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# Detection Of Human Papillomavirus By Chromogenic In Situ Hybridization And Expression Of P16 Protein In Malignant Lung Lesions: A Histomolecular Analysis

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## ABSTRACT

Lung cancer is the most prevalent and lethal malignant neoplasm worldwide, presenting high incidence and mortality rates. New studies have investigated the association between the Human Papillomavirus (HPV) and pulmonary carcinogenesis, suggesting that this virus may act as an additional risk factor for lung cancer, promoting the overexpression of protein p16(INK4a), a tumor suppressor and marker of cell cycle dysregulation, in malignant lung cells. However, the etiological relationship between HPV and lung cancer remains unclear. In this sense, the aim of this study was to detect and genotype HPV in lung malignant biopsy samples by nested-PCR, Chromogenic in situ Hybridization (CISH) and Multiplex-PCR with Reverse Line Blot Hybridization (PCR-RDB), correlating its presence with the immunohistochemical (IHC) expression of p16. 62 formalin-fixed paraffin-embedded biopsy samples were analyzed, especially adenocarcinomas and squamous cell carcinomas. 16.12% of samples were HPV-16 positive, while only 4.83% were HPV-18 positive. PCR-RDB identified the presence of HPV-16 E6 protein in 62.86% of cases. Positive expression of p16 by IHC was identified in 65.62% of lung biopsy samples. CISH assays of HPV-positive lung tumor samples were positive in 20% of cases. No significant differences were identified between comparisons by Mann-Whitney test. Significant differences were observed in Chi-Square and Fisher's analysis for HPV detection and p16 expression. In the Spearman's test significant correlations were observed, among others, between tumor stage and lesion (p = 0. 018), tumor stage and detection of HPV-16 E6 (p = 0.016), p16 expression and detection of HPV-16 E6 (p = 0.010) and between HPV detection and p16 expression (p = 0.030). For the diagnosis and differentiation of malignant lung lesions (SCCs or adenocarcinomas), p16 obtained excellent sensitivity (100%) and reasonable specificity (50%). Estimates for NPV were 100% and for PPV 82.10%. For the diagnosis and differentiation of HPV-positive tumors, p16 showed good sensitivity (67.74%) and excellent specificity (83.87%), with NPV corresponding to 55.32% and PPV to 89.82%. Our results reinforce the importance of HPV detection and genotyping, as well as p16 analysis as a complementary biomarker to the diagnosis of lung carcinomas and HPV-associated lesions. New studies, especially longitudinal and prospective ones, may improve p16 analysis by RT-qPCR or NGS, generating more accurate and robust results. HPV analysis should also include the evaluation of its oncogenes expression by quantitative methods, such as RT-qPCR, ensuring more accurate diagnoses and prognoses and a more assertive therapeutic approach.

**Keywords**: HPV; p16; Lung Cancer.

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## 1. INTRODUCTION

Lung cancer is the most prevalent and lethal malignant neoplasm worldwide, presenting high incidence and mortality rates. It is estimated that, annually, there are more than 2.4 million new cases and approximately 1.8 million deaths related to this disease (Bray *et al.*, 2024). In Brazil, projections from the National Cancer Institute (INCA, 2022) indicated, for each year of the 2023-2025 triennial, approximately 32,560 cases of lung cancer, corresponding to an estimated risk of 15.06 cases per 100,000 inhabitants, with 18,020 occurring in men and 14,540 in women.

Smoking is recognized as the primary risk factor for the development of lung cancer, accounting for about 85% of all cases. However, other factors, such as genetic susceptibility, inadequate diet, occupational exposures, and air pollution, may act independently or in conjunction with smoking, influencing the descriptive epidemiology of this neoplasm (Malhotra *et al.*, 2016; World Health Organization (WHO), 2023).

Recent studies have investigated the association between the presence of Human Papillomavirus (HPV) and pulmonary carcinogenesis, suggesting that this virus may act as an additional risk factor for the development of lung cancer. Researchers highlight possible mechanisms involving HPV oncoproteins E6 and E7, which are capable of modulating host tumor suppressor and apoptosis-related genes, such as p53 and retinoblastoma protein (pRb) leading to cellular immortality and carcinogenesis (Guo *et al.*, 2017; Sofiani *et al.*, 2023).

In this regard, there is evidence that the protein p16INK4a, a known tumor suppressor and marker of cell cycle dysregulation, is overexpressed in malignant lung cells. The possible expressions of HPV genes E6 and E7 in lung adenocarcinomas and squamous cell carcinomas (SCCs) could influence the behavior of p16, leading to disordered cell proliferation, as occurs in cervical carcinomas (Li *et al.*, 2023; Zhou *et al.*, 2019)

However, the etiological relationship between HPV and lung cancer remains unclear (Li *et al*., 2023). There is significant variability in HPV detection rates, with values ranging from 0 to 78%. It is believed that these differences may be explained by factors related to differential sensitivity among detection methods, as there is no gold standard method for HPV detection in human tissues. Furthermore, ethnic, lifestyle, and sociodemographic factors may also contribute to these discrepancies (Osorio *et al.*, 2022; Zhai; Ding; Shi, 2015)

Many studies have identified HPV presence on lung tissue, and the majority has been conducted in Asia, Europe and Latin-America (Tsyganov *et al.*, 2019). On the other hand, few studies have investigated HPV-positive lung neoplasms at E6 and E7 expression levels, correlation with p16 expression, and possible integration of the viral genome into the host cell genome. In this sense, the aim of this study was to detect and genotype HPV in lung malignant biopsy samples, correlating its presence with the immunohistochemical expression of p16 and the clinical-pathological characteristics of patients with lung cancer. HPV-positive samples were analyzed by chromogenic in situ hybridization (CISH) to investigate the possible integration of the viral genome into the host cell genome, especially in primary lung tumors.

## 2. MATERIAL AND METHODS

*Study Characterization and Ethical Aspects*

This is an observational, cross-sectional and retrospective study conducted through the analysis of 62 formalin-fixed, paraffin-embedded (FFPE) lung biopsy samples from the Pathology Department of the Santa Casa de Misericórdia de Alagoas, Brazil. All samples were from malignant lung neoplasms. The patients' clinical-pathological data were collected from medical records stored in the hospital's clinical documentation department. The selected FFPE samples were sectioned in a microtome, resulting in 4 µm thick sections. Some of the sections were used to prepare slides for hematoxylin and eosin (HE) staining, CISH and immunohistochemistry (IHC) for p16INK4a. Another part of the sections was placed in 2 µl microtubes for DNA extraction and subsequent Nested Polymerase Chain Reaction (Nested-PCR) and Multiplex-PCR/Reverse Line Blot Hybridization (PCR-RDB) for DNA-HPV detection and genotyping. HPV-positive samples with known viral subtypes were analyzed by CISH to identify whether the HPV genome had integrated into the host cell genome. This study was approved by the Ethics and Research Committee of the State University of Health Sciences of Alagoas (No. 2,729,138, CAAE: 79884317.4.0000.5011).

