Human Papillomavirus DNA and *p16* Gene in Squamous Cell Lung Carcinoma

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Abstract. *Aim: To investigate the presence of human papillomavirus (HPV) DNA in squamous cell carcinoma (SCC) of the lung, and to examine the protein expression and genomic status of p16 and their correlation. Materials and Methods: Fifty cases of surgically removed primary lung SCC were analyzed. HPV detection was performed by Polymerase Chain Reaction (PCR) of L1 region and E6/E7 region of high-risk viral genotype. p16 protein and gene analysis were carried out by immunohistochemistry and Fluorescence In Situ Hybridization (FISH), respectively. Results: HPV DNA was found in two out of 50 cases (4%, p>0.05). In five cases, p16 protein expression was positive. The data showed that in 45/50 cases (90%, p<0.05) HPV DNA and p16 were both negative, in 2/50 cases (4%) both were positive, and in 3/50 (6%) cases, HPV DNA was negative and p16 positive. FISH analysis for p16 gene showed aneusomia of chromosome 9 with or without loss of p16 gene in all cases (100%, p<0.05). Conclusion: Our study shows that in pulmonary SCC, there is no association between the presence of HPV DNA and the expression of p16 protein. Furthermore, the loss of the p16 gene and the instability of chromosome 9 were frequently found in HPV DNA-negative cases.*

Cigarette smoking is the major risk factor for the onset and development of lung cancer (1). In recent years, attention has been attributed on other risk factors, one of them is human papillomavirus (HPV). HPV DNA has been frequently observed in lung cancer and the expression of HPV oncoproteins E6/E7 has been documented (2). The presence of HPV has been demonstrated in all the subtypes of lung cancer, mainly in squamous cell carcinomas (SCC) (3).

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The role of HPV in bronchial squamous cell lesions was first suggested by Syrjanen in 1979 (4), who described the changes in bronchial carcinoma, comparing them to those of the genital tract. Subsequently, many authors have carried out experiments on HPV in squamous lung cancer, with inconclusive results. In 2009, Klein showed a large variability in the distribution of the virus-positive cancer cases in various countries (5). In particular, in Europe and America, the average incidence of HPV positivity was 15%, and in Asia 35%, with a particularly high frequency in Japan and Taiwan, with values up to 80%. These conflicting data have not yet confirmed nor denied the possibility of the involvement of HPV in bronchial carcinoma. Although many studies (6) consider that HPV has a causal role in the carcinogenesis of lung cancer, the evidence for cause and effect is still lacking. The association of HPV with uterine cervical SCC has been wel-documented. In the uterine cervix, the deregulation of the genes that control cell proliferation, including the *p16* gene, is one of the mechanisms of carcinogenesis caused by oncogenic viral proteins E6/E7. *p16* is a tumor suppressor gene (also known as *p16 INKA*), a cyclin-dependent kinase (CDK) inhibitor, which maps to the short arm of chromosome 9 (9q21), and is known to function as an inhibitor of CDK4 kinase, and regulates the G_1-S phase of the cell cycle (7). In dysplastic and neoplastic cervical lesions, the HPV inhibits the transcription of the *p16* gene, with abnormal expression of the p16 protein, deregulation of the cell cycle, and tumorigenesis (8).

In lung SCC the pathogenic mechanism induced by HPV could be identical to that seen in cervical cancer. The purpose of this study was twofold: firstly to assess the incidence of HPV in a series of lung SCC, and secondly, to test the hypothesis that HPV may play an oncogenic role in the expression of p16 protein in SCC. Finally, since the literature has documented alteration of the *p16* gene and the instability of chromosome 9 in lung SCC (9), molecular analysis of the *p16* gene and chromosome 9 was carried out using Fluorescence In Situ Hybridization (FISH).

