Human papillomavirus type 16 and 18 infection is associated with lung cancer patients from the central part of China

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Abstract. Infection with specific high-risk human papillomavirus (HPV) types 16 and 18 have been strongly associated with the genesis of various neoplasms in humans, though such study in lung cancer is limited and the results are controversial. In the present study, we collected and explored 313 fresh lung tumor specimens for the presence of HPV with polymerase chain reaction and non-isotopic *in situ* hybridization. We found that 44.1% of (138/313) non-small cell lung carcinoma (NSCLC) samples were positive for HPV detection, while 4.2% (4/96) of lung benign controls were positive for HPV 16 and 18 DNA. HPV infection was significant between lung squamous cell carcinoma and adenocarcinoma as well as smoking and non-smoking patients. In HPV-positive lung cancer tissues, abnormal p53 protein accumulation was seen in 97 of the 138 carcinomas (70.3%) and expression of pRb in 54 of the 138 carcinomas (39.1%). There was an obvious relationship between the presence of papilloma viral DNA and abnormal p53 protein accumulation and pRb depletion. Cell proliferation and apoptosis were correlated with HPV infection in NSCLC samples. Our data confirm the high prevalence of HPV in lung carcinomas in the central part of China and suggest the possible mechanism of the carcinogenic role of HPV in these carcinomas.

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Introduction

Lung cancer is the most common cancer and cause of cancer-related deaths in China and worldwide. The pathogenesis of lung cancer is complex and is believed to be due to the interaction between environmental and genetic factors (1). Although cigarette smoking is the most important environmental risk factor for the development of lung cancer, recently, the incidence of lung adenocarcinoma has increased and most individuals with lung adenocarcinoma (especially women) are non-smokers (2). Thus, the exploration of carcinogenic etiologies other than cigarette smoking is warranted.

Human papillomavirus (HPV) has been shown to be implicated in human neoplasms including the 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. The first indication of the possible involvement of HPV in bronchial squamous cell carcinogenesis was given by Syrjänen, based on histological data (4). Subsequently, HPV DNA was detected in several isolated cases of bronchial squamous cell carcinoma, although many biopsies have been screened (5-12). Many studies from Taiwan show that HPV infection is correlated with lung cancer of non-smoking females (13-16). In contrast to the foregoing positive results, other groups recently did not detect any HPV DNA sequences or found only low infection prevalence in lung carcinomas (17-24). These studies have been affected by varying geographical locations, race and different methods used, making the results difficult to compare.

In order to explore the relationship between HPV infection and lung cancer in the central part of China, we screened for HPV DNA in 313 fresh lung specimens using polymerase

chain reaction (PCR) and confirmed with non-isotopic *in situ* hybridization (ISH). Then, p53, pRb, Ki-67 proteins and cell apoptosis were detected by immunohistochemistry (IHC) and TdT (terminal deoxynucleotidyl transferase) mediated X-dUTP nick end-labeling (TUNEL) to evaluate the differences between the infected and non-infected groups of cancer in this study. These results provide more detailed information to support the association between HPV 16/18 infection and lung cancer development in China.

Materials and methods

Patients and samples. A total of 313 patients were diagnosed with NSCLC at Tongji Hospital (Wuhan, China). The mean age of the patients was 54.71 (range 35-83) years, with 76 women and 237 men. Lung tissue resected from primary tumors provided fresh samples, which were immediately frozen at -80˚C. Ninety-six noncancer patients with different lung diseases, including pneumothorax, tuberculosis, bullae lung, cryptococcal infection, lung cyst and inflammatory pseudotumor, all of whom had undergone thoracic surgery at Tongji Hospital, served as control subjects. None of the patients had received radiotherapy or chemotherapy prior to surgery. All specimens were diagnosed and classified by three experienced pathologists according to the World Health Organisation (WHO) classification. These included 215 squamous cell carcinomas (SCC) and 98 adenocarcinomas (AC). Information on smoking history was obtained from the patients by interview with informed consent. Smokers and non-smokers were defined as current smokers who had smoked up to the day of pulmonary surgery and life-time non-smokers, respectively. The Institutional Review Board of the Medical School of Huazhong University of Science and Technology approved the study. The study was carried out in accordance with the Declaration of Helsinki.

