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The Association of Human Papillomavirus 16/18 Infection with Lung Cancer among Nonsmoking Taiwanese Women[1]

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Abstract

Lung cancer is the leading cause of cancer death in Taiwanese women since 1982. High lung cancer mortality ratio of male:female in Taiwan (2:1) was observed, although less than 10% of female lung cancer patients are smokers. Until now, the etiological factor remains unknown. We hypothesize that high-risk human papillomavirus (HPV) 16/18 may be associated with lung cancer development based on high prevalence of p53 negative immunostainings in female lung tumors compared with that of male lung tumors. In this study, 141 lung cancer patients and 60 noncancer control subjects were enrolled to examine whether HPV 16/18 DNA existed in lung tumor and normal tissues by nested PCR and *in situ* hybridization (ISH), respectively. The concordant detection of HPV 16 and 18 DNA between nested PCR and ISH method was 73 and 85.5%, respectively. Our data showed that 77 (54.6%) of 141 lung tumors had HPV 16/18 DNA compared with 16 (26.7%; $P = 0.0005$) of 60 noncancer control subjects. In addition, ISH data showed that HPV 16/18 DNA was uniformly located in lung tumor cells, but not in the adjacent nontumor cells. When study subjects were stratified by gender, age, and smoking status, nonsmoking female lung cancer patients who were older than 60 years old had significantly high prevalence of HPV 16/18 infection. The odds ratio of HPV 16/18 infection of nonsmoking female lung cancer patients is much higher at 10.12 (95% confidence interval, 3.88–26.38) compared with 1.98 (95% confidence interval, 0.84–4.76) of nonsmoking male lung cancer patients. This result strongly suggests that HPV infection is associated with lung cancer development of nonsmoking female lung cancer patients. The high prevalence of HPV 16/18 infection may explain to a certain extent why Taiwanese women nonsmokers had a higher lung cancer mortality rate.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide and in Taiwan. Cigarette smoking is considered to be the most important risk factor of lung cancer, and about 90% of lung cancer worldwide can be explained by cigarette smoking. However, in Taiwan, only around 50% of lung cancer incidence can be associated with cigarette smoking, particularly because less than 10% of Taiwanese women are smokers. Furthermore, the gender discrepancy in lung cancer mortality in Taiwan (male:female ratio, 2:0) is lower than that of other areas of the world, in which the ratio ranges from 2.3 to 8.6 (1, 2). Thus, it is conceivable that environmental factors other than cigarette smoking may be associated with the development of lung cancer in Taiwan.

HPV[3] has been shown to be implicated in human neoplasm including uterine cervix, vulva, skin, esophagus, and head and neck. (3) According to the present data, HPV is most commonly associated with the development of cervical carcinomas and HPV 16/18 are the types most frequently detected in high-grade squamous intraepithelial lesions and invasive carcinomas. E6 proteins encoded by HPV can bind to the host cellular tumor suppressor protein, p53, and induce its degradation through the ubiquitin pathway (4, 5). In addition, the presence of homozygous arginine-72 in wild-type p53 is considered as a risk of cervical cancer because this genotype of p53 protein is more susceptible to HPV-E6-mediated degradation (6). Therefore, inactivation of p53 by HPV-E6 is considered to play a crucial role for human carcinogenesis.

Although a number of reports revealed that no or low frequency of HPV infection was found in lung tumors (7, 8), some data from Japan and Northern European countries, such as Finland and Norway, showed that significantly higher frequencies of HPV infection (69-78.3%) were determined in lung carcinomas (9–12). The association between HPV infection and lung cancer was then being reasonably suggested to be geography- and race-dependent. In our preliminary studies, the frequencies of p53 mutation and p53 positive immunostaining in nonsmoking female lung cancer patients were significantly lower than those of nonsmoking male lung cancer patients.[4] This phenomenon is quite similar to the pattern of *p53* gene alteration found in cervical cancer (13). Therefore, we hypothesized that high-risk HPV 16/18 infection may be involved in lung carcinogenesis of Taiwanese women nonsmokers. Previous reports regarding the association between HPV and lung cancer examined only whether HPV DNA was present in lung tumor specimens (7–12). To further address this association, we examined the HPV infection in 141 non-small cell lung cancer patients and 60 noncancer subjects and evaluated the difference between the cancer and noncancer groups in this study. These results will provide more detailed information to support the association between HPV 16/18 infection and lung cancer development in Taiwan.

