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Research Article

Correlation between HPV PCNA, p16, and p21 Expression in Lung Cancer Patients

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Purpose. Evaluate if human papillomavirus (HPV) infection in lung cancer patients might be helping cancer development by altering p16, p21, and PCNA, key human genes involved in cell proliferation and tumor development. *Methods.* 63 fresh-frozen (FF) and formalin-fixed paraffin-embedded (FFPE) samples from lung tumor patients were used to detect HPV by PCR, followed by genotype through sequencing. The host gene expressions of p21, p16, and PCNA were quantified by qPCR in both FF and FFPE samples, and the expression of viral oncogenes E5, E6, and E7 was also measured by qPCR in 19 FF samples. *Results.* 74.6% of samples were positive for HPV, 33/44 FFPE samples and 14/19 FF samples. HPV-16 and HPV-18 were detected in 31/33 and 7/33 FFPE, respectively, and HPV-16 was the only type in FF samples. E5, E6, and E7 were expressed in 10/19, 2/19, and 4/19 FF samples, respectively. The p16 RNAm expression was higher in FF HPV+ samples and FFPE+FF HPV+ samples, while p21 showed higher expression in all HPV- samples. In turn, the PCNA expression was higher in HPV+ FF samples; however, in FFPE and FFPE+FF samples, PCNA was higher in HPV- samples. In FF samples, PCNA, p16, and p21 showed a significant positive correlation as well as E5 and E7, and E5 was inversely correlated to p21. In FFPE, also, a positive correlation was observed between PCNA HPV+ and p21 HPV+ and PCNA HPV+ and p16 HPV. In FF+FFPE analysis, a direct correlation was found between PCNA HPV+ and p21 HPV+, p21 HPV+ and p16 HPV+, and PCNA HPV- and p16 HPV-, and an inverse correlation between PCNA HPV+ and p16 HPV+. Also, the p16 protein was positive in 10 HPV+ samples and 1 HPV-. *Conclusions.* Our data show that lung cancer patients from Northeast Brazil have a high prevalence of HPV, and the virus also expresses its oncogenes and correlates with key human genes involved in tumor development. This data could instigate the development of studies focused on preventive strategies, such as vaccination, used as a prognostic indicator and/or individualized therapy.

1. Introduction

The relationship between the human papillomavirus (HPV) infection and cervical tumor development is well known. However, other types of tumors have been associated with the activity of HPV [1, 2]. One of these possible HPV-related cancers is lung cancer; however, the presence and activity of the virus remain controversial [3–5].

This virus can disrupt several cell pathways through the work of E6 and E7 oncoproteins, and one of the paths is the cell cycle, which leads to cell proliferation and stops of cell differentiation through changes in the expression of p53 and pRb proteins, and these functions were already confirmed in lung infected cells [6].

Since HPV requires the host cell DNA replication machinery for its progeny production, the virus must ensure

that the S-phase of the cell cycle continues, and to do so, several key molecules suffer alteration, which is a fact in the role of HPV in cervical cancer development. In nonanogenital HPV-related tumors, such as head and neck cancers (HNC), it was already observed that cyclin-dependent kinase inhibitors (CKI) p16 and p21 are two of the disrupted factors [7, 8] together with the proliferating cell nuclear antigen (PCNA) in order to keep the cell in constant proliferation [9].

The p16 gene (INK4A/CDKN2A) in normal conditions is activated to cause G1 phase arrest by binding with cyclin-dependent kinases (CDK4 and CDK6) and consequent inhibition of retinoblastoma protein (pRB) phosphorylation [10]. If there is any damage in the DNA, p16 continues to be expressed until the cell is led to senescence [11]. Because of its action, p16 was found to be inhibited in numerous cancer types, including lung cancer, through methylation and deletions, which negatively affected lung cancer patient's survival [12–15]. However, the well knows the relation between HPV and p16 in cervical cancer might not be the same in lung cancer. Not all patients infected with HPV and expressing its oncoproteins have an expressively change in p16 expression, and some studies did not find an association between HPV and p16 expression, as is the case in cervical cancer [15–18]. In any case, p16 positivity showed significant prognostic usefulness in non-small lung cancer patients, but its association with HPV in lung cancer still needs further study [19].