Polymerase chain reaction (PCR) for HPV. Genomic DNA was prepared from frozen tissues by a conventional method. Briefly, the tissues were crushed and ultrasonicated in an extraction buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 20 μg/ml RNase and 0.5% SDS). After boiling for 10 min at 100˚C, the samples were digested with proteinase K (100 μg/ml) at 50˚C for 3 h and the supernants were then obtained by centrifugation. The genomic DNA was isolated by phenol-chloroform extraction and ethanol precipitation and finally dissolved in 50 μl sterile distilled water. HPV 16 and 18 genomic DNA were obtained by amplification with type-specific primers in a 50 μl reaction system consisting of 1 μl (10 μg) of genomic DNA as a template, 0.2 mM dNTP, 50 pM of each of the primers, 2.5 units of Taq DNA polymerase (Promega, Madison, WI, USA), 2 mM MgCl$_2$, 5 μl 10X buffer and overlaid with 10 μl mineral oil (Sigma, St Louis, MO, USA). The amplification reaction was carried out in a Minicycler Model PTC-100 (MJ Research Inc, Watertown, MA, USA) by 40 cycles consisting of three steps: denaturation at 94˚C for 1 min, annealing at 57˚C for 30 sec and extension at 72˚C for 1 min. The genomic DNA from SiHa and HeLa cells were loaded as a positive control for HPV 16 and 18 (25,26). The final PCR product of 10 μl was loaded onto a 1.5% agarose gel, stained with ethidium bromide and

visualized under UV illumination. The following were the primer sequences of HPV 16 and 18: HPV16 E6: forward 5'-CTGCAAGCAACAGTTACTGCGACG-3', reverse 5'-CATACATCGACCGGTCCACC-3', product of 315 bp; HPV 18 E7: forward 5'-GAGCCGAACCACAACGTCAC-3', reverse 5'-GGATGCACACCACGGACACA-3', product of 152 bp.

In situ hybridization (ISH) for HPV. Nucleic acid ISH for HPV 16/18 was performed using digoxin labelled DNA probes and a commercially available hybridization kit (Pan-path, Amsterdam, The Netherlands) following the manufacturer's instructions. In brief, the deparaffinised and rehydrated 3-5-μm sections were first digested with pepsase at 37˚C for 10 min, rinsed with Tris-buffered saline (TBS) and dehydrated. Hybridization probes (20 μl/section) were applied on the slides and the DNA was denatured at 95˚C for 5 min. The sections were hybridized in a humidified chamber at 37˚C overnight. After hybridization, the sections were washed with TBS and Pan Wash and then were blocked and added to 20-40 μl of the detection reagents (biotin conjugated anti-digoxin antibody and StreptAvidin Biotin-peroxidase Complex, SABC). The slides were then incubated in the substrate solution (3,3-diaminobenzidine, DAB) and counterstained with haematoxylin (1 min). After being rinsed briefly in absolute ethanol and xylene, the sections were mounted with Eukitt (Kindler, Freiburg, Germany). We used slides with acetone-fixed SiHa and HeLa cells containing HPV 16 and 18 DNA as positive controls. Slides without adding the probe were used as the negative controls.

Immunohistochemical (IHC) staining. Sections were cut into 5 μm from the specimens and placed on poly-L-lysine-coated slides (Sigma). The slides were dewaxed in xylene and rehydrated in graded alcohol. Then, the endogenous peroxidase was consumed and the sections were blocked for non-specific association. We used the avidin-biotin-peroxidase complex method for HPV 16/18 E6, p53, pRb and Ki-67 immunostaining. The sections were incubated with the primary antibodies (E6, at 1:100; p53 at 1:50; pRb at 1:50 and Ki-67 at 1:100) (all of the antibodies were the products of Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA) at 4˚C overnight, followed by a biotinylated secondary antibody (dilution at 1:100) (Santa Cruz) and the avidin-biotin-peroxidase complex (Santa Cruz). The color was developed with DAB, counterstained lightly with haematoxylin and mounted with Eukitt. Negative control staining was carried out by substituting phosphate-buffered saline (PBS) for the primary antibody. The known positive slides (provided by the reagent company) were used as a positive control.