*DNA Extraction, Viral DNA Detection and Genotyping*

The samples stored in microtubes, each containing 10 sections of 4 µm thick, underwent nucleic acid extraction. First, the samples were deparaffinized in xylene solution, homogenized in a vortex, and centrifuged at 13,000 rpm (2 minutes, 25°C). The samples were then washed in ethyl alcohol (96-100%) and again homogenized in a vortex and centrifuged at 13,000 rpm (2 minutes, 25°C). The ethanol residue was then carefully removed to avoid disturbing the pellet, and the open microtube was placed in an incubator at 37°C to evaporate all the ethanol. For isolation of genomic DNA from FFPE tissue sections, the QIAamp DNA FFPE Tissue Kit (Qiagen®) was used according to the manufacturer's protocol. After extraction, the samples underwent DNA quantification using the NanoDrop® Lite Spectrophotometer. After quantification, the samples were stored in a freezer at -80°C.

For DNA-HPV detection, a conventional PCR was initially performed with primers for MY09/11, resulting in fragments of approximately 450 bp. From the MY09/11 amplicons, a nested-PCR was performed with the primer pair for GP5/6, forming fragments of approximately 140 bp, representative of the L1 region of the HPV genome. All samples were amplified in the presence of positive controls (HeLa Cells, HPV18) and negative controls (DNAse-free water).

The reaction was performed using a PCR preparation mix following the manufacturer's guidelines. Each sample tube contained 2 µL of 5x FIREPOL® Master Mix Ready to Load (Solis BioDyne Data Sheet – 12.5 mM MgCl2), 0.3 µL of each primer (10 pmol/µL), 4 µL of DNA, and DNAse-free H2O to complete a volume of 10 µL. The thermocycling conditions were as follows: initial denaturation for 5 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C, with a final extension at 72°C for 5 minutes. The Nested-PCR products were separated by 2% agarose gel electrophoresis and stained with 10% ethidium bromide. The β-Actin gene was used as a control for DNA extraction, PCR reactions and electrophoresis. DNA integrity was assured by amplification of the β-Globin gene under the same PCR conditions.

The samples that tested positive for HPV detection by nested-PCR were genotyped using the PCR-RDB methodology. The in vitro diagnostic kit for HPV PCR-RDB detection and genotyping (Xgen Multi HPV Lyo Chip-HS12 auto, Vitro Grup®) detects 35 different HPV types, including high-risk HPV (16, 18, 26, 31, 33, 35 etc) and low-risk HPV (6, 11, 40, 42, 43, 44 etc). The assay principle is based on the amplification of the L1 region of HPV by PCR-RDB with specific DNA probes immobilized on a nylon membrane chip (Flow Chip Technology). The hybridization process allows the binding of biotinylated PCR products to complementary probes present on the chip, and the hybridization signal is developed by an immunoenzymatic colorimetric reaction (streptavidin-alkaline phosphatase and NBT-BCIP chromogen). All procedures were performed according to the manufacturer's instructions.

*Chromogenic in situ Hybridization for HPV Integration*

HPV-positive lung tumor samples were analyzed by CISH using the ZytoFast® PLUS CISH Implementation Kit HRP-DAB (ZytoVision GmbH, Bremerhaven, Germany). The ZytoFAST® HPV type 16/18 Probe was used, capable of detecting sequences that encode the L1, E6, and E7 proteins of HPV. Initially, the samples were pretreated by dewaxing in xylene baths, incubations in 100% ethyl alcohol and hydrogen peroxide ($H_2O_2$), and washing in distilled water. The slides were then incubated at 98°C for 15 minutes in a water bath in pre-warmed Heat Pretreatment Solution (EDTA). After further washing in distilled water, proteolysis was performed with Pepsin Solution in a humid chamber at 37°C. After the proteolytic step, the slides were dehydrated in increasing solutions of ethyl alcohol (70%, 90% and 100%).

For denaturation and hybridization, 10 µL of ZytoFAST HPV type 16/18 Probe was placed on each slide in a hybridizer at 75°C for 5 minutes. The slides were then transferred to a humid chamber and hybridized for 1 hour at 37°C. This was followed by washing in TBS buffer (1x) and incubation with Anti-Mouse-Digoxigenin (DIG) and Anti-Mouse-HRP-Polymer in a humid chamber at 37°C for 30 minutes each. The chromogenic solution containing 3,3-Diaminobenzidine (DAB) was applied to reveal the hybridization reactions. Finally, the slides were washed in distilled water, counterstained with Nuclear Blue Solution, dehydrated in ethyl alcohol, clarified with xylene, air-dried, and mounted with a coverslip and Entellan® (Merk).

The analysis was performed on a Zeiss® optical microscope by two independent pathologists in a double-blind scheme, with images being recorded and captured by the Zen Blue Edition System (Zeiss®). Positive hybridization reactions showed brownish staining. Only nuclear staining in lung neoplastic cells were considered, representing HPV DNA sequences. Positive controls included samples of cervical SCCs, while negative controls were performed without incubation with the ZytoFAST HPV type 16/18 Probe.

*Immunohistochemistry for p16INK4a*

Immunohistochemistry for detecting p16INK4a expression was performed on silanized slides containing 4 µm histological sections. Additionally, negative controls without incubation with primary antibody and positive controls with samples of cervical SCCs were used. The staining method used was the biotin-free and HRP-conjugated (ImmPRESS® Excel Amplified Polymer Staining Kit, Anti-Mouse IgG, Peroxidase, Vector Laboratories). The antibody used was the Anti-CDKN2A/p16INK4a (1:50 dilution, sc-56330, Santa Cruz Biotechnology®).

The slides were deparaffinized according to the manufacturer's established protocol and incubated for 4 hours at 60°C, followed by three xylene baths for 10 minutes each. After xylene treatment, the slides were immersed in a graded alcohol series (100% I, 100% II, 100% III, 90%, 80%, and 70%) for 5 minutes each. Antigen retrieval was performed in an electric pressure cooker with slides incubated in sodium citrate buffer (0.01M, pH 6.0). Endogenous peroxidase and non-specific binding sites blocking was done with BLOXALL, for 10 minutes, and 2.5% horse serum, for 20 minutes, respectively.

After removing the excess serum, the slides were incubated overnight in a dark chamber with the primary antibody anti-mouse p16. The following day, after washing with PBS, the samples were incubated for 15 minutes with the amplification antibody and with the HRP visualization reagent (ImmPRESS® Polymer Reagent) for 30 minutes. Finally, the slides were incubated for 5 minutes with the DAB chromogen solution from the kit and counterstained with Harris hematoxylin. After

drying the slides, they were mounted with coverslips and Entellan® (Merk).

The slides were analyzed using a Zeiss® optical microscope, and the images were captured and recorded with the aid of the Zen Blue Edition system (Zeiss®). The evaluation of the p16 immunoreactivity pattern in lung carcinoma samples was performed qualitatively by two independent pathologists, according to the double-blind model. For the purpose of immunoreaction positivity, only nuclear staining, with or without cytoplasmic staining, in neoplastic lung cells were considered. Marking areas were identified as DAB-positive regions, stained in brownish colors.

*Statistical Analysis*

Statistical analysis was performed using Prism software, version 10.4 (GraphPad Software®). Nonparametric tests were performed, with values expressed as median plus interquartile range, and differences considered significant when $p < 0.05$. The Mann-Whitney test was used to compare the data of categorical variables between two independent groups. Similarly, the Chi-square test of independence ($X^2$) and Fisher's exact test were applied to establish comparisons between the expected and observed frequencies of categorical variables, divided according to positivity or negativity of HPV detection, whether by nested-PCR or CISH, and p16 expression. Spearman's correlation was applied to investigate the strengths of association between categorical variables, whether directly or inversely proportional. Univariate logistic regression analyses were used to estimate the probability of predictor variables influencing HPV-16 detection positivity and p16 expression. Analyses of sensitivity, specificity and negative (NPV) and positive (PPV) predictive values estimated the potential of p16 for diagnosing malignant lung lesions and distinguishing their histological subtypes.