Similar mechanisms occur with p21. This protein is responsible for the transition of cell cycle S-phase to a cell differentiation stage and cellular senescence in a p53-dependent and p53-independent manner. Due to that, its expression was considered a tumor suppressor in several tumors like brain, lung, and colon [20]. p21 activation can stop tumor growth by the p53 pathway through inhibition of CDKs, PCNA, transcription factors, and coactivators [21]. By the phosphoinositide 3-kinase- (PI3K-) protein kinase B (AKT) pathway activation, p21 can be inhibited, and this path is stimulated by HPV E5 and E7 oncoproteins [22]. E7 can also decrease p21 expression through recombinant human protooncogene (c-Myc) and histone deacetylases (HDAC) activation (pRB path) [20], and in the lung cancer context, p21-decreased expression was associated with poor overall survival [23, 24].

As well as both previous genes, PCNA is also important in the cancer environment. It is involved in DNA replication by interaction with DNA polymerase; gene expression control through DNA- (cytosine-5-) methyltransferase 1 (DNMT1), HDAC1 e CREB ligand protein (p300), DNA repair through DNA mismatch repair proteins (Msh3/Msh6) proteins, and cell cycle control through p21 [25]. PCNA is overexpressed in a wide range of tumors like breast, liver, prostate, colon, head and neck, and lung, and it is a prognostic biomarker of aggressiveness and a therapeutic target [26–30]. This gene has been studied for over 20 years and pointed to as a biomarker for cancer alterations. However, to our knowledge, there is no study about PCNA and HPV infection in lung cancer samples. In lung tissue, PCNA was overexpressed in lung tumor tissue and connected with tumor cell invasiveness, and in cervical cancer, PCNA overexpression was linked to the differentiation between cervical lesions, metastasis, tumor invasiveness, and HPV infection [31, 32]. To our knowledge, PCNA and HPV were not yet evaluated in lung cancer patients.

As mentioned above, HPV is, directly and indirectly, involved in the alteration of p16, p21, and PCNA expression/activity, although none of these targets were evaluated in lung cancer patients positive for high-risk HPV. Therefore, this is the first study that evaluated these genes and their correlation with HPV infection in lung tumor patients.

2. Materials and Methods

2.1. Study Design. Patients were selected from the pneumology sector of the Oswaldo Cruz University Hospital, located on the Campus of Pernambuco University in Pernambuco, Brazil. This study enrolled lung biopsies from 63 patients with primary lung cancer, 44 archived formalin-fixed paraffin-embedded (FFPE), and 19 fresh-frozen (FF) biopsies. The mean age of the patients was 55 years (range 11-81years). At the time of enlistment, the patients received an explanation of the project and signed a consent form, observing ethical compliance and patient privacy.

2.2. Extraction of DNA. The FFPE sample slices were deparaffinized, and DNA was extracted using xylol (30 min at 65°C) and QIAamp DNA FFPE tissue kit (QIAGEN), respectively. Liquid nitrogen and 1 mL of TRIzol (Invitrogen) were used to macerate and homogenize FF biopsies (25-100 mg). Then, DNA was purified using DNeasy blood and tissue kits (Qiagen). The spectrophotometry (nanodrop LITE, Thermo Scientific) was used to quantify the extracted DNA, and quality control was checked through PCR amplification of the human beta-globin gene [33]. Thorough care was taken to avoid any cross-contamination or external contamination (individual protection equipment, meticulous cleaning of work area and analyst prior, during, and after sample manipulation, UV light incubation of cabin and work tools, few samples manipulated at a time, negative control for each nucleic acid extraction, and all work was performed inside the laminar flow cabin).