TdT-mediated X-dUTP nick end-labeling (TUNEL) assay. TUNEL assay was performed to detect cell apoptosis according to the manufacturer's instructions (Roche Diagnosis, Basel, Switzerland). In brief, the slides were dewaxed and rehydrated in graded alcohol, then quenched for the endogenous peroxidase. After being digested with proteinase K at 37˚C for 20 min, the sections were rinsed with PBS and incubated with a TUNEL reaction mixture (solution A: solution B=1:9) at 37˚C for 2 h. The slides were then blocked with normal goat serum and incubated with POD reagent in a

Table I. Relationships between the presence of HPV DNA in the study subjects and characteristic parameters.

| Features | Number | HPV16, 18 DNA[a] | | χ^2 | P-value |
		Positive	Negative		
Gender					
Male	237	110	127	2.14	0.1435
Female	76	28	48		
Histological type					
Squamous cell carcinoma	215	112	103	17.84	0.0002[b]
Adenocarcinoma	98	26	72		
Differentiation degree					
Low	113	43	70	0.17	0.6801
Moderate	123	66	57		
High	77	29	48		
Lymph node metastasis					
Yes	201	94	107	1.63	0.2017
No	112	44	68		
Smoking status					
Yes	208	107	101	13.60	0.0002[b]
No	105	31	74		

[a]Data present are detected by PCR. [b]$P<0.05$.

humidified chamber at 37˚C for 40 min. The tissues were reacted with DAB and counterstained with haematoxylin. The positive control was obtained from the Roche Diagnosis Company and the negative control was treated by substitution of the TdT enzyme solution (solution A) with PBS.

Image analysis. The positive slides had brown staining in the nucleus (p53, pRb, Ki-67 and TUNEL) and in the cytoplasm (HPV E6). We analyzed each positive slide with an HMIAS-2000 high definition image analysis system (Qianping Image Technology Co., Ltd., Wuhan, China) in >5 high magnification fields, calculating >1000 cells. The percentages of proliferation or apoptosis cells plus the apoptotic body in the total cells were defined as proliferation index (PI) and apoptosis index (AI), respectively.

Statistical analysis. Statistical analysis was performed using the SPSS 12.0 statistical software program. Differences of HPV 16, 18 infections between gender, smoking status, histological cell types, differentiation degree, lymph node metastasis, PI and AI were calculated using the Pearson's Chi-square test. The expression levels of p53, pRb and E6 in the HPV 16, 18-positive and negative groups were analyzed by the McNemar's test.

Results

Clinical characteristics of patients. The patients ranged from 35 to 83 years of age (mean 54.71 years), where 237 of the 313 were males. Two hundred and eight patients had a history of cigarette smoking, while 105 were non-smokers. A

histological examination of the tumors revealed 98 cases of AC and 215 cases of SCC. Tumor differentiation degrees of low, moderate and high were observed in 113 (36.1%), 123 (39.3%) and 77 (24.6%), respectively. Of the tumor cases 201 (64.2%) had lymph node metastasis. The characteristics of the study subjects of lung cancer patients are shown in Table I.

Detection of HPV 16/18 in lung tissues. The detection rates of HPV 16, 18 DNA by PCR were 44.1% (138/313) in the NSCLC samples. We re-examined the positive samples by ISH and IHC. Except for 15 cases, most cases were positive for both PCR and ISH, while only 67.7% (88/138) of the samples were positive for PCR expressed HPV E6 (Fig. 1). There were no differences of HPV infection in the clinico-pathological status including gender, differentiation degree and lymph node metastasis. However, patients of SCC had a significantly higher rate of HPV infection than patients of AC (52.1 vs. 26.5%, P<0.05). Likewise, smokers of the NSCLC patients also had a higher rate of HPV infection than non-smokers (51.4 vs. 29.5%, P<0.05) (Table I). The co-infection of HPV 16 and 18 was not significant (data not shown). Moreover, we found koilocytes existed in some cases of NSCLC, which were positive for HPV DNA (Fig. 1G).