## 3. RESULTS

Sixty-two formalin-fixed paraffin-embedded biopsy samples were analyzed, all representative of malignant lung neoplasms, especially adenocarcinomas (45.2%) and squamous cell carcinomas (45.2%), in addition to undifferentiated and metastatic tumors. In the tumor staging analysis, approximately 54.9% of cases were considered stage I, while stages II and III accounted for 29.03% and 14.51%, respectively. Approximately 16.12% of samples were HPV-16 positive, while only 4.83% were HPV-18 positive. Quantitative analyses by PCR-RDB detected the presence of HPV-16 E6 protein in 62.86% of cases. Positive expression of p16 by immunohistochemistry was identified in 65.62% of lung biopsy samples. CISH assays of HPV-positive lung tumor samples were positive in 20% of cases. Distributions of HPV and p16 positive cases based on histological lesion type, tumor stage and CISH are shown in Figure 1.

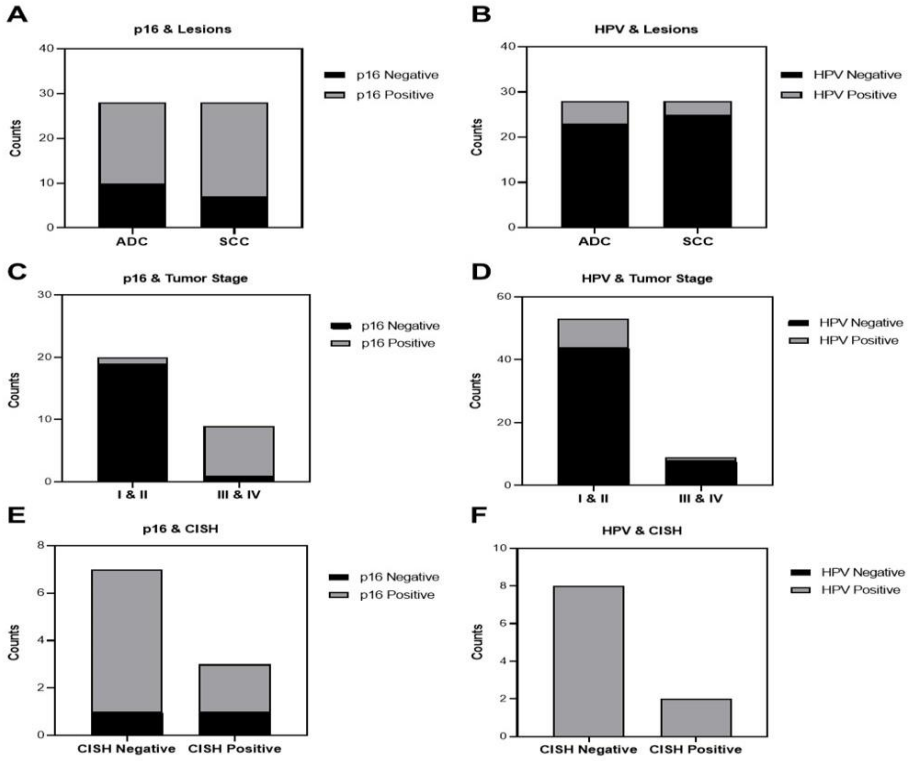


**Figure 1. HPV and p16 positive samples distributed by Lesions (A and B), Tumor Stage (C and D) and CISH (E and F), respectively. ADC: Adenocarcinoma; CISH: Chromogenic in situ Hybridization; HPV: Human Papillomavirus; SCC: Squamous Cell Carcinoma.**

*HPV Detection and Genotyping*

HPV DNA was detected by nested PCR in only 12 of the 62 samples analyzed (19.35%). Genotyping by PCR-RDB identified HPV-16, -18, -6, and -11 among the viral subtypes investigated in the 10 lung tumor samples. HPV-16 was identified in all 10 HPV-positive samples, with coinfection occurring in 40% of cases, mainly between HPV-16 and HPV-18. Of all HPV DNA-positive samples, 50% were adenocarcinomas and 30% were SCCs, 80% were classified as class I on tumor staging analysis, and 100% of cases were positive for p16 expression by IHC. Among HPV-positive cases, there was also a predominance of females (90%), individuals over 45 years of age (90%), brown-skinned individuals (70%), and individuals with comorbidities (70%).

*Comparison Tests and Regression Analysis*

Comparisons between two independent groups were performed using Mann-Whitney test, based on HPV detection by nested-PCR and p16 expression via IHC (Table 1). No significant differences were identified between the clinicopathological variables compared, except for HPV detection and p16 expression.

**Table 1. Mann-Whitney analysis based on HPV Detection and p16 Expression.**

| Categorical Variables (Lung & HPV) | HPV Detection (p Value) | p16 Expression (p Value) |
|---|---|---|
| Age (≤ 45 Years x > 45 Years) | >0.999 | 0.3170 |
| Sex (Female x Male) | 0.0758 | 0.2613 |
| Ethnicity (Black x White) | 0.2447 | 0.3903 |
| BMI (≤ 25 Kg/m² x > 25 Kg/m²) | >0.999 | 0.7863 |
| Smoking (No Smoking x Smoking) | >0.999 | 0.1655 |
| Comorbidity (No Comorbidity x Comorbidity) | 0.5111 | 0.1655 |
| Malign Lesion (ADC x SCC) | 0.7049 | 0.5619 |
| Tumor Stage (I+II x III+IV) | >0.999 | 0.2494 |
| E6 Detection by PCR (E6 Negative x E6 Positive) | >0.999 | >0.999 |
| p16 Expression by IHC (p16 Negative x p16 Positive) | 0.0230 | - |
| HPV Detection by Nested-PCR (HPV Negative x HPV Positive) | - | 0.0230 |
| CISH Detection (CISH Negative x CISH Positive) | - | >0.999 |

ADC: Adenocarcinoma; BMI: Body Mass Index; CISH: Chromogenic in situ Hybridization; IHC: Immunohistochemistry; PCR: Polymerase Chain Reaction; SCC: Squamous Cell Carcinoma.

Immunohistochemical reaction for p16 was performed on 62 lung biopsy samples. Nuclear staining, with or without cytoplasmic staining, was considered positive for p16 expression. Adenocarcinoma and SCC samples were analyzed, as well as negative and positive controls, performed using samples of normal squamous tissue and cervical SCCs (Figure 2).

