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# Human papillomavirus in lung carcinomas among three Latin American countries

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**Abstract.** The presence of human papillomavirus (HPV) genome in lung carcinomas has been reported worldwide but its frequency varies from country to country. We examined HPV genome in 36 lung carcinomas, consisting of 14 squamous cell carcinomas, 13 adenocarcinomas, and 9 small cell carcinomas, collected from Colombia, Mexico and Peru. PCR analysis using *GP5+/GP6+* primers, combined with Southern blot hybridization, found the presence of HPV genome in 10 (28%) of 36 cases. This percentage is similar to the value of 22% reported by Syrjänen, who conducted a meta-analysis of nearly 2500 lung carcinomas examined to date. Genotype analysis revealed that the most predominant genotype was HPV-16 (7 cases), followed by HPV-18 (2 cases) and HPV-33 (1 case). HPV-16 was more frequently found among female than male cases (P=0.008) but was not detected in any adenocarcinoma cases. On the other hand, HPV-18 and HPV-33 were detected only among male cases. These HPV genotypes were detected only in adenocarcinomas, and all the HPV genotypes detected in this histological type were HPV-18 or HPV-33. The frequency of HPV-16 positive cases among all the HPV positive cases differed in the sexes (P=0.033) and differed in the three histological types (P=0.017). The presence of HPV tended to be more frequent in well-differentiated tumors when squamous cell carcinomas and adenocarcinomas were combined. However, it was not statistically significant (P=0.093). Neither p16 nor p53 expression in carcinoma cells was related to the proportion of HPV-positive cases. In conclusion, high-risk HPV DNA was detected in 28% of lung carcinomas. The predisposition of HPV-16 to female cases and to non-adenomatous carcinomas warrants further investigation.

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## Introduction

Human papillomavirus (HPV) is a double stranded DNA virus, and is an established etiological agent causing cancer of the uterine cervix. A large-scale international study detected high-risk HPV in 99.7% of cervical carcinomas (1) and persistent infection with high-risk HPV is considered its 'necessary cause' (2,3). Furthermore, HPV is suspected to cause extragenital cancers, including cancers of the oral cavity, larynx, esophagus, and lung. However, the role of HPV infection in the pathogenesis of those malignancies is still controversial (4-6).

Lung cancer is the leading cause of death from malignant tumors, and its incidence is rapidly rising in developing countries (7). Although smoking is the most important risk factor of this malignancy, its worldwide epidemic is exacerbated by additional factors, including indoor radon exposure, environmental arsenic contamination and atmospheric pollution, and, possibly, by HPV infection (7). Etiological involvement of HPV in development of lung cancer was originally postulated by Syrjänen (8,9) but the cause-effect relationship is yet to be established (6). Most importantly, HPV DNA integration into host-cell DNA, which is considered an important process in development of cervical cancer, has not been confirmed in lung cancer (6).

Lung cancer harboring HPV DNA is not rare; the meta-analysis conducted by Syrjänen (10) revealed that HPV DNA is detected in as much as 21.7% of the 2468 lung carcinomas analyzed to date. HPV DNA can also be detected in epidermoid esophageal carcinomas. The frequency of HPV-related esophagus carcinomas varies widely worldwide (10,11). While the geographical distribution of HPV-related lung

Table I. Sequences of the oligonucleotides used as PCR primers.

| Gene | Primers | Sequences | Size (bp) |
|---|---|---|---|
| HPV L1 | *GP5+* | 5'-TTTGTTACTGTGGTAGATACTAC-3' | 150 |
| | *GP6+* | 5'-GAAAATAAACTGTAAATCATATTC-3' | |
| Human ß-globin | *PCO3* | 5'-ACACAACTGTGTTCACTAGC-3' | 110 |
| | *PCO4* | 5'-CAACTTCATCCACGTTCACC-3' | |

carcinoma is much less clear, a high frequency was reported by studies in southern Japan (11) and Taiwan (12). In a study conducted in Taiwan, Cheng *et al* reported that a high incidence of lung cancer among non-smoking women may be explained by a high prevalence of HPV-16/18 infection (12).