Relationship between HPV infection and expression levels of p53 and pRb. Since tumor suppressor proteins, p53 and pRb, are degraded and inactivated by HPV E6 and E7 (27,28), we examined their expression levels in NSCLC tissues by IHC. The mutated p53 protein, which lacks the physiological properties of the wild-type p53 protein, is more stable and
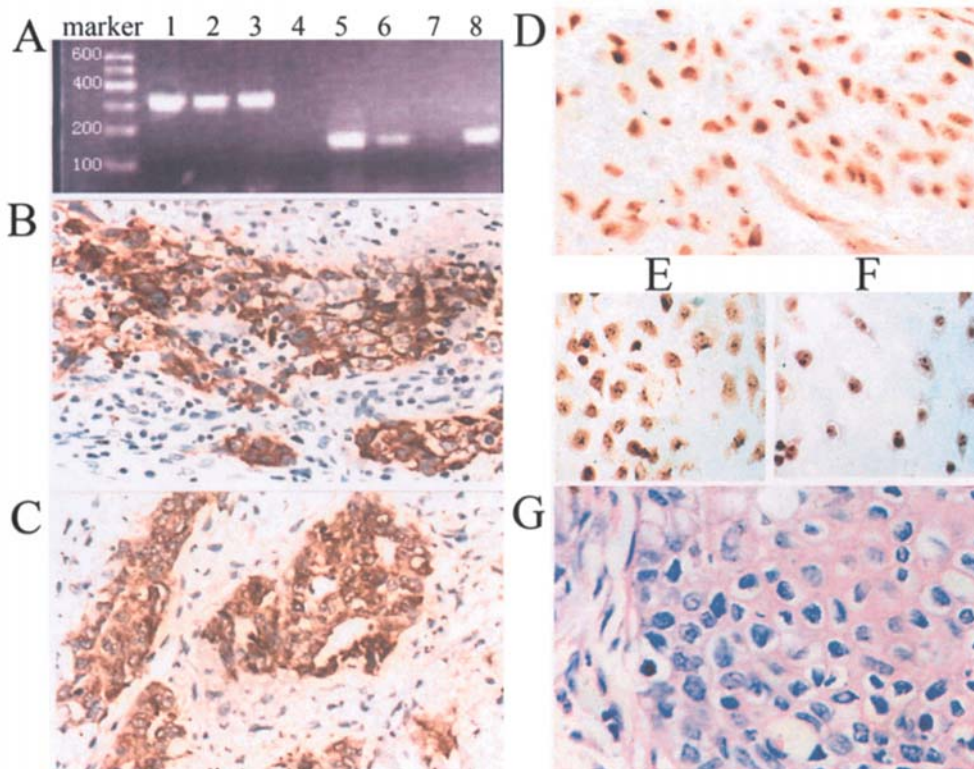
Figure 1. Presence of HPV DNA in human NSCLC. (A) Representation of positive and negative HPV 16 and 18 DNA were detected by PCR in lung tumor samples. Lanes 1 and 5: positive controls. DNA of SiHa (1) and HeLa (5) cell lines served as a positive control for HPV 16 and 18, respectively. Lanes 2 and 3: positive of HPV 16 DNA. Lanes 6 and 8: positive of HPV 18 DNA. Lanes 4 and 7: negative controls of HPV 16 and 18 DNA. (B and C) HPV E6 oncoprotein existed in SCC (B) and AC (C) of the lung. Magnification, x200. (D) HPV DNA was detected by ISH in SCC. HeLa (E) and SiHa (F) cells were used as positive controls for HPV 16 and 18 DNA. Magnification, x400. (G) The koilocytes are shown in lung SCC, which constituted nuclear angulation surrounded by perinuclear clear halo. Magnification, x400. All these figures show representative experiments from NSCLC samples which were repeated three times.

Table II. The presence of HPV DNA in relation to immunohistochemical results of p53 and pRb. Data of PI and AI represent the means of percentages, which indicate the ratios of positive staining cells for Ki-67 and TUNEL in total cells.