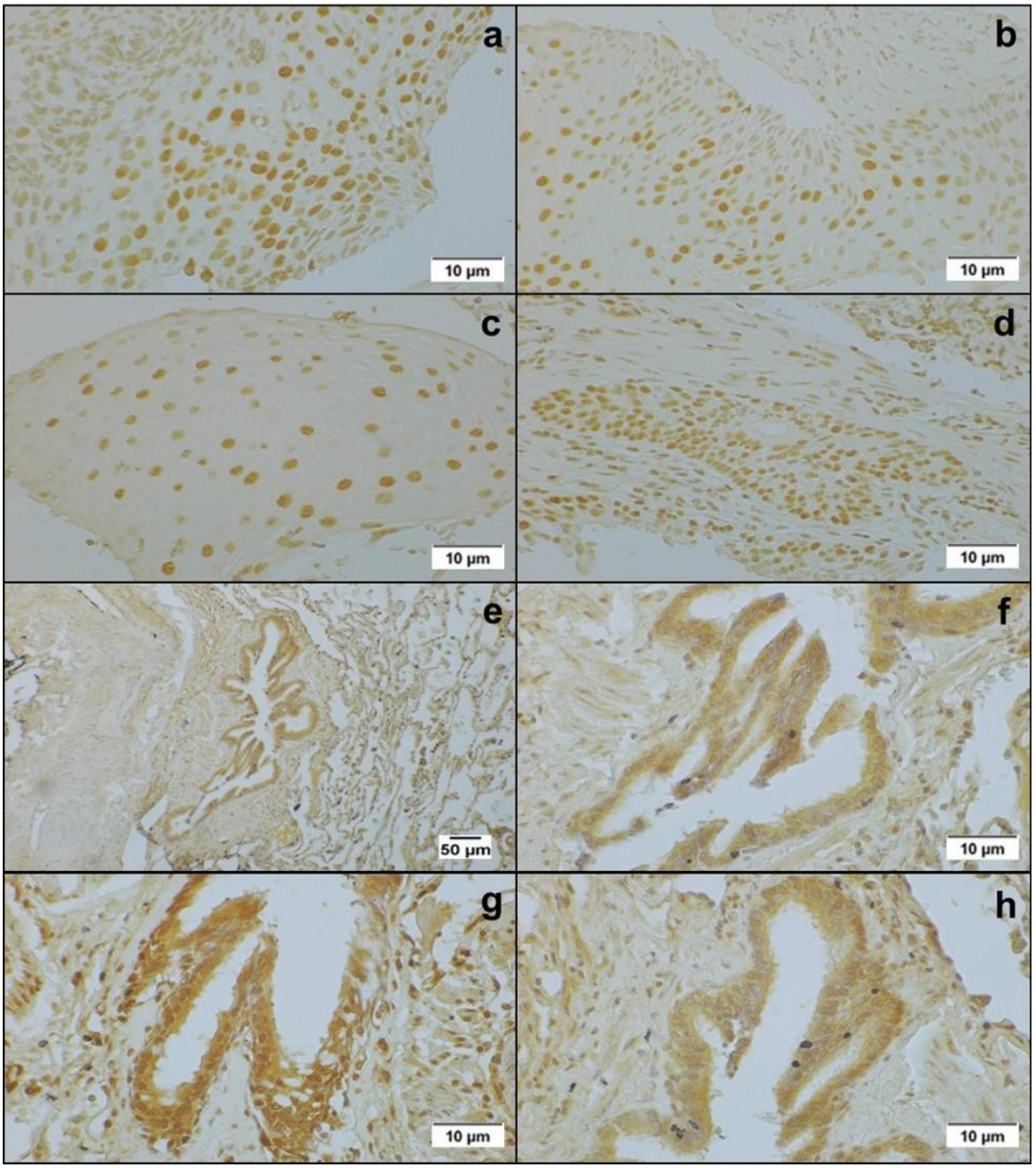


**Figure 2. Immunohistochemical reaction for p16 in lung biopsy samples. (A, B, C and D) Positive reaction for p16 in lung tumor, showing nuclear staining in cells (400×). (E) Positive reaction for p16 in lung tumor (100×). (F, G and H) Positive reaction for p16 in lung tumor with nuclear and cytoplasmic stainings.**

CISH assays were performed on the 10 lung tumor samples with positive HPV detection by nested PCR. Nuclear stainings for DNA sequences encoding L1, E6, and E7 genes were considered evidence of HPV genome integration into the host cell genome, occurring in 20% of cases, as shown in Figure 3.

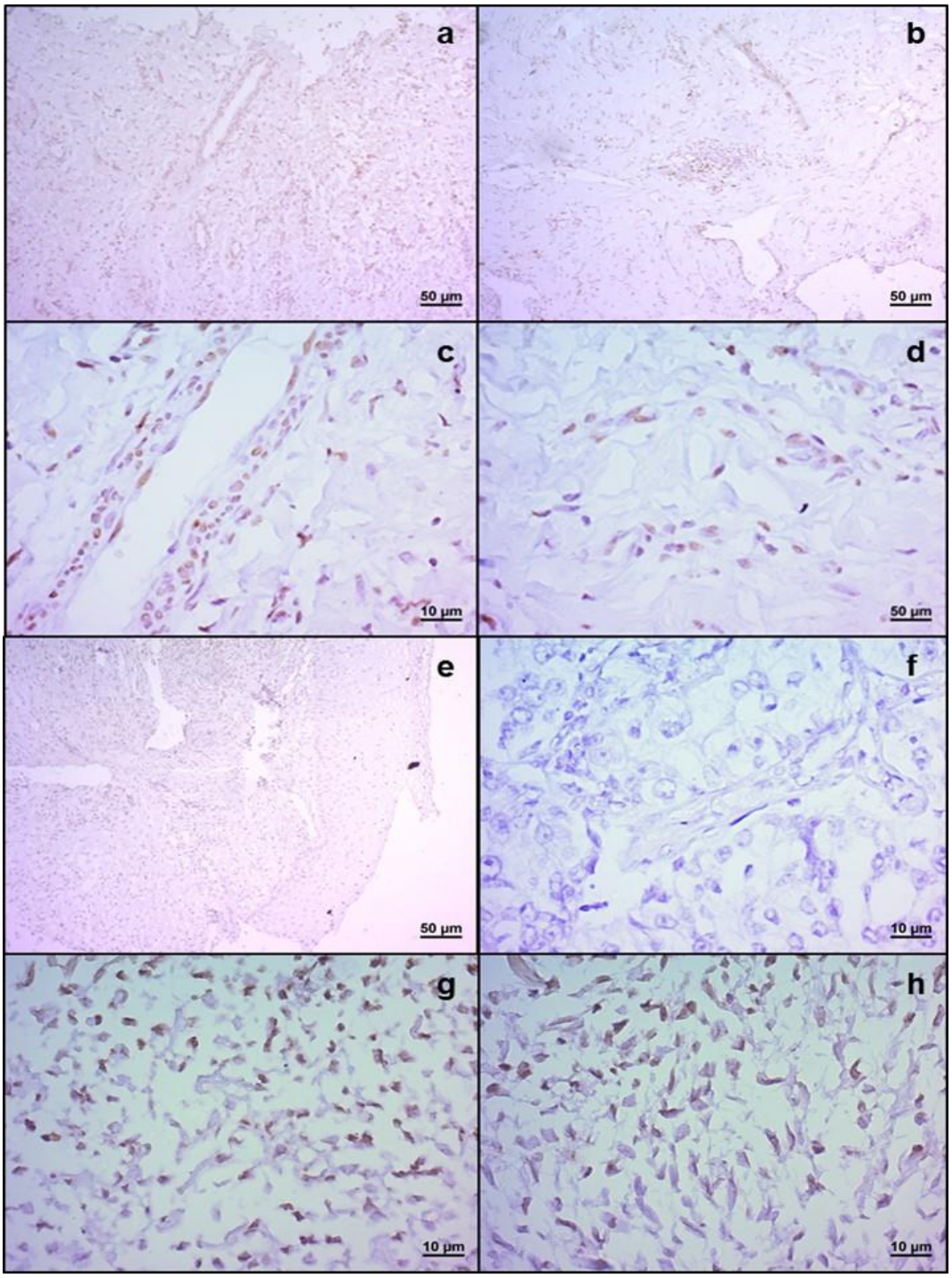


**Figure 3. CISH detection of HPV genes in lung biopsy samples. (A and B) Positive CISH reaction in lung cells (100×). (C and D) Positive CISH reaction in lung cells (400×). (E and F) Negative CISH reaction in lung cells (100× and 400×, respectively). (G and H) Positive CISH reaction in lung cells (400×).**