2.3. HPV Detection. Conventional PCR assay using MY09/MY11 and GP5/GP6 consensus primers, which amplify a conserved region within the L1 viral gene, was done to detect the HPV [34]. The PCR reaction contained 2.0 μL of sample (100 ng of DNA), 20 pmoles of oligonucleotide, 0.1 mM of dNTP, 1.0 μM of primer, and 1.0 U Taq DNA polymerase (Qiagen), in a total volume of 25 μL of reaction buffer (50 mMKCl, 10 mM Tris HCl and 0.1%Triton X-100). The "hot start" protocol was used for amplification as follows: DNA denaturation at 95°C for 5 min; followed by 35 cycles at 95°C for 30 seconds, 55°C for 40 seconds, 72°C for 45 seconds, and final extension at 72°C for 5 min. All amplicons were visualized through 2.0% agarose gel electrophoresis. The same strict measures mentioned above to avoid contamination were applied.

2.4. HPV Genotyping. The HPV type was determined by sequencing using ABI PRISM Big DyeTM Terminator Cycle Sequencing v 3.1 Ready Reaction kit (Applied Biosystems). The BLAST tool available at http://blast.ncbi.nlm.nih.gov/Blast.cgi was used to compare DNA sequences.

2.5. RNA Purification and cDNA Conversion. In a mixture of liquid nitrogen and 1 mL de TRIzol (Invitrogen), FF biopsies (25-100 mg) were macerated and homogenized, followed by the RNeasy tissue kit (QIAGEN) protocol guide to purify total RNA. The RNA quality and quantity were assured by 1% agarose gel electrophoresis and NanoDrop 2000 Spectrophotometer (Thermo Scientific Wilmington, USA), respectively [35–37]. 500 ng of purified RNA (OD260/280 from 1.8 to 2.1 and intact rRNA subunits -28S and 18S) was used for cDNA conversion according to FIRESript RT cDNA Synthesis Kit (Solis BioDyne) manufacturer' instruction. A negative RT reaction control (no reverse transcriptase enzyme) was prepared for each sample.

2.6. Primer's Design and Efficiency for qPCR. Primers for detection and quantification of human genes and HPV oncogenes were created in the CLCbio Main Workbench software version 5.7.1 (QUIAGEN); it is possible to see the sequences in supplementary Table S1. The reference genes used for relative quantification were EEF1A1 and ACTB, validated beforehand in lung tissue [38]. Primer pairs efficiency were evaluated by serial dilution of 10 potencies and were used an actual cDNA of a positive lung sample to exemplify the real assay condition (Supplementary Table S1).

2.7. Real-Time qPCR. All the targeted genes (p16, PCNA, and p21) were quantified in the 63 FFPE and FF samples using the QuantiTect SYBR Green PCR kit (Qiagen) and Rotor-Gene 6000 thermocycler (Qiagen, Hilden, Germany). The assay conditions were 95°C for 12 minutes for polymerase activation, 35 cycles of 94°C for 15 seconds for denaturation, 60 seconds of annealing temperature (Supplementary table S2), and extension at 72°C for 60 seconds. Therefore, the geometric mean of EEF1A and ACTB reference genes were used to calculate the relative expression of all targets [39]. Even in HPV-negative samples from the MY conventional PCR analysis, the qPCR to detect E5, E6, and E7 was performed to ensure accurate results. In order to detect and quantify E5, E6, and E7 from the FFPE samples, we performed a nested PCR based on two previous works [40, 41]. The first was a conventional PCR as follows: 95°C for 5 minutes, 15 cycles of 94°C for 15 seconds, 60 seconds of annealing (Supplementary Table S2, annealing temperatures for PCR and qPCR amplification of viral and human targets genes), and 72°C for 60 seconds. Then, with 1 μL of the PCR product, we performed the real-time PCR following the condition mentioned in the previous paragraph.