Patients and Methods

Study Subjects. One hundred forty-one primary lung cancer patients including 45 female and 96 male, who had undergone thoracic surgery at Veterans General Hospital-Taichung, were enrolled. Sixty noncancer patients with different lung diseases, including pneumothorax, tuberculosis, chest wall deformity, cryptococcal infection, and fibrosis, all of whom had undergone thoracic surgery at Chen-Kung University Hospital, Tainan, or Changhua Christian Hospital, Changhua, served as control subjects. None of the subjects had received radiation therapy or chemotherapy prior to surgery. Information on smoking history was obtained from the patients by interview with informed consent. Smokers and

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[3] The abbreviations used are: HPV, human papillomavirus; ISH, *in situ* hybridization; DIG, digenoxenin; OR, odds ratio; CI, confidence interval.

[4] Unpublished data.

Table 1 *Characteristics of study subjects*[a]

Parameter	Lung cancer (n = 141)	Noncancer (n = 60)	P
Age (yr ± SE)	63.02 ± 9.61	49.95 ± 15.14	<0.001
Gender			
Female	45 (31.9)[b]	11 (18.3)	
Male	96 (68.1)	49 (81.7)	0.059
Smoking status			
Nonsmoking	78 (55.3)	48 (80.0)	
Smoking	63 (44.7)	12 (20.0)	0.001
Tumor type			
Adenocarcinoma	83 (58.9)		
Squamous cell carcinoma	58 (41.1)		
Tumor stage			
I	46 (32.6)		
II	42 (29.8)		
III	53 (37.6)		

[a] The difference of age between lung cancer and non-cancer cancer patients was calculated by the Wilcoxon rank sums test. The other status differences between lung cancer and non-cancer cancer patients were calculated by the χ^2 test.
[b] Numbers in parentheses are percentages.

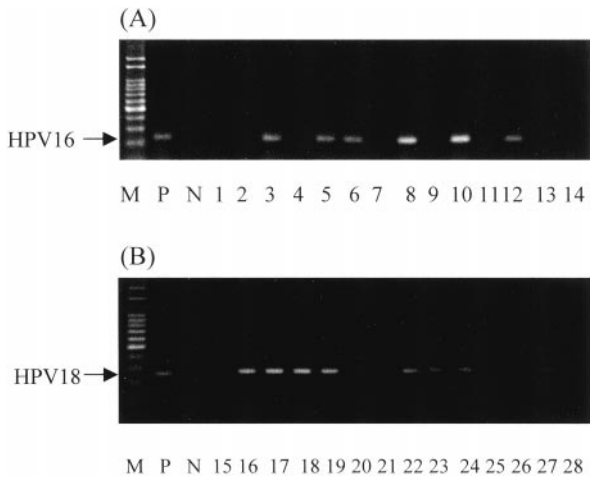

Fig. 1. Representatives of positive and negative HPV 16 (*A*) and HPV 18 (*B*) DNA detected by nested PCR in lung tumors of lung cancer patients (*Lanes 1–28*). *M*, 100-bp ladder DNA marker. *P*, positive control; DNA of SiHa or HeLa cell line served as positive control for HPV 16 and 18, respectively. *N*, negative control, the DNA template was replaced with distilled water.

nonsmokers were defined as current smokers who had smoked up to the day of pulmonary surgery and life-time nonsmokers, respectively. The characteristics of study subjects including age, gender, smoking status, tumor type, and tumor stage of lung cancer patients are shown in Table 1.