Chi-square ($X^2$) and Fisher's exact tests were used to compare, in a contingency and binary model, the observed and expected frequencies between two groups of clinical-pathological variables, based on HPV detection (negative or positive) and p16 expression via IHC (negative or positive). When analyzing the comparisons between the groups, organized according to the presence/absence of HPV and expression of p16, no significant differences were identified for the clinical-pathological variables and CISH detection. However, when comparing the observed and expected proportions between

**Table 2. Categorical Variables compared by HPV detection and p16 expression.**

| Categorical Variables | HPV (-) | HPV (+) | p16 (-) | p16 (+) | $X^2$ (p Value) | | Fisher (p Value) | |
|---|---|---|---|---|---|---|---|---|
| | OF (EF) | OF (EF) | OF (EF) | OF (EF) | HPV | p16 | HPV | p16 |
| Age | | | | | 0.806 | 0.166 | >0.99 | 0.317 |
| ≤ 45 Years | 04 (4.19) | 01 (0.80) | 03 (1.61) | 02 (3.38) | | | | |
| > 45 Years | 48 (47.8) | 09 (9.19) | 17 (18.3) | 40 (38.6) | | | | |
| Sex | | | | | 0.052 | 0.173 | 0.075 | 0.261 |
| Female | 30 (32.7) | 09 (6.29) | 15 (12.5) | 24 (26.4) | | | | |
| Male | 22 (19.2) | 01 (3.70) | 05 (7.42) | 18 (15.5) | | | | |
| Ethnicity | | | | | 0.169 | 0.326 | 0.244 | 0.384 |
| Black | 32 (33.7) | 07 (5.28) | 11 (12.6) | 29 (27.3) | | | | |
| White | 19 (17.2) | 01 (2.71) | 08 (6.33) | 12 (13.6) | | | | |
| BMI | | | | | >0.99 | 0.530 | >0,99 | 0.595 |
| ≤ 25 Kg/m² | 26 (26) | 05 (05) | 11 (9.84) | 20 (21.1) | | | | |
| > 25 Kg/m² | 26 (26) | 05 (05) | 09 (10.1) | 23 (21.8) | | | | |
| Smoking | | | | | 0,981 | 0.104 | >0.99 | 0.165 |
| No | 21 (20.9) | 04 (4.03) | 11 (8.06) | 14 (16.9) | | | | |
| Yes | 31 (31.0) | 06 (5.96) | 09 (11.9) | 28 (25.0) | | | | |
| Comorbidity | | | | | 0,467 | 0.104 | 0.726 | 0.165 |
| No | 22 (20.9) | 03 (4.03) | 11 (8.06) | 14 (16.9) | | | | |
| Yes | 30 (31.0) | 07 (5,97) | 09 (11.9) | 28 (25.0) | | | | |
| Malign Lesion | | | | | 0.445 | 0.383 | 0.704 | 0.561 |
| ADC | 23 (24) | 05 (04) | 10 (8.50) | 18 (19.5) | | | | |
| SCC | 25 (24) | 03 (04) | 07 (8.50) | 21 (19.5) | | | | |
| Tumor Stage | | | | | 0.658 | 0.142 | >0.99 | 0.249 |
| I+II | 44 (44.4) | 09 (8.54) | 19 (17.0) | 34 (35.9) | | | | |
| III+IV | 08 (7.54) | 01 (1.45) | 01 (2.90) | 08 (6.09) | | | | |
| E6 Detection | | | | | 0.783 | 0.783 | >0.99 | >0.99 |
| Negative | 10 (9.65) | 03 (3.34) | 10 (9.65) | 03 (3.34) | | | | |
| Positive | 16 (16.3) | 06 (5.65) | 16 (16.3) | 06 (5.65) | | | | |
| p16 Expression | | | | | 0.017 | - | 0.023 | - |
| Negative | 20 (16.7) | 0 (3.22) | - | - | | | | |
| Positive | 32 (35.2) | 10 (6.77) | - | - | | | | |
| HPV Detection | | | | | - | 0.017 | - | 0.023 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Negative | - | - | 20 (16.7) | 00 (3.22) | | | | |
| Positive | - | - | 32 (35.2) | 10 (6.77) | | | | |
| CISH Detection | | | | | - | 0.490 | - | >0.99 |
| Negative | - | - | 01 (1.4) | 06 (5.6) | | | | |
| Positive | - | - | 01 (0.6) | 02 (2.4) | | | | |

HPV detection and p16 expression by IHC, statistical significance was observed, as shown in Table 2.

ADC: Adenocarcinoma; BMI: Body Mass Index; CISH: Chromogenic in situ Hybridization; EF: Expected Frequencies; HPV: Human Papillomavirus; IHC: Immunohistochemistry; OF: Observed Frequencies; PCR: Polymerase Chain Reaction; SCC: Squamous Cell Carcinoma.

Univariate logistic regression analyses estimated the probabilities of clinical-pathological variables, including malignant lesion subtypes (adenocarcinoma or SCC) and tumor staging levels (I-III), influencing the presence/absence of HPV, detected via nested-PCR, and positive or negative expression of p16 by IHC. No statistically significant results were observed in the regression analysis, either for HPV detection or p16 expression (Table 3). Odds ratio (OR) estimates evaluated the possibilities of HPV identification and p16 immunoreactivity varying between different lesion subtypes, tumor staging levels and clinical-pathological characteristics, such as age and smoking, when compared to the absence of HPV detection or p16 expression.