HPV	Mutant p53		pRb		PI (%)	AI (%)
	+	-	+	-		
+	97	41	54	84	36.24±0.5	1.09±0.9
-	113	62	130	45	16.59±9.1	5.87±2.64
	$\chi^2=33.66$	P<0.05	$\chi^2=9.89$	P<0.05	P<0.05	P<0.05

consequently has a longer half-life (29). The accumulation of such a mutated dysfunctional protein can be detected immunohistochemically in the affected cells in contrast to the wild-type protein which has a short half-life (30,31). P53 and pRb immunostaining was evaluated as positive (+) (Fig. 2) or negative (-) (data not shown) according to the presence or absence of nuclear immunostaining in tumor cells. In the HPV positive group, we found that 70.3% (97/138) of the cases were positive for p53 staining, while 39.1% (54/138) were positive for pRb (Table II). There were significant differences of p53 and pRb immunostaining in HPV-positive or negative NSCLC cases.

Relationship between HPV infection and cell proliferation and apoptosis. Both p53 and pRb are negative regulators of the cell cycle and have an important role for normal cell growth. HPV infection inactivates p53 and pRb, then disrupts the cell cycle and causes proliferation and anti-apoptosis (32,33). We detected cell proliferation and apoptosis *in situ* by Ki-67 immunostaining and TUNEL and then defined the proliferation index (PI) and apoptosis index (AI). The results were analyzed with a HMIAS-2000 high definition image analysis system. In our study, the PI of HPV-positive groups was higher than that of the negative groups, though the AI was observed as a reverse result (Fig. 3, Table II).