Materials and Methods

Collection of tissues of patients and controls. For the current study we analyzed 50 cases collected from the archives of the Second Department of Pathology, Spedali Civili, University of Brescia, Italy, from 2005 to 2010. Specimens were obtained from 50 consecutive surgically-excised primary pulmonary SCCs. Samples were fixed in 10% buffered formalin and processed according to a standardized protocol. Sections were stained with haematoxylin-eosin for histological diagnosis based on the recent classification of pulmonary adenocarcinoma proposed by the World Health Organization (WHO) classification of tumors (10); 23 cases of wedge resection for non-neoplasm-associated pneumothorax were used as controls. All cases were smokers (≥10 pack/years).

DNA isolation, HPV Polymerase Chain Reaction (PCR). Genomic and viral DNA was obtained from two 25 μm-thick sections from the paraffin blocks of the tumours. All sections were placed into 2 ml microcentrifuge tubes and DNA was extracted by Tissue NucleoSpin Kit (Macherey-Nagel, GmbH & Co., Duren, Germany). The HPV PCR method was applied to amplify the 139-150 bases of HPV/L1 region and for 208-245 bases of the E6/E7 gene of the high-risk (HR) viral genotype (types 16, 18, 33, 35, 52, 58). Briefly, 2.5 μl of the DNA were used to amplify the target gene with the consensus primers (MY09/11) (Experteam, Venice, Italy) in a two-step nested PCR. All amplification reactions were performed in a total volume of 25 μl with 0.2 units of ExperTaq (Experteam). Cycling conditions for the amplification of the HPV/L1 region were 30 sec at 95˚C, 30 sec at 53˚C (first step) or 45˚C (second step) and 30 sec at 72˚C for 30 or 40 cycles. Cycling conditions for the amplification of the E6/E7 regions were 1 min at 95˚C, 1 min at 55˚C and 1 min at 72˚C for 40 cycles. One negative control was used for each amplification. The primers of the human β-globin gene (268 bp) were used for testing the quality of the extracted DNA in order to exclude false-negative results derived from DNA degradation of long-fixed samples.

Immunohistochemistry. For immunohistochemical analysis, the monoclonal antibody to p16 INK4A (clone E6H4; Novocastra Laboratories, Ltd, Newcastle, UK), was used. Antigen unmasking was performed with three cycles of 5 min each in 3-EDTA buffer (pH 8, 1 mM) at 750 W. After antigen unmasking, the slides were cooled in the solution for 20 min at room temperature. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. The slides were first stained with the primary antibody (1:100) for 30 min at room temperature, followed by incubation with Post Primary Block for 8 min (LEICA, Menarini, Florence, Italy) and polymer (LEICA). Staining was carried out with 3,3'-diaminobenzidine chromogen and counterstaining with Mayer's haematoxylin (Dako, Glostrup, Denmark). In the evaluation of the p16 immunohistochemically stained slides, both nuclear and cytoplasmic staining were evaluated. Immunoreactivity was considered positively when ≥10% of the neoplastic cells were positive stained (11, 12).

FISH analysis. We performed interphase FISH analysis using a specific probe mapping to chromosome 9p21 and the centromeric probe CEP9 (9p21 CEP9, Vysis, Downers Grove, IL USA). Sections, cut at 3 μm of thickness were prepared for FISH analysis, the paraffin was removed from the sections with three 10-min washes in xylene, and washed twice in ethanol 100% for 5 min. The sections were then processed with pre-treatment kit I (Vysis). Briefly, the sections were treated with 0.2 N HCl for 20 min, washed with Buffer 1 and 2 (Vysis) for 3 min, than were bathed in pre-treatment solution for 30 min at 81˚C. After washing with distilled water and buffer 1, sections were digested with protease solution for 45 min at 37˚C. Next, the slides were rinsed with Buffer 1 and 2 for 5 min and dried. The slides were fixed with 10% buffered formalin for 1 min at room temperature, next they were rinsed with Buffer 1 and 2 for 5 min, dehydrated in consecutive 70%, 80% and 100% ethanol solutions for 1 min each and then dried. Next 10 μl of 9p21 CEP9 probe was applied to each slide and the section cover slipped. Denaturation was achieved by incubating the slides at 72˚C for 5 min in the Hybridizer; the hybridization was undertaken at 37˚C for 14 h. The coverslips were then removed, and slides were immersed, at room temperature, in SSC2X/0.3% NP40 for 1 min and at 72˚C in SSC2X/0.3% NP40 for 2 min. The slides were dehydrated, dried and counterstained with 10 μl of 4',6-diamidino-2-phenylindole antifade (DAPI) (Vysis). Slides were examined with appropriate filter for SpectrumGreen and a UV filter for DAPI nuclear counterstain. The signals were recorded with a Nikon CCD camera. Two hundred nuclei were analyzed for each preparation (13, 14).