To our knowledge, there is only one study from Latin America reporting the presence of HPV in lung carcinomas (13). In this Chilean study, 6 of 13 squamous cell carcinoma cases (42%) had HPV DNA. The present study examined the HPV genome and genotype in lung carcinoma specimens among patients from Colombia, Mexico and Peru.

## Materials and methods

*Subjects*. The present study examined HPV genome in carcinoma tissues obtained from 36 lung cancer cases diagnosed in the following institutes of three Latin American countries: 7 squamous cell carcinoma and 8 adenocarcinoma cases diagnosed at Unidad de Patologia, Hospital General de Mexico, Mexico, during the period from 1975 to 1981; 3 squamous cell carcinoma, 2 adenocarcinoma and 6 small cell carcinoma cases diagnosed at Hospital Universitario del Valle in Cali, Colombia, during the period from 1996 to 2001; and 4 squamous cell carcinoma, 3 adenocarcinoma and 3 small cell carcinoma cases diagnosed at National Cancer Institute, INEN of Peru, in 1996. The institutional review board of the Graduate School of Medical and Dental Sciences, Kagoshima University, Japan, approved the present study.

*DNA extraction*. Sections (10 $\mu$m) were prepared from each formalin-fixed paraffin-embedded sample. 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 $\mu$g of Proteinase K (Invitrogen) and incubated at 56˚C for 24 h. 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.

*PCR, Southern blot hybridization and sequencing*. HPV amplification with *GP5+/GP6+* primers (Table I) (14,15) was made in a reaction mix containing 2.5 $\mu$l of template DNA, 200-$\mu$M dNTP, 0.5 $\mu$M of each primer and 1.0 U Taq DNA polymerase (Takara, Japan) in a total volume of 25 $\mu$l of reaction buffer (50 mM KCl, 20 mM Tris-Cl, pH 8.3). The conditions of amplification were as follows: initial denaturation to 95˚C for 4 min; subsequent 45 cycles consistent of 95˚C for 1 min, 40˚C for 2 min and 72˚C for 1.5 min and final extension at 72˚C for 5 min.

ß-globin amplification with *PCO3/PCO4* (110 bp) primers was used as the internal positive control (Table I). 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-18 was used as external positive control.

The amplified products were revealed through electrophoresis with 3.0% agarose gels at 100 volts for 30 min. After electrophoresis, the DNA was transferred onto Hybond $N^+$ nylon transfer membrane (Amersham, UK) by capillary blotting using 0.4 N NaOH. The generic $GP5^+/GP6^+$ PCR products amplified from the cloned HPV-6 and -18 were purified from agarose gel by QIAEX II Extraction Kits (Qiagen GmbH and Qiagen Inc., Hilden, Germany) and used as probes to detect HPV-6/11 and HPV-16/18, respectively. The hybridization was made at 42˚C overnight and then the membranes were washed at 42˚C with solution containing 6 M urea, 0.4% SDS and 0.5 X SSC buffer. For the detection of HPV genome, hybridization was carried out using the ECL direct labeling and detection kit (Amersham, UK) according to the instructions of the manufacturer.

Amplified PCR products that appeared as a visible band after ethidium bromide staining were purified using QIAGEN PCR purification kit and were directly sequenced by fluorescent dye-labeled dideoxynucleotides and cycle sequencing methods using the Big DyeTerminator Cycle Sequencing Kit (PE Applied Biosystems, NJ, USA). In the samples where positive signal was seen only after Southern blot analysis but not in the agarose-gel electrophoresis, the second round of PCR was conducted. Sequence analysis was performed on the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). The nucleotide sequences were aligned and compared with those of known HPV types available through the GenBank database (NCBI, National Institute of health, Bethesda, MD, USA) by using BLAST 2.2 (http://www.ncbi.nih.gov/BLAST/).