Nested PCR. Genomic DNA was prepared from a tissue section and isolated by conventional phenol-chloroform extraction and ethanol precipitation and was finally dissolved in 20 μl of sterile distilled water. HPV viral DNA was first amplified with type consensus primers MY09 and MY11 (10) followed by a second round of amplification with type-specific primers flanking the L1 region to identify the subtype. Ten μl of the final PCR product were loaded onto a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. Appropriate negative and positive controls were included in each PCR reaction. A part of the *β-actin* gene in all of the samples was amplified to exclude false-negative results whereas DNA preparations from the SiHa cells (containing HPV 16) and the HeLa cells (containing HPV 18) were used as positive controls.

ISH. ISH for the detection of HPV type 16 and 18 DNA was performed by using DIG-labeled oligonucleotide probes and a commercially available hybridization kit (Boehringer Mannheim, Indianapolis, IN). Briefly, the hybridizing probes were prepared by PCR amplification using HPV 16 or 18 type-specific primers (16UP, 5′-TACTAACTTTAAGGAGTACC-3′; 16DN, 5′-GTGTATGTTTTTGACAAGCAATT3-′; 18UP, 5′-CCAAATTTAAGCAGTATAGC-3′; 18DN, 5′-TTGTACAAAACGATATGTATCCA-3′) with DIG-labeled dUTP as substrate following the manufacturer's instructions. The deparaffinized and rehydrated 5-μm sections were digested with proteinase K, rinsed with PBS, and dehydrated. The hybridization was performed in a humidified chamber at 48°C for 16 h followed by washing with SSC. Thereafter, the detection reagent (anti-DIG antibody conjugated with alkaline phosphatase) was applied to the sections, and then the sections were incubated with the NBT/BCIP solution to allow the signals to develop. After the signal development, the sections were counterstained with methyl green, rinsed briefly in absolute ethanol, mounted, and observed for signals under a microscope.

Statistical Analysis. The differences of HPV 16/18 infection among gender, smoking status, tumor type, tumor stage, grade, and TMN value were calculated by the χ^2 test. Logistic regression analysis was used to assess which variable was important for HPV infection and which factor may act as a risk factor of lung cancer.

Results and Discussion

To elucidate the association between HPV infection and lung cancer development, nested PCR with two different sets of primers was used to examine the existence of HPV 16/18 DNA in lung tissues of lung cancer patients and noncancer control patients. To establish the specificity of nested PCR used in this study, we performed PCR amplification with two sets of primers, one type 16-specific and the other type 18-specific, on both the SiHa and the HeLa cell lines. The results proved the adequate specificity of this methodology because SiHa cells were amplified by type-16 primer but not by type-18 primers and HeLa cells were amplified by type-18 primers but not by

Table 2 *The differences of HPV infection between lung cancer and noncancer patients with different gender and smoking habits*

	Lung cancer		Noncancer		
Parameter	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	P
HPV 16					
Gender					
Female	18 (40.0)	27 (60.0)	9 (81.8)	2 (18.2)	0.031
Male	73 (76.0)	23 (24.0)	42 (85.7)	7 (14.3)	0.253
P	0.00007		0.664		
Smoking					
No	40 (51.3)	38 (48.7)	41 (85.4)	7 (14.6)	0.0002
Yes	51 (81.0)	12 (29.0)	10 (83.3)	2 (16.7)	1.0
P	0.0005		1.0		
HPV 18					
Gender					
Female	12 (26.7)	33 (73.3)	10 (90.9)	1 (9.1)	0.0002
Male	71 (74.0)	25 (26.0)	43 (87.8)	6 (12.2)	0.089
P	0.0000003		1.0		
Smoking					
No	33 (42.3)	45 (57.3)	42 (87.5)	6 (12.5)	<0.000001
Yes	50 (79.4)	13 (20.6)	11 (91.7)	1 (8.3)	0.444
P	0.00002		1.0		

Table 3 *Comparison of HPV 16/18 DNA detection results between nested PCR and ISH methods*

This study was done on sections of lung cancer patients because no paraffin sections of lung tissues from noncancer patients were available.