2.8. p16 Immunohistochemical Stain. The 19 FF samples were formalin-fixed and paraffin-embedded for detecting p16 through immunohistochemistry analysis and the 44 FFPE samples. After a maximum time of 24 hours in formalin buffer 10%, the tumor fragments were washed under running water for 2 hours and then stored in alcohol 70% for further processing. Slices of 4 μm were cut from the 63 blocks, using strict procedures to avoid cross-contamination. After deparaffinization and dehydration, the sections were washed with tap water, and the slices were incubated in PBS. The p16 monoclonal antibody (Santa Cruz Biotechnology, Brazil) was used (sc56330 with 1 : 100 dilution). The sections were washed with running water and incubated in PBS, each step for 5 min. In sequence, they were incubated for 20 min in citrate buffer 10 nM and kept at room temperature. After cooling down, blockade of peroxidases for 30 min at room temperature was performed, and sections were washed in running water for 5 min and twice with PBS for 5 min. The sections were then incubated for 2 hours in a wet camera at 37°C with primary antibody, then incubated with secondary antibody (AdvanceTM HRP Link) for 45 min at room temperature, tailed by tertiary antibody (AdvanceTM HRP Enzyme) for 45 min at room temperature, finalized with the addition of DAB solution for 3 min, counterstained with hematoxylin, and visualized on the microscope. The semiquantitative analysis was used to test reactivity to immunohistochemical staining, considering region and intensity. Staining intensities were scored on the following scale: negative reaction sign (-); weak (reactivity in less than 10% of the field); moderate (reactivity between 10% and 50% of the field); and strong (reactivity above 50% of the field). Region reactivity analysis was done by protein detection in one or more sites, and the ZEISS image capture system was used for scanning. A cervical cancer sample was used as a positive control, and negative controls were used by omitting primary antibodies.

2.9. Statistical Analysis. Statistical analysis was performed using the GraphPad Prism (version 8.3.0) software. The Shapiro-Wilk test was applied to enquire if the data had a Gaussian distribution. The Kruskal-Wallis test, Dunn's comparison test, and Mann-Whitney-Wilcoxon test were applied to compare gene expression in different types of lung cancer and in the presence and absence of HPV infection time. Spearman's correlation test was also used to evaluate if the analyzed genes were correlated with each other. Were considered statistically significant p values lower than 0.05 ($p < 0.05$).

3. Results

3.1. Sample Characterization and HPV Detection. The present study analyzed 63 patients, presenting a median of 59 years (ranging from 11 to 81 years old). The patients were 60.32% male sex and 39.68% female.

A total of 33 from 44 FFPE samples were positive for HPV (HPV+) at the initial screening by conventional PCR. HPV 16 was present in 81.81%, while HPV 18 was present in 18.19%. The 19 FF samples were screened separately, and five samples were positive for hrHPV on the first screening, 80% positive for HPV 16, and 20% for HPV 18.

To confirm the screening results and infection, we also evaluated HPV oncogenes expression in all samples. HPV

TABLE 1: Histopathological classification of all FFPE and FF samples and HPV type detection.

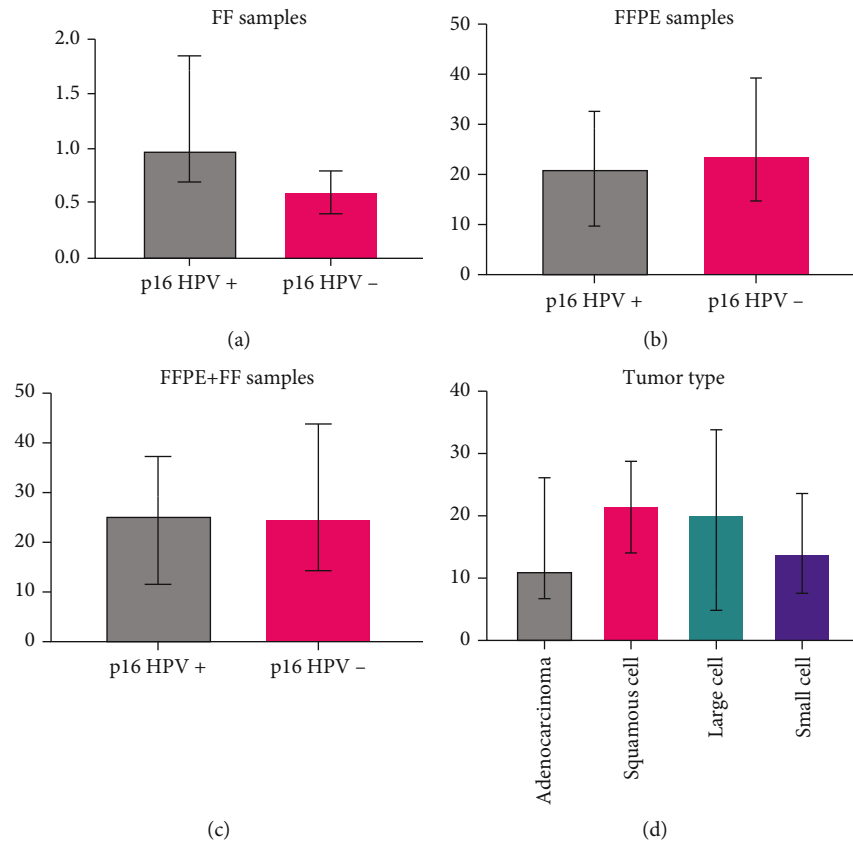| Tumor type	Subtype	HPV 16	HPV 18
	Squamous cell carcinoma	20 (42.55%)	2 (4.26%)
Non-small cell carcinoma	Adenocarcinoma	14 (29.79%)	2 (4.26%)
	Large cell carcinoma	1 (2.13%)	2 (4.26%)
Small cell carcinoma		5 (10.64%)	1 (2.13%)
		47 total positive samples	