*Immunostaining for p16INK4a and p53 proteins*. Sections of paraffin-embedded block (2-3 $\mu$m) were placed on silane-coated glass slides, and deparaffinized by passage through xylene. The endogenous peroxidase activity was blocked with 0.3% $H_2O_2$/methanol, and the slides were then rehydrated with 0.01 mol/l sodium phosphate/citrate buffer, pH 8.0. For antigen retrieval, the slides were heated in 0.01 M-citrate

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Demographic characteristics and histological types of nomas.

| | No. | Mexico | Colombia | Peru |
|---|---|---|---|---|
| Gender | | | | |
| Male | 22 | 10 | 6 | 6 |
| Female | 14 | 5 | 5 | 4 |
| Age | | | | |
| ≤60 | 12 | 6 | 4 | 2 |
| 60-74 | 16 | 5 | 5 | 6 |
| 75+ | 8 | 4 | 2 | 2 |
| Histology | | | | |
| Squamous cell carcinoma | 14 | 7 | 3 | 4 |
| Adenocarcinoma | 13 | 8 | 2 | 3 |
| Small cell carcinoma | 9 | 0 | 6 | 3 |
| Total | 36 | 15 | 11 | 10 |

buffer, pH 6.0, at 95˚C for 30 min. After rinsing in 0.01 mol/l phosphate-buffered saline (PBS), pH 7.4, nonspecific antibody binding was reduced by incubating the sections with 10% fetal bovine serum in PBS for 30 min. Then, the sections were incubated overnight at 4˚C with a mouse monoclonal antibody of p16 (1:200 dilutions, GST-p16$^{INK4}$, PharMingen International) or p53 (1:50 dilutions, DO-7, Dako Japan Co., Ltd.). After washing thoroughly with PBS, the slides were incubated with biotinylated horse anti-mouse IgG for 30 min followed by a 1:100 dilution of the avidin-biotin-peroxidase complex (Vectastain elite ABC kit, Vector Laboratories, Burlingame, CA) for an additional 30 min. The peroxidase signal was visualized by treatment with DAB substrate-chromogen system (Dako) for 8 min. Finally the sections were stained lightly with hematoxylin. Nuclear staining was considered positive for p16 and p53 expression. In statistical analysis, the cases with less than 10% carcinoma cells stained positive were classified as negative and the other cases were regarded as positive for the expression in both p16 and p53 proteins (16,17).

*Statistical analysis*. Fisher's exact test was applied to examine associations between HPV status and each clinicopathological factor including p16 and p53 expressions. We used Wilcoxon rank-sum test for the comparison of age distribution. All the P-values presented are two-sided.

## Results

We analyzed 36 lung carcinomas, consisting of 14 squamous cell carcinomas, 13 adenocarcinomas, and 9 small cell carcinomas collected from Mexico, Colombia and Peru (Table II). Twenty-two patients were male (61%) and 14 were female (39%). The mean age of the patients was 65±11 years.

HPV was detected using *GP5*$^+$/*GP6*$^+$ primers for PCR and its presence was confirmed by Southern blot analysis. In total, we detected HPV in 10 (28%) out of 36 cases (Table III).

Table III. Detection of HPV genome in lung carcinomas.

| | No. | HPV$^+$ (%) | HPV$^-$ (%) | P-value[a] |
|---|---|---|---|---|
| Total | 36 | 10 (28) | 26 (72) | |
| Country | | | | 0.405 |
| Mexico | 15 | 5 (33) | 10 (67) | |
| Colombia | 11 | 4 (36) | 7 (64) | |
| Peru | 10 | 1 (10) | 9 (90) | |
| Gender | | | | 0.140 |
| Male | 22 | 4 (18) | 18 (82) | |
| Female | 14 | 6 (43) | 8 (57) | |
| Histology | | | | 0.899 |
| Squamous cell carcinomas | 14 | 4 (29) | 10 (71) | |
| Adenocarcinomas | 13 | 3 (23) | 10 (77) | |
| Small cell carcinoma | 9 | 3 (33) | 6 (67) | |
| p16INK4a protein expression (%) | | | | 0.687 |
| ≥80 | 9 | 2 (22) | 7 (78) | |
| <80 | 7 | 3 (43) | 4 (57) | |
| <10 | 20 | 5 (25) | 15 (75) | |
| p53 expression (%) | | | | 0.645 |
| ≥80 | 5 | 2 (40) | 3 (60) | |
| <80 | 7 | 1 (14) | 6 (86) | |
| <10 | 24 | 7 (29) | 17 (71) | |