Statistical analysis. Statistical analysis was performed using the Chi-square test with Yates correction. A value of $p<0.05$ was considered as being statistically significant.

Table I. *Demographic and histological data of patients with lung SCC included in the study.*

Gender	Age, years	Grading	
		G2	G3
Male, 48	68.22±7.76	46%	54%
Female, 2	72±0	50%	50%

[1]No G1 cases were encountered. Grading according to WHO 2004.

Results

Demographic and histological data of the studied cases are summarized in Table I. The expression of the p16 protein was detected in 5 out of 50 (10%, $p>0.05$) total cases of SCC (Figure 1).

In all cases included in the study PCR was performed to identify viral DNA and the high-risk genotypes. The amplification with primers in the E6-E7 region, identified 2 out of 50 (4%, $p>0.05$) positive cases of the main high-risk genotypes. In both those two cases, p16 protein was detected by immunohistochemistry (4%). Three cases were p16-positive and HPV-negative (6%). A total of 45 out of 50 cases were both p16-negative and HPV-negative (90%, $p<0.05$). Out of the 23 control cases analyzed for HPV DNA, one was positive.

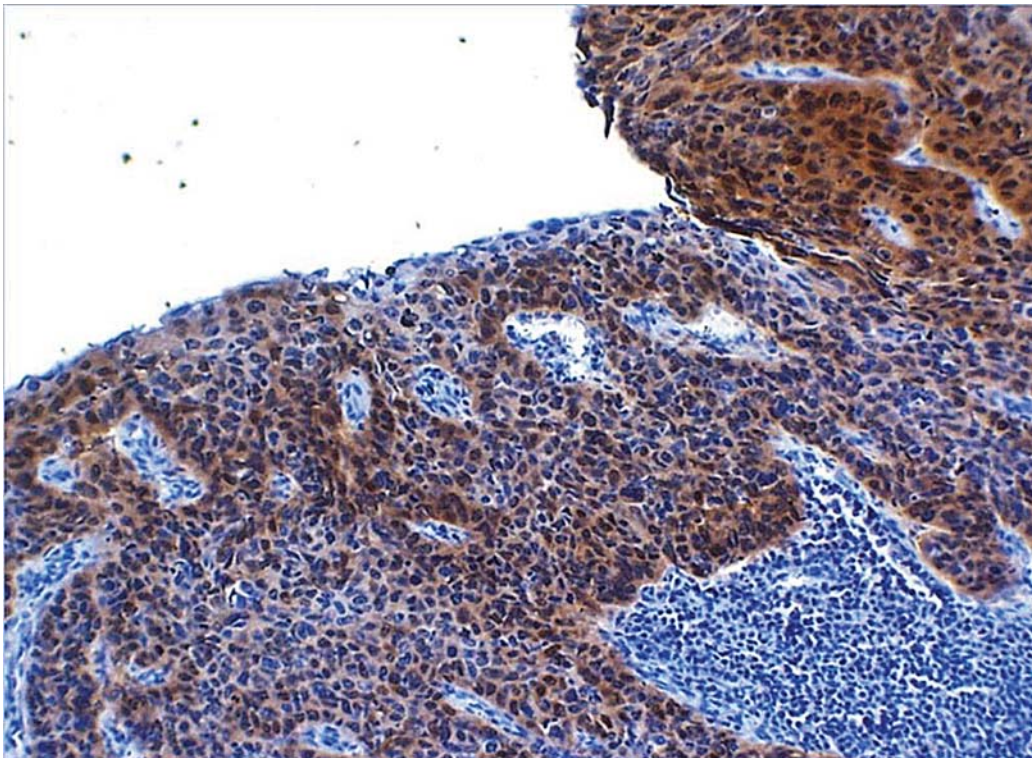

Figure 1. *Immunohistochemical expression of p16 protein in squamous cell carcinoma of the lung. The image shows that the staining of the p16 protein is nuclear and cytoplasmic. Magnification ×100.*