FIGURE 1: p16 expression profile. (a) p16 expression in HPV+ FF samples. Differences between both groups were compared by the Mann–Whitney test. No statistical significance was found for $p < 0.05$, and the medians were 0.9752 for HPV+ and 0.5960 for HPV -; (b) p16 expression in HPV+ and HPV- FFPE samples. Rank plot from the Mann–Whitney test with median and interquartile range. The medians were 45.20 for HPV+ and 46.81 for HPV-, and no statistical significance was found for $p < 0.05$; (c) p16 expression in HPV+ and HPV- FFPE and FF samples. The medians were 41.32 for HPV+ and 39.16 for HPV-; no statistical difference was found for $p < 0.05$; (d) p16 expression difference in all tumors—median and interquartile rank analysis by the Kruskal-Wallis test. The medians of p16 expression were as follows: adenocarcinoma 32.33; squamous cell carcinoma 53.10; large cell carcinoma 50.01; and small cell carcinoma 42.92. No significant statistical difference was found for $p < 0.05$.

oncogenes were expressed in 14/19, 13/14 positive for HPV 16, and 1/14 for HPV 18. Because of that, all the subsequent analysis was performed assuming 14/19 samples as HPV+.

We analyzed the prevalence of HPV in all types of tumors in both FF and FFPE samples (Table 1). The higher prevalence was in squamous cell carcinoma, succeeded by adenocarcinoma and small cell and large cell carcinoma, all samples inclusive.

3.2. HPV Oncogenes Expression. Unfortunately, the FFPE samples were difficult to analyze the viral oncogenes by

qPCR with the proper quality; then, we analyzed the human gene expression based on the HPV+ and HPV- status. On the other hand, we analyzed the expression of E5, E6, and E7 oncogenes from all FF samples (19 samples). 10/19 were positive for E5, 2/19 were positive for E6 oncogene, E7 was detected in 4/19 samples, and all four were previously negative for HPV by the conventional PCR results. The only oncogenes expressed were from HPV 16.