[a]P-values were obtained by Fisher's exact test.

ß-globin gene was successfully amplified by PCR in all subjects.

The proportion of HPV-positive cases did not differ by gender. The mean age at diagnosis among HPV-positive and negative cases were 67±11 and 64±8, respectively (data not shown in the table). Although HPV-positive cases tended to be older than HPV-negative cases, the difference was not statistically significant (P=0.297, Mann-Whitney test). In comparison among the three countries, the frequency of HPV-positive cases in Colombia appeared to be lower than the other two countries but the difference among countries was not statistically significant (Table III). The histology of lung cancer or the expression of p16 and p53 proteins in carcinoma cells was not related to the proportion of HPV-positive cases, either.

Genotypes for HPV-16/18 was determined by Southern blot hybridization of PCR products, and confirmed by sequencing. Southern blot analysis could not determine HPV genotype in one case and, therefore, the base sequence of its PCR product was analyzed. Blast analysis of the obtained base sequence revealed its genotype to be HPV-33. We could not detect any HPV-6/11 in the subjects.

Analysis of HPV genotype revealed that the predominant genotype was HPV-16 (7 cases), followed by HPV-18 (2 cases) and HPV-33 (1 case). Table IV-A shows the results examining the frequency of HPV-16 detection. Females accounted for

Table IV. A. Detection of HPV-16 in lung carcinomas.

| | No. of the cases (%) | | | |
|---|---|---|---|---|
| | No. | HPV-16$^+$ | HPV-16$^-$ [a] | P-value[b] |
| Total | 36 | 7 (19) | 29 (81) | |
| Country | | | | 0.772 |
| Mexico | 15 | 3 (20) | 12 (80) | |
| Colombia | 11 | 3 (27) | 8 (73) | |
| Peru | 10 | 1 (10) | 9 (90) | |
| Gender | | | | 0.008 |
| Male | 22 | 1 (5) | 21 (95) | |
| Female | 14 | 6 (43) | 8 (57) | |
| Histology | | | | 0.060 |
| SQ | 14 | 4 (29) | 10 (71) | |
| AD | 13 | 0 | 13 (100) | |
| SCC | 9 | 3 (33) | 6 (67) | |
| p16INK4a protein expression (%) | | | | 1.000 |
| ≥80 | 9 | 2 (22) | 7 (78) | |
| <80 | 7 | 2 (29) | 5 (71) | |
| <10 | 20 | 3 (15) | 17 (75) | |
| p53 expression (%) | | | | 0.468 |
| ≥80 | 5 | 2 (40) | 3 (60) | |
| <80 | 7 | 1 (14) | 6 (86) | |
| <10 | 24 | 4 (17) | 20 (83) | |

B. Comparison between HPV-16 and non-16-related HPV positive tumors.

| | No. of the cases (%) | | |
|---|---|---|---|
| | HPV-16 | Non-16-related HPV | P-value[b] |
| Total | 7 (70) | 3 (30) | |
| Country | | | 1.000 |
| Mexico | 3 (60) | 2 (40) | |
| Colombia | 3 (75) | 1 (25) | |
| Peru | 1 (100) | 0 | |
| Gender | | | 0.033 |
| Male | 1 (25) | 3 (75) | |
| Female | 6 (100) | 0 | |
| Histology | | | 0.017 |
| SQ | 4 (100) | 0 | |
| AD | 0 | 3 (100) | |
| SCC | 3 (100) | 0 | |
| p16INK4a protein expression (%) | | | 1.000 |
| ≥80 | 2 (100) | 0 | |
| <80 | 2 (67) | 1 (33) | |
| <10 | 3 (60) | 2 (40) | |
| p53 expression | | | 0.650 |
| ≥80 | 2 (100) | 0 | |
| <80 | 1 (100) | 0 | |
| <10 | 4 (57) | 3 (43) | |

