Correlation of HPV-16/18 infection of human papillomavirus with lung squamous cell carcinomas in Western China

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Abstract. Prevalent inconsistency of human papillomavirus (HPV) infection in lung cancer was found among recent studies from different countries with racial and geographic variations. Even in Chinese populations, the prevalent discrepancy of HPV infection in lung cancer patients was also found with the geographic variations and tumor types. To study the difference of HPV-16/18 infection in lung squamous cell carcinomas and non-cancer controls, we conducted this study to verify whether there was a similar HPV infection prevalence pattern in lung squamous cell carcinoma patients from the Western part of China. Paraffin-embedded samples (180), of 72 lung squamous cell carcinomas, 37 lung adenocarcinomas and 71 non-cancer controls, were analyzed by INNO-LIPA Genotype polymerase chain reaction (PCR) and real-time PCR analysis. The data showed that 51.4% (37/72) of lung squamous cell carcinoma samples, 16.2% (6/37) of adenocarcinoma, and 22.5% (16/71) of non-cancer controls were HPV DNA positive. The risk of lung squamous cell carcinomas was 3.5 times higher among people HPV-positive (odds ratio 3.5, 95% CI 1.6-7.3, p<0.001) compared with the HPV-negative population. Adjusted by smoking status, the risk of lung squamous cell carcinomas was 3.5 times higher among people HPV-positive (odds ratio 3.5, 95% CI 1.7-7.5, p<0.001) compared with the HPV-negative population. The risk of lung squamous cell carcinomas was 16.9 times higher for patients with positive HPV-16 (odds ratio 16.9, 95% CI 3.8-75.3, p<0.0001) than negative HPV-16. Adjusted by smoking status, the risk of lung squamous cell carcinomas was 17.4 times higher among people HPV-positive (odds ratio 17.4, 95% CI 3.9-77.5, p<0.0001) compared with HPV-16 negative people. INNO-LIPA Genotype analysis revealed that the frequency of high risk HPV-16 and 18 genotype in lung squamous cell carcinoma cases appeared to be significant higher than that in the non-cancer controls (P<0.001), and the most frequent genotype was HPV-16 (21 cases), followed by HPV-18 (6 cases), HPV-16/18 (4 cases) in lung squamous cell carcinomas, respectively, and the majority genotype was HPV-6 (9 cases) in controls. The HPV-16 E6/E7 ratio was significantly lower than the unity, suggested that the main presence of integrated form of HPV-16 genome (16/27) may contribute more to lung squamous cell carcinomas, despite its viral load estimated to be only <1 to 2 copies per cell.

Introduction

Human papillomavirus (HPV) is a double stranded DNA virus, and is an established etiological agent causing cervix cancer. Its high-risk genotypes, including HPV-16 and -18, are associated with cervix cancer and other genital cancers (1,2). Lung cancer is a major health problem and the leading cause of cancer deaths in the world. The pathogenesis of lung cancer is complex, and is believed to be due to the interaction between environmental and genetic factors. Various evidence shows that HPV might be associated with bronchial carcinogenesis and lung cancer (3-6).

Although smoking was the most frequent risk factor of lung cancer, the mechanism is not clear (7). Etiological involvement of HPV in development of lung cancer was originally postulated (8-10). Inconsistency in prevalence of human papillomavirus (HPV) infection in lung cancer was found among recent studies from different countries with racial and geographic variations. A large sample investigation reported that HPV DNA infection rate was 21.7% in 2468 lung carcinomas (11). HPV infection is considered a co-carcinogen of lung cancer and its contribution to pathogenesis has been evaluated (12-18). However, even in Asian populations, the prevalence discrepancy of HPV infection in lung cancer patients was also found with the geographic variations and tumor types. A study in Taiwan (13) reported that 77 (54.6%) of 141 lung tumors had HPV-16 or -18 DNA compared with 16 (26.7%) of 60 non-cancer control subjects among non-smoking Taiwanese women, and a recent study from mainland China suggested that the rates of HPV-16 and/or -18 infections in patients with 73 lung tumors were significantly higher than in 34 non-cancer control subjects (27.7 vs. 5.9%) (19). In the central part of China
investigators have found that 44.1% of (138/313) NSCLC samples are positive, while 4.2% (4/96) of lung benign controls are positive (20).

To study the role of integration of HPV-16 DNA into the host genome which may contribute to lung squamous cell carcinoma development, we conducted this study to verify whether there was a similar HPV infection prevalence pattern and HPV-16 integration status in lung squamous cell carcinoma patients from Western China.

