected in lung tumor tissue from Asian countries. For Western populations, however, this study found no data to support that HPV is associated with lung carcinogenesis.

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