3.3. Individual Human Genes Expression: p16, p21, and PCNA. The expression of p16 was analyzed in all FF and
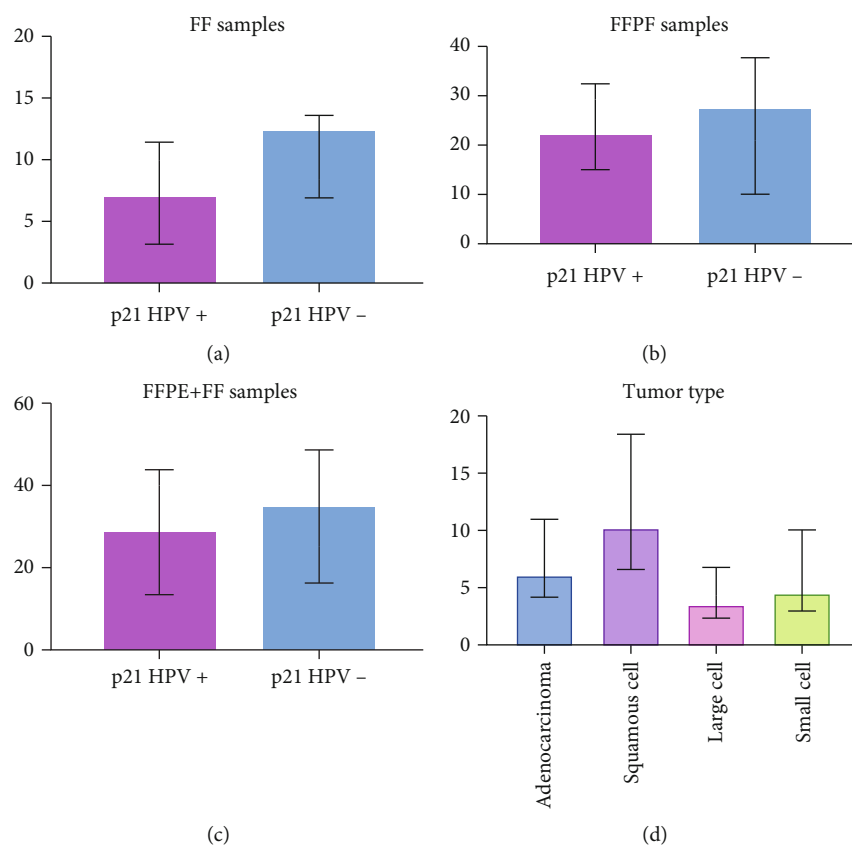
FIGURE 2: (a) p21 expression in HPV+ and HPV- FF samples. Rank plot from the Mann–Whitney test with median and interquartile range. The expression medians were 0.835 for HPV+ and 5.120 for HPV-; no statistical significance was found for $p < 0.05$; (b) p21 expression in HPV+ and HPV- FFPE samples. Rank plot from the Mann–Whitney test with median and interquartile range. The expression medians were 6.703 for HPV+ and 8.553 for HPV-, and no statistical significance was found for $p < 0.05$; (c) p21 expression in HPV+ and HPV- in FFPE and FF samples. The expression medians were 6.216 for HPV+ and 7.008 for HPV-. The Mann–Whitney analysis was performed, and no statistical difference was found for $p < 0.05$; (d) p21 expression profile distribution in all types of tumors—median and interquartile rank analysis by the Kruskal-Wallis test. The p21 expression medians were adenocarcinoma 6.548, squamous cell carcinoma 10.160, large cell carcinoma 3.279, and small cell carcinoma 4.213. No significant difference was found for $p < 0.05$.

FFPE samples. It was positive in 7/19 FF samples, five were HPV+, and 2 were HPV-. The expression was higher in HPV+ samples.

In FFPE samples, p16 was positive in all 33 HPV+ and 10 HPV- FFPE samples. However, the medians were almost the same between the groups.

All the FF and FFPE samples were also analyzed together, and still no statistical difference was found for p16 expression between HPV+ and HPV- groups. p16 was positive in 50/63 samples, 38/50 in HPV+, and 12/50 in HPV- samples. Still, almost no difference was found between the groups.

The p16 expression also varied from the different types of tumors. It showed higher expression in squamous cell carcinoma, large cell carcinoma, small cell carcinoma, and adenocarcinoma, from highest to lowest, although no statistical significance was found for $p < 0.05$ (Figure 1).

The p21 expression was obtained from 16/19 FF samples; 12/16 were HPV+, and 4/16 were HPV-, but the HPV- samples presented a higher p21 expression median than HPV+. In turn, p21 was detected in all FFPE samples. 33/44 were HPV+ and 11/44 were HPV-. As well as in FF

samples, the p21 expression median was higher in HPV- than HPV+. When analyzed together, FF and FFPE samples, p21 was detected in 60/63 samples, 45/60 were HPV+ and 15/60 HPV-, and p21 still presented a higher median rate in the HPV- group.