Materials and methods

Samples. The 180 paraffin-embedded samples, of 72 lung squamous cell carcinomas, 37 lung adenocarcinomas and 71 non-cancer controls were collected and diagnosed in the first affiliated hospital of Xi’an Jiaotong University, China. The 71 non-cancer controls included tuberculomas 41, hyperplasia 19, granuloma 8, and pseudoplasm 3, respectively. All of these cases were from the West part of China during the period from 2005 to December 2007. The demographic features review and statistical analysis were approved by the Department of Public Health, Xi’an Jiaotong University School of Medine, China. The study was approved by the Ethics Committee of Xi’an Jiaotong University, China.

DNA extraction. Formalin-fixed paraffin-embedded samples were cut into 10 μm slices and prepared according to the method described before (21). The specimens were treated with 1 ml of xylene, and then with 1 ml of ethanol. After centrifugation, the pellet was resuspended in digestion buffer (50 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 0.5% Tween-20) containing 200 μg of proteinase K (Invitrogen) and incubated at 56˚C overnight. After the incubation, the solution was heated at 100˚C for 10 min and centrifuged. An aliquot of the supernatant was directly used for PCR.

β-globin PCR. β-globin amplification with PCO 3/PCO 4 (110 bp) primers (PCO 3 primer 5'-ACACAACTGTGTTCA and PCO 4 primer 5'-CAATTGTTCAGGACC; E 2F: AACGAAGTATCCTCTCCTGTC ACC-3') was used as the internal positive control (22). PCR condition was as follows: the initial denaturation at 95˚C for 4 min, 40 cycles with the cycling profile of 95˚C for 1 min, 52˚C for 1 min and 72˚C for 2 min and the final extension for 5 min at 72˚C. DNA purified from HeLa cells containing, HPV-16 was used as external positive control.

INNO-LIPA genotyping system. A 65 bp region of L1 gene of the HPV genome was amplified by PCR using short PCR fragment (SPF10) biotinylated primers. The PCR products were visualized on 4% agarose gel with ethidium bromide staining by electrophoresis and (10 μl) of the PCR product was denatured and hybridized with specific oligonucleotide probes (25 HPV type-specific probes) immobilized as parallel lines on a nitrocellulose membrane strips, following the manufacturer’s instructions (INNO-LIPA HPV genotyping kit, Inno-genetics, Belgium). The 28 probes for all samples. The HPV-positive signal was not only detected in the cases and controls was 63.6±11.7 years and 63.3±12.4, respectively (data not shown).

The β-globin gene was successfully amplified by PCR in all samples. The HPV-positive signal was not only detected in the agarose-gel electrophoresis but was confirmed by INNO-LIPA HPV genotyping analysis kit. Thirty-seven cases were detected in the lung squamous cell carcinoma group, 6 cases were detected in 37 lung adenocarcinomas, while 16 cases were detected in non-cancer control group. The frequency of HPV-positive cases (51.4%) was higher in lung squamous cell carcinomas than in adenocarcinomas, while 16 cases were detected in non-cancer control group. The frequency of HPV-positive cases (51.4%) was higher in lung squamous cell carcinomas than in adenocarcinomas and non-cancer control group (16.2 and 22.5%) (P<0.001) (Table I).

Real-time PCR. Real-time PCR was performed with the ABI Prism 7000 Sequence Detection System and SYBR-Green PCR master mix (PE. Applied Biosystems). The amplification conditions were 15 cycles at 95˚C and a two-step cycle of 95˚C for 30 sec and 60˚C for 60 sec for a total of 40 cycles. The used primer sets were as follows: E 6F: GAGAAACTG CAATGTTCAGGACC and E 6R: TGTATAGTGTTTTGC AGCTCTGTGC; E 6F: AACGAAGTATCCTCCTCCTGAA ATTATTAG, and E 6R: CCAAGGCGACGGCTTTG. Those primers amplify a fragment of E 6 (81 bp) and E 6 (76 bp) ORFs, respectively (24). The final concentration of primers was 0.5 μM. All the experiments were made in duplicate. The specificity of amplification was confirmed using dissociation analysis starting at 60˚C and agarose gel electrophoresis of amplified products.

Statistical analysis. The central focus of our analysis was to examine the independent associations between HPV infection and lung squamous cell carcinomas, modeled as categorical variable. Significant differences in demographic characteristics and smoking status across case and control were assessed using χ² test or Fisher’s exact test for the equality of proportions. The associations between HPV status, HPV-16 status and lung squamous cell carcinomas were assessed using logistic regression and were expressed as the odds ratio (OR) and its 95% CI. We evaluated possible effect modification by smoking status by adding product terms to the regression models. Differences were considered significant at P<0.05. We used Stata version 9.2 (Stata /SE 9.2 StataCorp, College Station, TX) for all analyses.