	ISH		
Nested PCR	Negative	Positive	Concordant %[a]
HPV 16			
Negative	83	8	
Positive	30	20	73.0
HPV 18			
Negative	74	9	
Positive	11	47	85.8

[a] Concordant % = (Number of patients with the same HPV DNA detection result by both methods)/(number of total patients) × 100.

type-16 primers (data not shown); therefore, we used SiHa and HeLa cells as positive controls for HPV 16 and HPV 18, respectively, and distilled water instead of DNA templates as the negative control. These appropriate control tests were performed in every set of experiment. Because the accuracy of results is the basis of this study, we evaluated the results with precaution. An internal type-specific probe was used to hybridize the PCR products of a few cases, and the results were consistent with those from nested PCR (data not shown). Furthermore, we performed *in situ* RT-PCR to amplify the E6 region, besides the L1 region, on the paraffin sections (data not shown) and, thus, further confirmed the accuracy of our methodology. The representative data of positive and negative HPV 16/18 DNA detection in lung tumors by nested PCR are shown in Fig. 1. As shown in Table 2, the detection frequency of HPV 16/18 DNA in lung cancer patients [77 (54.6%) of 141] was significantly higher than that of noncancer (control) patients [16 (26.7%) of 60; $P = 0.0005$]. When the study subjects were stratified by gender, age, and smoking status, HPV 16/18 DNA detection frequency among lung cancer patients who were female, ≤60 years old, and nonsmokers was statistically higher than that in noncancer patients, but the same difference was not observed among patients who were male, >60 years old, and smokers. Meanwhile, among the lung cancer group, female patients >60 years old and nonsmokers had significantly higher HPV 16/18 infection frequency than male patients >60 years old and smokers ($P = 0.000003$ for gender; $P = 0.018$ for age; $P = 0.000005$ for smoking status). However, no such difference between these three parameters was seen among the noncancer (control) group. The significantly high prevalence of HPV 16/18 in nonsmoking female lung cancer patients suggests that HPV infection may play a role in lung carcinogenesis among Taiwanese women nonsmokers.

In the literature, the prevalence of HPV infection in squamous cell lung carcinomas varies from 0 to 79% (7, 8). The high prevalence of HPV infection in lung carcinomas in countries such as Japan, China, Greece, and Finland reflects the possibility that the geographic vari-

Table 4 *Associations between the presence of HPV 16/18 DNA and clinicopathological parameters*[a]

	HPV16		HPV18	
Parameter	− (%)	+ (%)	− (%)	+ (%)
Tumor type				
Adenocarcinoma	47 (56.6)	36 (43.4)	42 (50.6)	41 (49.4)
Squamous cell carcinoma	44 (75.9)	14 (24.1)	41 (70.7)	17 (29.3)
P	0.030		0.027	
Tumor stage				
I	26 (56.5)	20 (43.5)	26 (56.5)	20 (43.5)
II	34 (81.0)	8 (19.0)	27 (64.3)	15 (35.7)
III	31 (58.5)	22 (41.5)	30 (56.6)	23 (43.4)
P	0.029		0.696	

[a] The HPV 16/18 DNA detection was performed by nested PCR.