Table 3. Logistic regression for HPV detection and p16 expression in lung lesions.

| Categorical Variables (Lung) | Biomarker | p Value | OR | Confidence Interval (95%) | |
|---|---|---|---|---|---|
| | | | | Lower Limit | Upper Limit |
| Age (≤ 45 Years x > 45 Years) | HPV | 0.8067 | 1.333 | 0.06423 | 10.42 |
| | p16 | 0.1879 | 0.283 | 0.03488 | 1.854 |
| Sex (Female x Male) | HPV | 0.0836 | 0.151 | 0.00792 | 0.894 |
| | p16 | 0.1788 | 2.250 | 0.72090 | 7.962 |
| Ethnicity (Black x White) | HPV | 0.1984 | 0.240 | 0.01238 | 1.502 |
| | p16 | 0.3608 | 0.589 | 0.18820 | 1.859 |
| BMI (≤ 25 Kg/m² x > 25 Kg/m²) | HPV | >0.999 | 1.000 | 0.25030 | 3.995 |
| | p16 | 0.5874 | 0.743 | 0.25060 | 2.164 |
| Smoking (No Smoking x Smoking) | HPV | 0.9819 | 1.016 | 0.25830 | 4.383 |
| | p16 | 0.1079 | 2.444 | 0.82830 | 7.455 |
| Comorbidity (No Comorbidity x Comorbidity) | HPV | 0.4709 | 0.584 | 0.11590 | 2.363 |
| | p16 | 0.1079 | 0.409 | 0.13410 | 1.207 |
| Malign Lesion (ADC X SCC) | HPV | 0.5604 | 2.077 | 0.18800 | 46.26 |
| | p16 | 0.3852 | 1.667 | 0.53100 | 5.461 |
| Tumor Stage (I+II x III) | HPV | 0.6607 | 0.611 | 0.03100 | 3.981 |
| | p16 | 0.1729 | 4.471 | 0.73740 | 86.15 |
| E6 Detection by PCR (E6 Negative x E6 Positive) | HPV | 0.7840 | 0.800 | 0.14310 | 3.801 |
| | p16 | 0.8322 | 1.222 | 0.20190 | 9.884 |

ADC: Adenocarcinoma; BMI: Body Mass Index; HPV: Human Papillomavirus; OR: Odds Ratio; PCR: Polymerase Chain Reaction; SCC: Squamous Cell Carcinoma.

*Correlation and Diagnostic Metrics Analysis*

Spearman's nonparametric test investigated the strengths of association between different groups of categorical variables, ranging from clinical-pathological variables, including age, gender, ethnicity, BMI, smoking and presence of comorbidities, to distinct subtypes of histological lesions, tumor staging levels, presence of HPV and expression of p16. Correlation intensities, expressed in Rho values, ranged from -1 to 1, with values from 0 to -1 indicating inversely proportional associations, 0 to 1 directly proportional, and 0 indicating no correlation.

As shown in Figure 4 (A-D), significant correlations were observed between age and smoking (p = 0.037), sex and tumor stage (p = 0.002), BMI and lesion (p = 0.019), tumor stage and lesion (p = 0. 018), tumor stage and detection of HPV-16 E6 by PCR (p = 0.016), p16 expression and detection of HPV-16 E6 (p = 0.010), and between HPV detection and p16 expression (p = 0.030).

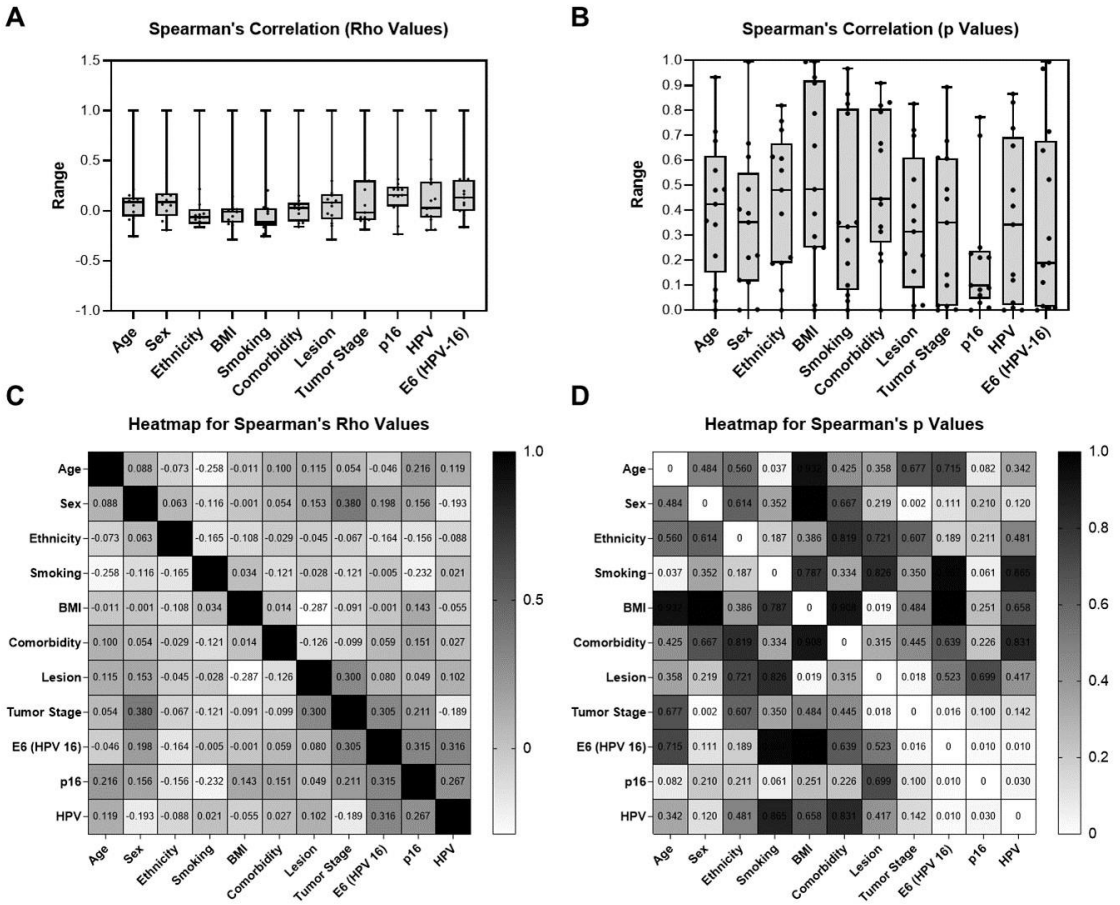


**Figure 4. Spearman's Correlation (Rho and p values). (A) Rho values for variables correlated by Spearman's test. (B) p values for variables correlated by Spearman's test. (C) Heatmap for Spearman's correlated Rho values. (D) Heatmap for Spearman's correlated p values.**

Accuracy analyses of diagnostic metrics for p16 were performed to assess the diagnostic potential and distinction between malignant histological subtypes (SCCs vs. adenocarcinomas) in lung biopsy samples, as well as between HPV-positive and HPV-negative tumors. ROC curves were constructed to graphically represent the performance of p16 in both proposed hypotheses, based on estimates of sensitivity, specificity, Youden's index, and area under the curve (AUC).

For the diagnosis and differentiation of malignant lung lesions (SCCs or adenocarcinomas), p16 obtained excellent sensitivity (100%) and reasonable specificity (50%), with a Youden index corresponding to 0.50 and AUC of 0.875. Estimates for NPV were 100% and for PPV 82.10%. For the diagnosis and differentiation of HPV-positive tumors, p16 showed good sensitivity (67.74%) and excellent specificity (83.87%), with NPV corresponding to 55.32% and PPV to

89.82%. The Youden index was 0.52 and the AUC was 0.824. The ROC curves, both for the performance of p16 for the diagnosis of lung lesions and for the identification of HPV-positive tumors, are shown in Figure 5 (A-B).