Results

Demographic features of lung carcinoma cases and controls are summarized. Male patients accounted for 66.7% (48 patients) of the 72 lung squamous cell carcinomas cases, 64.9% (24 patients) in 37 lung adenocarcinomas and 63.4% (45 patients) in 71 non-cancer control cases. The mean age of the cases and controls was 63.6±11.7 years and 63.3±12.4, respectively (data not shown).

The β-globin gene was successfully amplified by PCR in all samples. The HPV-positive signal was not only detected in the agarose-gel electrophoresis but was confirmed by INNO-LIPA HPV genotyping analysis kit. Thirty-seven cases were detected in the lung squamous cell carcinoma group, 6 cases were detected in 37 lung adenocarcinomas, while 16 cases were detected in non-cancer control group. The frequency of HPV-positive cases (51.4%) was higher in lung squamous cell carcinomas than in adenocarcinomas and non-cancer control group (16.2 and 22.5%) (P<0.001) (Table I).

Statistical analyses revealed that HPV infection was not correlated with gender, age, tumor stage and grades, but the positive rate of HPV is 42.5% (48/113) and 15.8% (3/19) in the smoker and non-smoker, respectively, and the frequency of HPV-positive in the cases was significantly associated with smoking habit (p<0.034) (Table II).

The risk of lung squamous cell carcinomas was 3.5 times higher among people HPV-positive (odds ratio 3.5, 95% CI 1.6-7.3, p<0.001) compared with HPV-negative people.
Adjusted by smoking status, the risk of lung squamous cell carcinomas was 3.5 times higher among people HPV-positive (odds ratio 3.5, 95% CI 1.7-7.5, p=0.001) compared with HPV-negative people.

The risk of lung squamous cell carcinomas was 16.9 times higher for people with positive HPV-16 (odds ratio 16.9, 95% CI 3.8-75.3, p<0.0001) than those with negative HPV-16. Adjusted by smoking status, the risk of lung squamous cell carcinomas was 17.4 times higher for people with positive HPV-16 (odds ratio 17.4, 95% CI 3.9-77.5, p<0.0001) than that with negative HPV-16. There was no association between smoking status and lung squamous cell carcinomas (Table III).

Analysis of HPV genotypes revealed that the predominant genotype was HPV-16 (21 cases), followed by HPV-18 (6 cases), HPV-16/18 (4 cases), HPV-6 (4 cases) and other genotype (2 cases) in lung squamous cell carcinomas cases, but the major genotype was HPV-6 (9 cases), followed by other genotype (3 cases), HPV-16 (2 cases), HPV-6/18 (1 cases) and HPV-18 (1 case) in non-cancer control cases. The high risk HPV genotypes 16 and/or 18 were detected in 43.06% (31/72) and 5.63% (4/71) in lung SQC cases and non-cancer control cases, respectively (P<0.001) (Table IV).

Results of real-time PCR analysis are summarized in Table V. The quantity of HPV-16 DNA ranged from 0.003 to 44.942 copies per nanogram of genomic DNA. Results from repeated analyses showed a difference less than 5% in all the cases. E2/E6 ratio was determined by real-time PCR.
E2 DNA was not detected. In the rest of the cases, E2 DNA was detected but the E2/E6 ratio was less than the unity.

### Discussion

The frequency of lung carcinomas with HPV genomic sequences showed a wide range variation, from none to almost 80% (11,25,26). A review by Syrjanen (11) showed that HPV was detected in 536 of 2468 (22%) bronchial carcinomas. Meanwhile, a study from Okinawa, Japan found positive HPV in 79% of lung squamous cell carcinomas (12).

In our study, HPV was detected not only using SPF10 primer PCR, but was confirmed by INNO-LIPA HPV genotyping analysis. Thirty-seven cases were detected in the 72 lung squamous cell carcinoma group, 6 cases were detected in 37 lung adenocarcinomas, while 16 cases were detected in 71 non-cancer controls. The frequency of HPV-positive cases (51.4%) was higher in lung squamous cell carcinomas than in non-cancer control group (22.5%). The positive rate of HPV was 42.5% (48/113) and 15.8% (3/19) in the smoker and non-smoker, respectively. It seemed that the frequency of HPV-positivity is significantly associated to smoking habit (P=0.034), and HPV detection rate was not associated with gender or age. This percentage was higher than the value of 22% reported by Syrjanen in bronchial carcinomas (11), and lower than 79% reported from Okinawa of Japan in lung squamous cell carcinomas (12).

Lung squamous cell carcinoma development was strongly associated to smoking habit because more than 90% of individuals undergoing squamous cell carcinomas were heavy smokers (27). To find the correlation between the smoking status and the risk of lung squamous cell carcinomas in HPV-positive cases, the associations between HPV status, HPV-16 status and lung squamous cell carcinomas were assessed using a logistic regression model. We evaluated the possible effect modification by smoking status of the regression models.