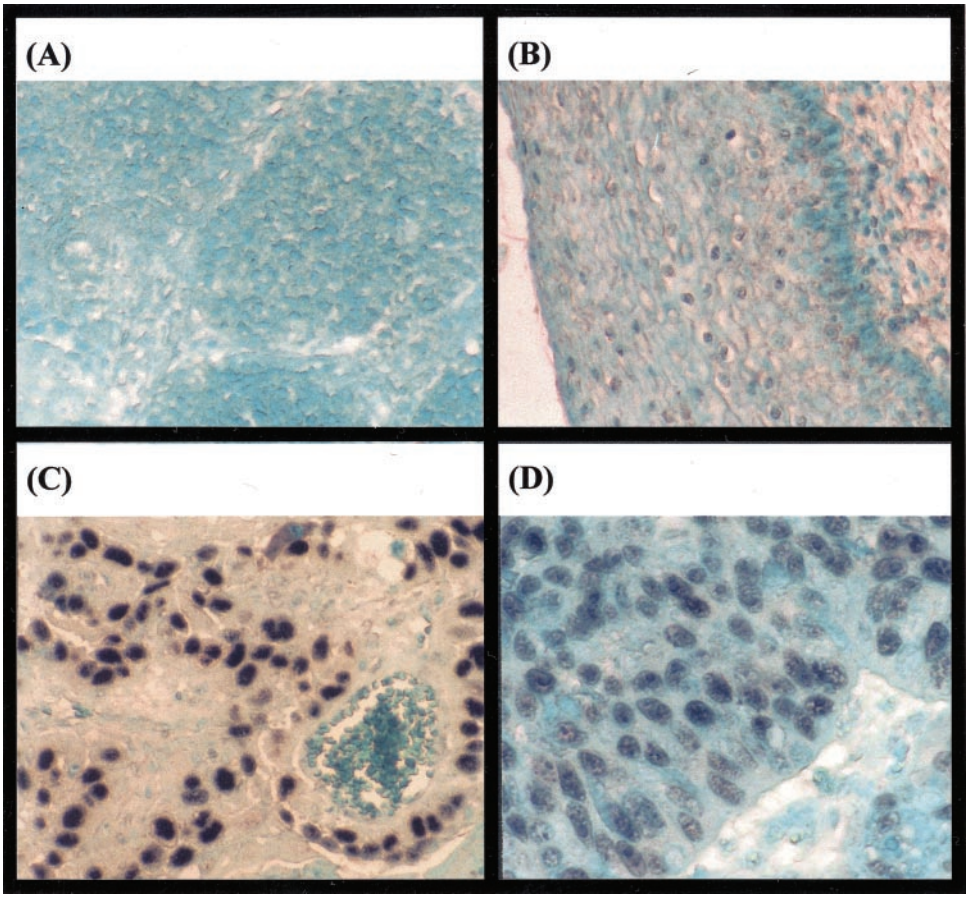

Fig. 2. Representative negative and positive HPV 16/18 DNA detection by ISH in paraffin sections of tumors. *A*, negative control; *B*, known positive cervical cancer; *C*, lung adenocarcinoma; *D*, squamous cell lung carcinoma.

Table 5 *OR of the presence of HPV in tumor tissues from lung cancer patients according to gender and smoking status*

		HPV16		HPV18	
Gender	Smoking	OR (95% CI)	AOR[a] (95% CI)	OR (95% CI)	AOR (95% CI)
Male	Yes	1	1	1	1
Male	No	2.13 (0.81–5.54)	1.77 (0.48–6.50)	2.20 (0.86–5.60)	2.30 (0.61–8.68)
Female	No	6.38 (2.68–15.17)	3.98 (1.13–13.98)	10.58 (4.30–26.0)	11.66 (2.94–46.27)

[a] AOR, OR after adjustment for age, tumor type, and tumor stage.

ability of HPV infection is associated with these lesions (9, 11, 14–16). However, the detection rates of HPV in squamous cell carcinomas reported from four previous large-scale studies with the number of cases being 185, 108, 94, and 85, were determined as 2.7, 4.6, 7%, and 0%, respectively (8, 11, 17, 18). The heterogeneity of the results can be reasonably explained by the different sampling modes for the tumor specimens: fresh-frozen or formalin-fixed, paraffin-embedded tissues (9–11,14–16, 18). To exclude such possibilities and to further confirm the specificity and sensitivity of nested PCR on the detection of HPV DNA in lung tumors, fresh-frozen and paraffin-embedded tissues were used in this study to detect HPV DNA by nested PCR and ISH, respectively. The concordant detection of HPV 16 and 18 DNA were seen in 73% (108 of 141) and 85.8% (121 of 141) of cases, respectively (Table 3). As shown in Fig. 2, DNA of HPV 16/18 was uniformly located in lung tumor cells but not in the adjacent nontumor cells. Unfortunately, the paraffin sections of lung tissues from noncancer subjects were not available; we could not locate HPV DNA in normal lung tissues. However, the high prevalence of HPV in Taiwanese lung carcinomas detected in this study does not seem to have been caused by the different modes of tumor specimen sampling.