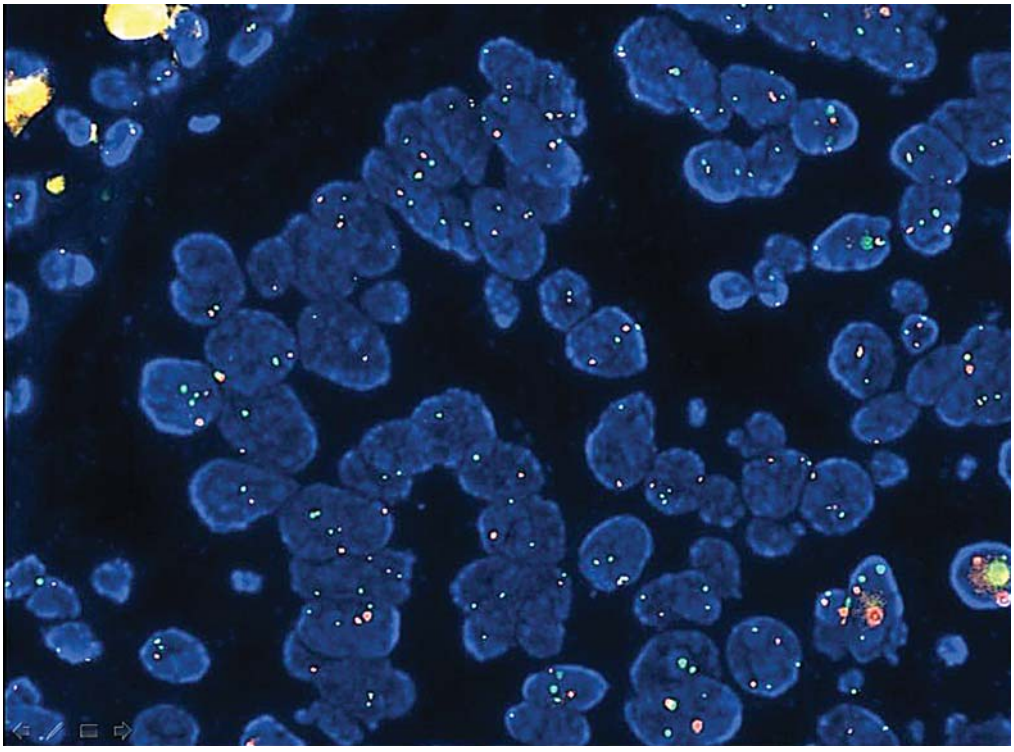

Figure 2. *Molecular analysis of chromosome 9 and p16 gene in lung squamous cell carcinoma. Fluorescence microscopy image of the nuclei of tumor cells presenting visible signs of chromosome 9 (Cep9 probe, green) and p16 gene (9p21 probe, red). Characteristic pattern observed: chromosomal instability (no. of chromosome 9 signals >2) and loss of p16 gene (no. of p16 signals <no. of chromosome 9 signals). Magnification ×600.*

Table II. *Patterns of HPV DNA/ p16 protein, p16 gene and chromosome 9 in lung SCC.*

HPV DNA and p16 protein pattern		Loss of *p16* gene		Instability of chromosome 9
		n	Yes/No	Yes/No
HPV^+p16^+	n=2	1:	Yes*	Yes
		1:	No**	No
HPV^+p16^-	n=0	0		0
HPV^-p16^+	n=3	2:	Yes*	Yes
		1:	No**	No
HPV^-p16^-	n=45	10:	Yes	Yes
		12:	Yes	No
		17:	No	Yes

*Heterozygosous. **Signals >2.