[a]HPV-16$^-$, HPV negative cases and non-16-related HPV positive cases; [b]P-values were obtained by Fisher's exact test; SQ, squamous cell carcinoma; AD, adenocarcinoma; SCC, small cell carcinoma.

Table V. HPV genotype distribution and the grade of tumor differentiation.

| Differentiation grade | No. | HPV$^+$ | HPV-16 | HPV-18 | HPV-33 |
|---|---|---|---|---|---|
| Squamous cell carcinomas | 14 | 4 | 4 | | |
| Well | 1 | 1 | 1 | | |
| Moderate | 6 | 1 | 1 | | |
| Poor | 7 | 2 | 2 | | |
| Adenocarcinomas | 13 | 3 | | 2 | 1 |
| Well | 1 | 1 | | 1 | |
| Moderate | 2 | | | | |
| Poor | 10 | 2 | | 1 | 1 |

6 out of 7 HPV-16 positive cases. The observed gender difference in the detection rate of HPV-16 was statistically significant (P=0.008, Fisher's exact test). HPV-16 was not detected in any adenocarcinoma cases. HPV-16 was more frequently found in squamous cell carcinomas and small cell carcinomas of the lung but the observed histological difference was not statistically significant (P=0.060, Fisher's exact test). On the other hand, HPV-18 and -33 were detected only among male cases, and all the HPV types detected in adenocarcinomas were 18 or 33, and these HPV genotypes were detected only in this histological type (Table IV-B). The frequency of HPV-16 positive cases among all the HPV positive carcinomas differed in the sexes (P=0.033, Fisher's exact test) and in the three histological types (P=0.017, Fisher's exact test).

Table V shows the results examining the relationship between tumor differentiation levels and HPV presence. The level of tumor differentiation was not related to the presence of HPV-16 (P=0.336, Fisher's exact test) or non-16-related HPV (P=0.266, Fisher's exact test). When squamous cell carcinomas and adenocarcinomas were combined, the presence of HPV genome tended to be higher in well-differentiated tumors. However, the tendency was not statistically significant (P=0.093, Fisher's exact test).

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We found the HPV genome in 28% of lung carcinoma collected from three Latin American countries (Mexico, Colombia and Peru). The frequency was slightly lower than that reported from Chile (13) where the presence of HPV genome was observed in 6 of 13 squamous cell carcinoma cases (42%). In the present study, HPV-16 predisposed to female cases (P=0.008) and to carcinomas other than adenocarcinomas (P=0.060). Its predisposition to non-adenocarcinomas became more evident when histological distributions of HPV-16 positive cases and other HPV positive cases were directly compared (P=0.017).

We could not detect HPV-6 or 11 genotype in any tumors examined. A population-based study conducted in Santiago, Chile, another Latin American country, showed a relatively low prevalence of HPV-6 and 11 genotypes (18). The four most common genotypes of low-risk HPV were HPV-42, 67, 70, and 81. Further investigations are necessary to examine common genotypes of low-risk HPV among the general populations of the three countries.

In the literature, the frequency of lung carcinomas with HPV genomic sequences shows a wide range, from none to almost 80%, as reviewed by Gillison and Shan (6). Although these observations suggest the presence of geographic and demographic differences in HPV-related lung carcinoma, a factor involved in these inconsistent results is the method used for HPV detection. The review made by Syrjänen enables us to compare the detection rate by the methods used in those studies. PCR-agarose gel electrophoresis has higher detection rates than *in situ* hybridization. In the present study, HPV DNA was detected by PCR analysis using *GP5*$^{+}$/*GP6*$^{+}$ primers, and the resulting products were analyzed by Southern blot hybridization with L1 gene labeled probe. When compared to PCR-agarose gel electrophoresis and *in situ* hybridization, which were frequently used in the studies reported so far (10) this method is considered to be more sensitive and enables us to detect HPV even when carcinoma cells have a small number of HPV copies.