The infection of HPV 16/18 has been well associated with the progression of cervical adenocarcinoma, and the infection rate was about 70% for cervical adenocarcinoma, which is lower than that for cervical squamous carcinomas (19). Surprisingly, similar phenomena were not observed in this study because a higher prevalence of HPV 16/18 was detected in the patient group with lung adenocarcinoma than in the group with squamous cell carcinoma [HPV 16 was detected in 36 (43.4%) of 83 cases of adenocarcinomas and in 14 (24.1%) of 58 cases of squamous cell carcinoma; HPV 18 was detected in 41 (49.4%) of 83 cases of adenocarcinoma and in 17 (29.3%) of 58 cases of squamous cell carcinoma; Table 4]. This unexpected finding is also in contrast to previous reports showing that HPV 16/18 was more prevalent in lung squamous cell carcinomas cases than in lung adenocarcinomas (11). To realize which influence factor among gender, tumor type, and smoking status was the most important to HPV infection in lung cancer patients, multivariate logistic regression analyses were performed and showed that gender is a much more important factor in HPV infection than are smoking status and tumor type (data not shown). We consider that such high prevalence of HPV infection in lung adenocarcinoma may be attributable to the high HPV prevalence among the nonsmoking lung cancer patients who have mostly lung adenocarcinomas. Nevertheless, the role of HPV 16/18 infection in lung adenocarcinoma development needs further investigation.

The OR of HPV 16/18 infection in lung tumors according to gender and smoking status was statistically analyzed (Table 5). Nonsmoking female lung cancer patients had a much higher OR of HPV 16/18 infection as 10.12 (95% CI, 3.88–26.38) compared with 1.98 (95% CI, 0.84–4.76) of nonsmoking male lung cancer patients. After adjustments for age, tumor type, and tumor stage, nonsmoking female lung cancer patients still had a significantly high OR value (OR, 8.82; 95% CI, 2.28–34.16; Table 5). These results suggest that HPV 16/18 infection is strongly associated with the lung cancer development of nonsmoking female lung cancer patients but not with that of male lung cancer patients in Taiwan. This surprising observation may be related to the fact that Taiwanese women have a history of cervical intraepithelial lesions and/or HPV 16/18 infection (12). In a case-control study on the association between Taiwanese cervical neoplasia and HPV infection, it has been shown that HPV DNA was detected in 91% of high-grade cases, 54% of low-grade cases, and 9% of controls. HPV 16 was the predominant type among HPV-positive high-grade cases as reported in Western countries (20). A recent study showed that HPV E6 mRNA was detected in the peripheral blood of advanced cervical cancer patients. It seems to reflect the possibility that HPV infection in lung tissues may originate in the cervix and would then be mediated by blood circulation (21). This speculation can be supported to a degree by the observation that HPV DNA was detected in the peripheral lymphocytes on the lung tumor section (data not shown). More detailed studies are under way in this laboratory and should provide more information to address this issue. Our preliminary data regarding p53 mutation and immunostaining results from female lung cancer patients lead us to consider the possibility that HPV infection may be associated with lung tumorigenesis through a HPV E6-dependent p53 degradation pathway, similar to the tumorigenesis of HPV-associated cervical cancer. In our series, 26 (68.4%) of 38 female lung cancer patients with HPV infection had shown p53-negative immunostaining. Only seven female lung cancer patients had HPV-negative infection, but six of these seven patients showed p53-negative immunostaining. We need to increase the sample size to further elucidate the role of HPV infection in p53-negative immunostaining. We considered that HPV infection was not responsible for p53 inactivation in the study population, and this partly explained p53-negative immunostaining in female lung cancer patients. Nevertheless, HPV 16/18 infection may partly play a role in lung tumorigenesis in nonsmoking Taiwanese women. Furthermore, the detection of HPV E6 mRNA in lung tumors will provide more evidence for understanding whether p53 inactivation in lung tumor cell lines is mediated through HPV E6 protein. In conclusion, HPV infection may be associated with the development of nonsmoking female lung cancer in Taiwan, and the high prevalence of HPV 16/18 infection may explain to a certain extent why Taiwanese nonsmokers have a higher lung cancer mortality rate.

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