Discussion

Since HPV condylomatous changes in the lung neoplasm was first reported by Syrjänen, HPV DNA was found in lung cancer worldwide (16,34). Our study of a large series of 313 NSCLC demonstrated the detection of HPV DNA in 138 cases
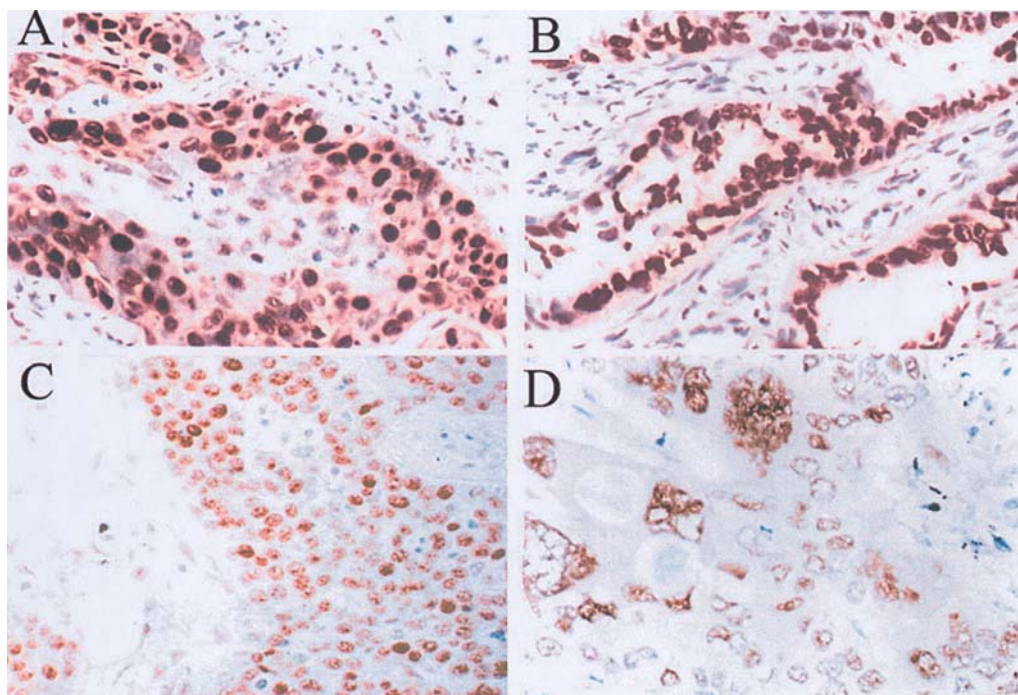
Figure 2. Examples of immunohistochemical results for mutant p53 and pRb in human NSCLC. Mutant p53 is shown as a positive staining in SCC (A) and AC (B) and pRb is seen as positive in SCC (C) and AC (D). These figures show representative experiments from NSCLC samples, which were independently repeated three times. Magnification, x200.
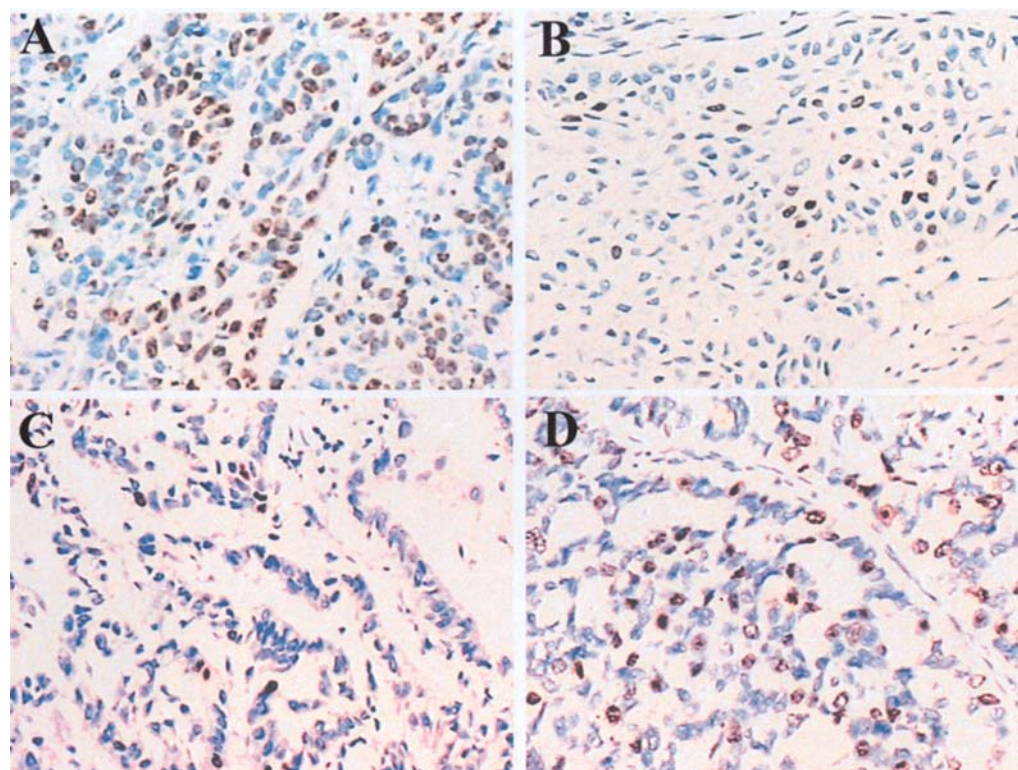

Figure 3. Detection of proliferation and apoptosis in human NSCLC with or without HPV infection. (A and B) The immunohistochemical staining results of Ki-67 are shown in HPV-positive (A) and -negative (B) samples from NSCLC patients. (C and D) Cell apoptosis was detected by TUNEL in HPV-positive (C) and -negative (D) samples. These figures show representative experiments from NSCLC samples, which were repeated three times. Magnification, x200.

(44.1%). In the literature, the infection rate of HPV DNA in lung cancer patients varies significantly from 0 to 79% (9,35). The high prevalence of HPV detection in lung

carcinomas in countries such as Japan (35), Taiwan (16), Greece (8), Iran (12) and Chile (11) may reflect the possibility of a geographic variability of HPV infection associated with