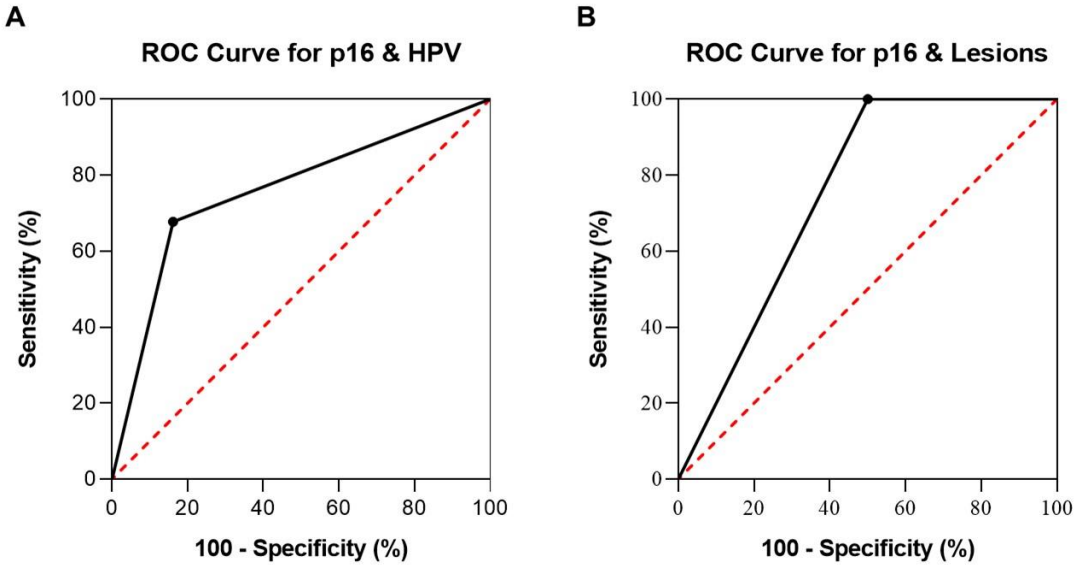


**Figure 5. ROC Curves for p16 expression by IHC. (A) ROC curve for the performance of p16 in the diagnosis of HPV-positive lung tumors. (B) ROC curve for the performance of p16 in the diagnosis and differentiation of malignant lung lesions.**

## 4. DISCUSSION

In this study, statistically significant results were observed for the presence of HPV and p16 immunoreactivity either by comparison analyses using Mann-Whitney, Chi-square and Fisher tests, or by Spearman's correlation test. Logistic regression analyses, however, did not show significant results when considering the influence of clinical-pathological variables on HPV detection and p16 expression. Evaluations of diagnostic metrics showed promising results, with satisfactory performance of p16 as a biomarker for malignant lung lesions and HPV-positive tumors. However, no significant differences were observed when comparing HPV detection by CISH and the IHC expression of p16.

With regard to the histological subtypes of lung lesions, this study observed a higher prevalence of ADCs and SCCs, in line with the research by Roosta; Valizadeh; Abbasi (2023), who identified, between 2016 and 2021, through the analysis of 567 histological samples of lung carcinomas, a prevalence of 25.6% for ADCs and 9.3% for SCCs. The study by Nguyen *et al.* (2022), in this sense, shows that SCC was the most prevalent histological subtype in the 1990s, having been surpassed by ADC, probably due to its higher incidence among non-smokers and the fact that smoking has decreased in recent decades, as proposed by Meza *et al.* (2023).

HPV infection is also an important risk factor for benign and malignant lesions, both in the genital tract and in other anatomical sites, such as the head and neck and, possibly, the lungs (Akhtar; Bansal, 2017; McBride, 2024). According to Tsyganov *et al.* (2019) and Sequeira *et al.* (2024), the frequency of HPV detection in lung samples has varied widely, ranging from 0 to 75%, possibly due to geographical and methodological differences between the various studies. Globally, the highest prevalence of HPV in lung cancer occurs in Asia, followed by Europe and America (De Freitas *et al.*, 2016). According to Karnosky *et al.* (2021), countries such as Brazil, Greece, and South Korea have the highest rates of HPV detection in lung cancer.

In our study, HPV DNA was detected in 16.12% of the 62 lung biopsy samples. HPV-16, a high-risk oncogenic subtype, was detected in 100% of positive samples, with coinfection by HPV-18 occurring in 30% of cases, HPV-11 in 20%, and HPV-6 in 10%. Our findings also showed no significant associations between the presence of HPV and the histomorphological subtypes of the lesions. Similarly, the meta-analysis by Shikova *et al.* (2017) identified that HPV-16/18 is detected in about 25% of lung lesions, with prevalence ranging from 0 to 48.1% for SCCs and 0 to 44.4% for ADCs. However, the role of HPV in these lesions is still poorly understood, and its presence may be associated with both hematogenous dissemination and the expression of oncogenic genes, such as E6 and E7, responsible for the mechanisms of cell cycle dysregulation after virus integration into host cells (Hu *et al.*, 2020).

In this sense, in our study, HPV-positive malignant lung lesions were analyzed by CISH in order to identify the integration of the viral genome into the host cell genome. However, only 20% of HPV-positive tumors were also CISH-positive. It is possible that the higher prevalence of CISH-negative lesions was influenced by low viral load, the spread of HPV via the bloodstream or the dissemination of metastatic foci of HPV-positive cancers, such as cervical cancer. On the other hand, despite the integration of the viral genome into CISH-positive tumors, it was not possible to establish HPV as prominent in lung carcinogenesis, given that viral transcripts were not analyzed and, therefore, the tumorigenic activity of HPV in malignant lung neoplasms cannot be confirmed, even though the virus influences the molecular modulation of the tumor microenvironment.

Thus, as proposed by Li *et al.* (2023), the possible carcinogenic mechanisms of HPV in the development and progression of SCCs and ADCs still need further elucidation, whether in terms of cause or distinction between transient infections and active oncogenic processes. In these cases, the analysis of biomarkers associated with cell cycle dysregulation, such as that induced by HPV oncogenes E6 and E7, may aid in understanding the etiopathogenesis of lung lesions. In our study, 100% of HPV-positive samples showed p16 expression, indicating probable dysregulation of cell cycle checkpoint mechanisms and possible errors in the mitotic division process, culminating in disordered cell proliferation and tumor formation (Li *et al*., 2023).

Although p16 expression is a good indicator of active HPV infection and probable integration of the viral genome into the host cell, especially in the squamous tissues of the uterine ectocervix, isolated analysis of p16 as a biomarker is not sufficient to establish HPV as a definitive risk factor for pulmonary carcinogenesis (Kataoka *et al.*, 2023). In this sense, according to Li *et al.* (2023), p16 is frequently used as an alternative molecular marker and potential indicator of HPV infection, even though both HPV DNA detection by PCR and IHC expression of p16 in lung tumors show high variability between different studies.

In this perspective, Zhou *et al*. (2019) suggest that p16 may be an excellent immunohistochemical biomarker for the diagnosis and prognosis of primary lung adenocarcinomas, indicating more favorable survival times than those of p16-negative tumors. Marcos *et al.* (2022) propose that HPV, by inducing changes in the tumor microenvironment, may also accentuate the expression of p16 and other molecular effectors, even though it is not a direct cause of carcinogenesis. Therefore, despite the outstanding potential of p16 as a biomarker, it is important to emphasize that its isolated analysis may present several limitations, ranging from difficulties in distinguishing between benign and malignant lesions to implications for the estimated prognosis for patients (Gonçalves *et al.*, 2017).