We found that the risk of lung squamous cell carcinomas was 3.5 times higher among people HPV-positive (odds ratio 3.5, 95% CI 1.6-7.3, p<0.001) compared with HPV-negative people. Adjusted by smoking status, the risk of lung squamous cell carcinomas was 3.5 times higher among people HPV-positive (odds ratio 3.5, 95% CI 1.7-7.5, p=0.001) compared with HPV-negative people. The risk of lung squamous cell carcinomas was 16.9 times higher among people HPV-16 positive (odds ratio 16.9, 95% CI 3.8-75.3, p<0.0001) compared with HPV-16 negative people. Adjusted by smoking status, the risk of lung squamous cell carcinomas was 17.4 times higher among people HPV-16 positive (odds ratio 17.4, 95% CI 3.9-77.5, p<0.0001) compared with HPV-16 negative people. Interestingly, there was no association between smoking status and lung squamous cell carcinomas, while HPV-16 positive had a predominantly higher risk than HPV-16 negative for the development of lung squamous cell carcinomas in Western China.

Cheng et al reported that lung carcinomas with high risk HPV was much more frequently found among females than males (4,13). The study from Japan (17) also reported that HPV-16 was more frequently found among female than male cases, and the frequency of HPV-16 positive cases among all the HPV-positive cases differed among the sexes. But in our study, no difference was found in gender and age. It was similar to the report from Noutsou et al (28) that no correlation was found between HPV infection with sex, age and clinical outcome of the patient, or the histological type and the differentiation grade of the tumor.

Meanwhile, some reports revealed that the higher risk of HPV-16 and HPV-18 were more strongly associated with non-small cell lung cancer (14,15,29). But inconsistent...
results were found among these studies. A study in Taiwan (13) reported that 77 (54.6%) of 141 lung tumors had HPV-16 or -18 DNA compared with 16 (26.7%) of 60 non-cancer control subjects among non-smoking Taiwanese women, and a report from Central China found that the rates of HPV-16 and/or -18 infections in patients with 73 lung tumors were significantly higher than in 34 non-cancer control subjects (27.7 vs. 5.9%) (19). Also they found that 44.1% of (138/313) NSCLC samples were positive, while 4.2% (4/96) of lung benign controls were positive (20). Our data showed that the high risk HPV genotypes 16/18 were 43.1% and 5.6% in lung squamous cell carcinoma cases and non-cancer control cases, respectively (P<0.001). It is very similar to the study from the central part of China in NSCLC (20), but our study focused on lung squamous cell carcinomas cases.

In the majority of carcinomas associated with high-risk HPV and some advanced HPV-associated precancerous lesions, the whole viral genome or its fragment is integrated into the chromosomal DNA of the host cell whereas the HPV genome was retained as episomal molecules in early dysplastic low-grade lesions (17). Although the mechanism involved in switching from episomal state to viral integration was yet unclear, it was suspected that methylation pattern on HPV genome was different in episomal and integrated states. Kim et al reported that the overall methylation status of the LCR was greater than that seen in the more highly differentiated cells (30).

Since HPV integration may result in deletion of the E2 gene, the status of HPV in the host cells was determined on the basis of the E2/E6 ratio (24). When the ratio was equal to or higher than 1, all the HPV genome was considered to be in an episomal form and not integrated. The lack of amplified HPV E2 genome was considered to indicate the integration of all the HPV genome into the host genome. When the E2/E6 ratio was larger than zero and smaller than 1, the condition was considered as the mixture of episomal and integrated forms, where a portion of HPV genome was integrated into the host genome. In our study, real-time PCR was used in 27 samples to confirm the presence of HPV-16. E2 was not found in 59.3% (16/27) of the samples, suggesting that at least a partial HPV-16 genome was integrated into the host genome, which is similar to the 66.7% reported in Chile (31).

Although the viral load was low in our study, ranging from 0.003 to 44.942 copies per nanogram of genomic DNA, which corresponds to <1 to 2 copies/cell, our data were very similar to the reports from a Chinese study on esophageal cancer tissues (<1 to 157 copies/cell) (32) and a Finnish study on head and neck SCC (4.6 to 49 copies/cell) (33). The low viral load may be sufficient to promote carcinogenesis especially if viral DNA is integrated into the cell (34). Our data emphasize the importance of high-risk HPV DNA integration into the host genome contributing to lung squamous cell carcinogenesis.
In summary, there is a significant difference of HPV-positivity between lung squamous cell carcinomas and non-cancer controls, and the HPV-16 positive had higher risk than HPV-16 negative in the development of lung squamous cell carcinomas. A possible mechanism is the integration of HPV-16 into the host genome.

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References