these lesions. The disparities between the positive results may be related to the different sizes, sample modes and detection methods of the series reported. To our knowledge, our study is at the present time the most important considering the number of lung tumors explored. Many previous studies have been performed with 218 (24), 185 (22), 149 (14), 141 (13) and 121 (10) different carcinomas, reporting HPV detection in 1.8, 2.7, 47.7, 54.6 and 33.9%, respectively. Moreover, the heterogeneity of the results is largely explained by the mode of the sampling of tumor specimens of fresh-frozen, formalin-fixed and paraffin-embedded tissues. To avoid modified or fragmented DNA from archival tissue due to formalin fixation, we chose to work with fresh and immediately frozen tumor samples. Furthermore, the HPV detection method interferes with the quality and quantity of the results obtained. *In situ* hybridization localizes the cells infected within the tumor, though its sensitivity is weak and detects ~20-50 copies per cell. Most studies have used PCR with 'consensus' or specific type primers amplifying HPV DNA. Some authors have used two sets of primers and nested PCR to improve the rate of HPV detection (18). Each of these PCR methods have their own drawbacks, with the difficulty of obtaining large amplimers, especially when formalin-fixed material is used. In the current study, we used PCR with specific type primers to detect HPV DNA from the fresh-frozen samples and then confirmed the results by ISH. As noted above, the sensitivities of ISH and IHC are weaker than PCR. Our results showed that the prevalence of HPV in lung cancer was 44.1% (138/313) by PCR, 39.3% (123/313) by ISH and 28.1% (88/313) by IHC, which corresponds with literature. More importantly, we found that typical koilocytes existed in certain cases of NSCLC, which were positive for HPV DNA (Fig. 1G). In our study, the prevalence of HPV detection in lung carcinomas in the central part of China is high.

The positive rates of HPV DNA were not significant in the clinicopathological status including gender, differentiation degree and lymph node metastasis except for histological type and smoking status. Patients of SCC or smokers had significantly higher rates of HPV infection than patients of AC or non-smokers (Table I). Squamous-columnar junctions (SCJs) between respiratory epithelium (columnar cells) and stratified squamous epithelium are present in the mucosa of the pharynx and larynx, respectively (3). SCJs are detected in bronchial spurs and thus may play an important role in the spread of HPV. This pulmonary infection pathway may be comparable to the pathway associated with HPV infection of the uterine cervix, in which HPV infects the transformation zone, where benign condylomas and cervical carcinoma precursor lesions arise (3). Cigarette smoking induces squamous metaplasia of respiratory epithelium and produces more SCJs. Our results suggested that smokers brought more of an infection pathway for HPV and so had high rates of infection, which might be related to SCC.

In HPV-infected cells, oncoprotein E7 interacts with pRb, increases in active E2F and induction of DNA synthesis (28). Rb-E2F complexes dissociate and free E2F becomes available as a transcriptional activator. Accordingly, E7 promotes cell proliferation by stimulating the transcription of E2F-responsive genes, which in turn propel the cell through the cell cycle (33). Similarly, HPV E6 proteins abrogate the p53

oncosuppressive functions by inducing its degradation through the ubiquitin-proteasome pathway (27). There were obvious relationships between the presence of papilloma viral DNA and abnormal p53 protein accumulation and pRb depletion (Table II). Furthermore, the interaction between E6 and p53 is preventing p53-induced apoptosis (32) and E7 resists apoptosis by the inactivation of pRb together with p21 to inhibit cell cycle arrest (33). Our results suggested that PI rather than AI increased in NSCLC patients with HPV infection (Table II, Fig. 3), which might be a mechanism of carcinogenesis for HPV.

In conclusion, our data demonstrated that HPV was associated with NSCLC in China, especially with patients of SCC or smokers. In these cases, HPV degraded pRb and increased mutated p53, inducing cell proliferation and resisting apoptosis. The loss of heterozygosity of the FHIT gene, p16^{INK4a} promoter hypermethylation and p53 inactivation were frequently found in HPV-infected non-smoking female lung cancer patients in Taiwan (36-38) and transfection of HPV DNA into cultured adenocarcinoma cells induces squamous metaplasia (39). Thus far, it is difficult to expand on an understanding of the role of HPV in bronchial carcinogenesis *in vivo*. More effort, including clinical and epidemiological studies, should be focused on assessing the role of HPV in bronchial carcinogenesis.

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