In the present study, we detected 7 carcinomas with HPV-16 DNA; 6 of them were female cases. On the other hand, carcinomas with non-16-related HPV were limited to male cases. Cheng *et al* reported that lung carcinomas with HPV-16/18 were much more frequently found among females than males (12). Their subsequent studies, using blood samples, tumor tissue and vaginal smear collected from the same lung cancer patients, made the following interesting findings: i) blood samples and lung tumors had similar HPV genotype distributions (19); ii) lung tumors and blood cells showed identical sequences of L1 and E6 of HPV-16/18 in their preliminary analysis and iii) the presence of HPV-16/18 in cervical pap smears of female lung cancer patients was correlated with those present in their lung tumors. On the basis of these findings, they suspect the possibility of HPV-16/18 transmission from uterine cervix to lung via blood circulation. Their hypothesis is not at variance with the predominance of HPV-16 in female lung carcinoma cases observed in the present study.

HPV E6 and E7 are considered to play important roles in the development and maintenance of malignant cells, and their expressions are regulated by cis-active elements in LCR, a long control region (20). HPV E6 interacts with p53 tumor suppressor and E6-associated protein, a host cell ubiquitin ligase, and induces accelerated proteasomal degradation of p53 (21). In lung carcinomas, Soini *et al* showed an inverse relationship between the presence of HPV DNA and abnormal p53 protein accumulation (22). On the other hand, binding of HPV E7 and pRb resulted in the release of E2F factors and the induction of high levels of p16 (23), and marked p16 over-expression was observed in cervical cancers (24). In the present study, however, we observed no tendency of p53 down-regulation or p16 up-regulation among HPV positive 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 is retained as episomal molecules in early dysplastic low-grade lesions. Although the mechanism involved in switching from episomal state to viral integration is yet unclear, it is suspected that methylation pattern on HPV genome is 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 (25). Wu *et al* also suspect that the involvement of HPV infection in lung tumorigenesis may be mediated at least in part through the increase of hypermethylation to cause p16INK4a inactivation (26). In addition, Sano *et al* pointed out that early-stage carcinomas showed p16 down-regulation, probably through hypermethylation, while its up-regulation is induced in late stages (24). We observed no relationship between HPV presence and p16 immunostaining.

In the uterine cervix, HPV-16 and -18 are the most commonly detected HPV genotypes in squamous cell carcinoma whereas HPV-18 is the most prevalent type in adenocarcinomas (2). In the present study, HPV-18 was detected in 2 adenocarcinoma cases and was not found in squamous cell carcinomas or small cell carcinomas of the lung, confirming the predisposition of HPV-18 to adeno-carcinomas.

Miyagi *et al* reported that HPV involvement in cancer of the lung is most evident in well-differentiated squamous cell carcinomas (11), whose frequency has declined over the years (11). Overall, our findings are in agreement with what was reported by Miyagi *et al* (11). Indeed, we found HPV-16 only in squamous cell carcinomas and small cell carcinomas but not in adenocarcinomas. Due to a small number of cancer cases, it was difficult to examine the relationship between grades of tumor differentiation and HPV presence in each histological type. However, in the analysis where adeno-carcinomas and squamous cell carcinomas were combined, HPV presence seemed to be related to tumor differentiation although the relationship was not statistically significant.

In conclusion, we detected high-risk HPV DNA in 28% of lung carcinomas. This percentage is similar to the value of 22% reported by a meta-analysis analyzing nearly 2500 lung carcinomas examined to date. HPV-16 predisposition to female cases and to carcinomas other than adeno-carcinomas observed in the present study warrants further studies.

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