This study also had some limitations, primarily due to its retrospective and observational nature, without the possibility of long-term follow-up of patients with lung tumors or evaluation of the clinical outcome of the lesions. In addition, the samples analyzed, obtained from FFPE material, may be of reduced quality for DNA extraction and HPV detection and genotyping, due to formalin fixation and paraffin impregnation. Similarly, the analysis of p16 expression by IHC alone, although effective, leaves room for improvement of results by more complex, quantitative and accurate methods, such as Real-Time PCR, Reverse Transcription PCR (RT-qPCR) and Next Generation Sequencing (NGS).

Therefore, for a more robust and accurate analysis of lung lesions, it is necessary to evaluate diagnostic panels with several biomarkers, including p16, which could assist in determining the nature of the lesions (whether benign or malignant), histological subtype, and tumor staging level. In this sense, the joint analysis of biomarker expression could improve the sensitivity and specificity rates associated with the identification of benign or malignant and HPV-positive or HPV-negative lesions. The evaluation of mutagenic patterns of lung carcinomas, such as EGFR-positive, added to the IHC expression of p16, as well as HPV detection and genotyping, could increase diagnostic accuracy, individualizing the clinical-therapeutic approach according to the histological and molecular classifications of the tumors.

## 5. CONCLUSION

Our results reinforce the importance of HPV detection and genotyping by PCR followed by in situ hybridization, as well as p16 analysis as a complementary biomarker to the histopathological diagnosis of lung carcinomas and HPV-associated lesions. New studies, especially longitudinal and prospective ones, may improve p16 analysis by quantifying its expression values by RT-qPCR or NGS, generating more accurate and robust results. Similarly, HPV analysis should not be restricted to virus detection and genotyping, but should also include the evaluation of its oncogene expression, both by quantitative methods, such as RT-qPCR, and qualitative methods, such as immunofluorescence. Finally, the analysis of a wider variety of histomorphological subtypes of lung carcinomas, in addition to ADCs and SCCs, may improve the specificity of p16 and other biomarkers, ensuring more accurate diagnoses and prognoses and a more assertive therapeutic approach.

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## REFERENCES

[1] AKHTAR, N.; BANSAL, J. G. Risk factors of Lung Cancer in nonsmoker. Current Problems in Cancer, [s. l.], v. 41, n. 5, p. 328–339, 2017.

[2] BRAY, F. et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians, [s. l.], v. 74, n. 3, p. 229–263, 2024.

[3] DE FREITAS, A. C. et al. Human papillomavirus and lung cancinogenesis: an overview. Journal of Cancer Research and Clinical Oncology, [s. l.], v. 142, n. 12, p. 2415–2427, 2016.

[4] GONÇALVES, J. E. S. et al. The role of p16 as putative biomarker for cervical neoplasia: A controversial issue?. Medical Express, [s. l.], v. 4, n. 6, 2017. Disponível em: http://www.gnresearch.org/doi/10.5935/MedicalExpress.2017.06.01. Acesso em: 12 nov. 2025.

[5] GUO, L. et al. Human papillomavirus infection as a prognostic marker for lung adenocarcinoma: a systematic review and meta-analysis. Oncotarget, [s. l.], v. 8, n. 21, p. 34507–34515, 2017.

[6] HU, Y. et al. Possible Oncogenic Viruses Associated with Lung Cancer. OncoTargets and Therapy, [s. l.], v. Volume 13, p. 10651–10666, 2020.

[7] INCA. Incidência — Instituto Nacional de Câncer - INCA. [S. l.], 2022. Disponível em: https://www.gov.br/inca/pt-br/assuntos/gestor-e-profissional-de-saude/controle-do-cancer-do-colo-do-utero/dados-e-numeros/incidencia. Acesso em: 22 mar. 2024.

[8] KARNOSKY, J. et al. HPV and lung cancer: A systematic review and meta-analysis. Cancer Reports, [s. l.], v. 4, n. 4, p. e1350, 2021.

[9] KATAOKA, Y. et al. A case of primary lung cancer requiring differentiation from metastatic cervical cancer. Respirology Case Reports, [s. l.], v. 11, n. 9, p. e01196, 2023.

[10] LI, X. et al. Detection of Human Papillomavirus DNA, E6/E7 Messenger RNA, and p16INK4a in Lung Cancer: A Systematic Review and Meta-analysis. The Journal of Infectious Diseases, [s. l.], v. 228, n. 8, p. 1137–1145, 2023.

[11] MALHOTRA, J. et al. Risk factors for lung cancer worldwide. European Respiratory Journal, [s. l.], v. 48, n. 3, p. 889–902, 2016.

[12] MARCOS, B. F. S. et al. Correlation between HPV PCNA, p16, and p21 Expression in Lung Cancer Patients. Cellular Microbiology, [s. l.], v. 2022, p. 1–13, 2022.

[13] MCBRIDE, A. A. Human malignancies associated with persistent HPV infection. The Oncologist, [s. l.], v. 29, n. 6, p. 457–464, 2024.

[14] MEZA, R. et al. Trends in US Adult Smoking Prevalence, 2011 to 2022. JAMA Health Forum, [s. l.], v. 4, n. 12, p. e234213, 2023.

[15] NGUYEN, P. T. et al. Trends in lung cancer incidence by gender, histological type and stage at diagnosis in Japan, 1993 to 2015: A multiple imputation approach. International Journal of Cancer, [s. l.], v. 151, n. 1, p. 20–32, 2022.

[16] OSORIO, J. C. et al. High-Risk Human Papillomavirus Infection in Lung Cancer: Mechanisms and Perspectives. Biology, [s. l.], v. 11, n. 12, p. 1691, 2022.

[17] ROOSTA, Y.; VALIZADEH, M. A.; ABBASI, M. R. Determining the Prevalence of Primary Lung Malignancies by Histopathological Types and Gender: A Five-year Study. Hormozgan Medical Journal, [s. l.], v. 27, n. 4, p. 219–226, 2023.

[18] SEQUEIRA, T. et al. HPV and Lung Cancer: A Systematic Review. Cancers, [s. l.], v. 16, n. 19, p. 3325, 2024.

[19] SHIKOVA, E. et al. Human papillomavirus prevalence in lung carcinomas in Bulgaria. Microbiology and Immunology, [s. l.], v. 61, n. 10, p. 427–432, 2017.

[20] SOFIANI, V. H. et al. The complexity of human papilloma virus in cancers: a narrative review. Infectious Agents and Cancer, [s. l.], v. 18, n. 1, p. 13, 2023.

[21] TSYGANOV, M. M. et al. Human papillomavirus and lung cancer: an overview and a meta-analysis. Journal of Cancer Research and Clinical Oncology, [s. l.], v. 145, n. 8, p. 1919–1937, 2019.

[22] WORLD HEALTH ORGANIZATION (WHO). WHO report on the global tobacco epidemic, 2023: protect people from tobacco smoke. Genebra, Suíça: World Health Organization, 2023. Disponível em: https://www.who.int/publications/i/item/9789240077164. Acesso em: 11 nov. 2025.

[23] ZHAI, K.; DING, J.; SHI, H.-Z. HPV and lung cancer risk: A meta-analysis. Journal of Clinical Virology, [s. l.], v. 63, p. 84–90, 2015.

[24] ZHOU, Y. et al. Expression of P16 and P53 in Non-Small-Cell Lung Cancer: Clinicopathological Correlation and Potential Prognostic Impact. Biomarkers in Medicine, [s. l.], v. 13, n. 9, p. 761–771, 2019.