High instability of chromosome 9 and loss of *p16* gene were detected with high frequency in our series (Figure 2). The data are summarized in Table II. The cases were then divided into four groups according to the presence of HPV DNA and protein expression of p16: HPV^-p16^-, HPV^+p16^-, HPV^+p16^+, and HPV^-p16^+. Cases positive for HR-HPV and positive for p16 protein revealed: one case with the loss of *p16* gene in heterozygosity and chromosomal instability, and the other with amplification of the *p16* gene in a clone of cells. The cases negative for viral DNA, and not expressing the p16 protein revealed loss of the gene and chromosomal instability. For the three cases without HPV, which positive for p16 protein expression, the genetic cause behind this was investigated. In one case, about 20% of nuclei showed a number of p16 signals greater than those of chromosome 9. For the other cases, there was loss of the gene loading to heterozygosity, and chromosomal instability. Most of the cases in which HPV was not detected and which did not express the p16 protein, had gene loss with and without chromosome instability.

Discussion

After the study of Syrjänen, many authors carried out experiments on SCC with conflicting results (14-19). Various differences were found among different geographical areas. In addition, significant heterogeneity was reported among certain countries and regions. HPV/high-risk genotypes (16, 18, 31 and 33) were more frequent, but low-risk genotypes (6 and 11) were also found. These conflicting data have not confirmed nor excluded the possibility of HPV involvement in lung carcinoma.

To investigate the presence of HPV DNA and the expression of the HPV-deregulated cell cycle protein p16, we studied 50 consecutive cases of surgically removed primary SCC in the lung. We conducted a double investigation: a study of molecular biology (using PCR to detect the presence of HPV DNA) and an immunohistochemical profiling (to investigate the expression of p16 protein).

We found HPV DNA in only two out of 50 SCCs. In our cases the percentage of co-existence of HPV DNA and expression of p16, by the tumor, is very low.

On the other hand, in contrast to what has been reported for uterine cervical cancer, in the SCC of the lung, loss of p16 protein expression is more frequent. We therefore examined by FISH the loss of the *p16* gene and the instability of chromosome 9, where the gene itself is located. The analysis showed alterations in a high proportion of cases in the form of aneusomia of chromosome 9 or loss of the *p16* gene. The expression of p16 protein appears to depend on gene deletion in heterozygosity, amplification of isolated clones, or chromosome instability.

In summary our data show that in SCC of the lung there is no association between the presence of HPV DNA and expression of p16; the *p16* gene frequently has molecular changes in HPV DNA-negative cases. Smoking might have a major role in de-regulation of cell cycle gene products (20). The role of the HPV in the early stages of carcinogenesis, and in cases of lung cancer patients who are nonsmokers, still needs further investigation. Furthermore, we found that the vast majority of the SCC samples carried genetic abnormalities on chromosome 9, suggesting their role in lung tumorigenesis.

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References

1 Jemal A, Siegel R, Ward E, Hia Y, Xu J, Muray T and Thun MJ: Cancer Statistics. Cancer J Clin *58*: 71-96, 2008.
2 Kim YT and Zhao M: Aberrant cell cycle regulation in cervical carcinoma – review. Yonsei Med J *46*: 597-613, 2005.
3 Yu Y, Yang A, Hu S, Zhang J and Yan H: Significance of human papillomavirus 16/18 infection in association with *p53* mutation in lung carcinomas. Clin Respir J 2011.
4 Syrjänen KJ: Bronchial carcinoma and its regional lymph nodes in relation to immunological functions. Z Immunitatsforsch Immunobiol *155(3)*: 212-222, 1979.
5 Klein F, Amin Kotb Waleed FM and Petersen I: Incidence of papilloma virus in lung cancer. Lung Cancer *65*: 13-18, 2009.
6 Petersen I, Schewe C, Schlüns K, Dietel M, Speich N, Schmitt C, Bollmann M,Sotlar K, Bültmann B, Dours-Zimmermann MT, Padberg B and Zimmermann DR: Interlaboratory validation of PCR based HPV detection in pathology specimens. Virchows Arch *451(3)*: 701-716, 2007.
7 Sano T, Oyama T, Kashiwabara K, Fukuda T and Nakajima T: Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. Am J Pathol *153*: 1741-1748, 1998.

8 Psyrri A and Dimaio D: Human papillomavirus in cervical and head-and-neck cancer. Nat Clin Pract Oncol *5*: 24-31, 2008.
9 Demirhan O, Taştemir D, Hastürk S, Kuleci S, Hanta I: Alterations in *p16* and *p53* genes and chromosomal findings in patients with lung cancer: fluorescence *in situ* hybridization and cytogenetic studies. Cancer Epidemiol *34*: 472-7, 2010.
10 Travis WD, Brambilla E, Muller-Hermelink HK and Harris CC (Eds): World Heath Organization Classification of the Tumours. Pathology and Genetics of Tumors of the Lung, Pleura, Thymus and Heart. IARC Press: Lyon 2004.
11 Kim DH, Nelson HH, Wiencke JK, Zheng S, Christiani DC, Wain JC, Mark EJ and Kelsey KT: p16 INK4A and histology-specific methylation of CpG islands by exposure to tobacco smoke in non-small cell lung cancer. Cancer Res *61*: 3419-3424, 2001.
12 Dessy E, Rossi E, Berenzi A, Tironi A, Benetti A and Grigolato P: Chromosome 9 instability and alterations of p16 gene in squamous cell carcinoma of the lung and in adjacent normal bronchi: FISH and immunohistochemical study. Histopathology *52*: 475-482, 2008.
13 Gazzeri S, Gouyer V, Vour'ch C, Brambilla C and Brambilla E: Mechanisms of *p16 INK4A* inactivation in non small-cell lung cancers. Oncogene *16*: 497-504. 1998.
14 Aguayo F, Castillo A, Koriyama C, Higashi M, Itoh T, Capetillo M, Shuyama K, Corvalan A, Eizuru Y and Akiba S: Human papilloma virus-16 is integrated in lung carcinomas: a study in Chile. Br J cancer *97*: 85-91, 2007.
15 Aguayo F, Castillo A, Koriyama C, Higashi M, Itoh T, Capetillo M, Shuyama K, Corvalan A, Eizuru Y and Akiba S: Human papilloma virus-16 is integrated in lung carcinomas: a study in Chile. Br J cancer *97*: 85-91, 2007.
16 Ciotti M, Giuliani L, Ambrogi V, Ronci C, Benedetto A, Mineo TC, Syrjänen K and Favalli C: Detection and expression of human papilloma virus oncogenes in non-small cell lung cancer. Oncol Rep *16*: 183-189, 2006.
17 Castillo A, Aguayo F, Koriyama C, Shuyama K, Akiba S, Herrera-Goepfert R, Carrascal E, Klinge G, Sánchez J and Eizuru Y: Human papillomavirus in lung carcinomas among three Latin American countries. Oncol Rep *15*: 883-888, 2006.
18 Shamanin V, Delius H and de Villiers EM: Development of a broad spectrum PCR assay for papillomaviruses and its applications in screning lung cancer biopsies. J Gen Virol *75*: 1149-1156, 1994.
19 Koshiol J, Rotunno M, Gillison ML, Van Doorn LJ, Chaturvedi AK, Tarantini L, Song H, Quint WG, Struijk L, Goldstein AM, Hildesheim A, Taylor PR, Wacholder S, Bertazzi PA, Landi MT and Caporaso NE: Assessment of human papillomavirus in lung tumor tissue. J Natl Cancer Inst *103*: 501-507, 2011.
20 Landi S, Gemignani F, Canzian F, Gaborieau V, Barale R, Landi D, Szeszenia-Dabrowska N, Zaridze D, Lissowska J, Rudnai P, Fabianova E, Mates D,Foretova L, Janout V, Bencko V, Gioia-Patricola L, Hall J, Boffetta P, Hung RJ and Brennan P: DNA repair and cell cycle control genes and the risk of young-onset lung cancer. Cancer Res *15;66(22)*: 11062-11069, 2006.

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