The expression profile was analyzed among the tumor types, and the median expression was higher in squamous cell carcinoma, then adenocarcinoma, small cell carcinoma, and large cell carcinoma, but no significant difference were found for $p < 0.05$ (Figure 2).

The same analysis was performed for PCNA expression. PCNA was detected in 15/19 FF biopsies; 3/15 were HPV-, and 12/15 were HPV+. PCNA median was just a little higher in HPV+ than HPV- samples but with no statistically significant difference. In FFPE samples, PCNA was detected in 33/44 HPV+ and 11/44 HPV- samples, and the expression median was higher in HPV- than HPV+ samples.

In FFPE and FF samples, the PCNA expression was obtained from 59/63 samples, 45/59 HPV+, and 14/59 HPV-, and the median expression in HPV- samples was higher. PCNA expression was higher in squamous cell carcinoma, followed by small cell carcinoma, large cell
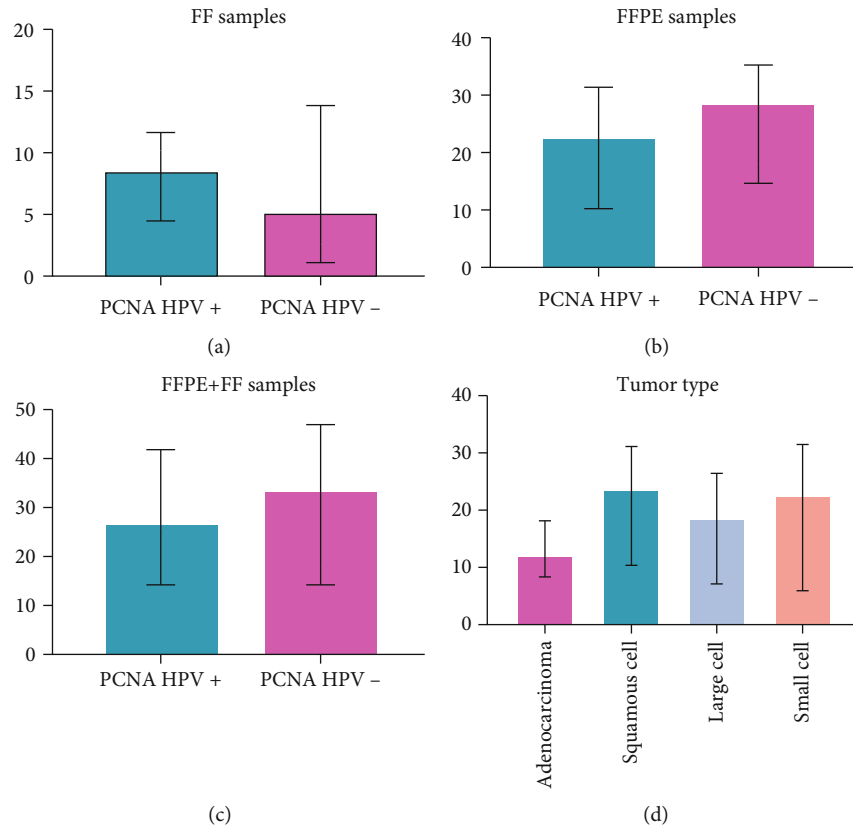
(a)

(b)

(c)

(d)

Figure 3: (a) PCNA expression in HPV+ and HPV- FF samples. Rank plot from the Mann–Whitney test with median and interquartile range. No statistical significance was found for $p < 0.05$ between the medians: 0.0807 for HPV+ and 0.0418 for HPV-; (b) PCNA expression in HPV+ and HPV- FFPE samples. Rank plot from the Mann–Whitney test with median and interquartile range. No statistical significance was found for $p < 0.05$ between the medians: 1.682 for HPV+ and 2.103 for HPV-. (c) PCNA expression in HPV+ and HPV- FFPE and FF samples. The expression medians were 0.5560 for HPV+ and 1.546 for HPV-. The Mann–Whitney analysis was performed, and no statistical difference was found for $p < 0.05$. (d) PCNA expression distribution in all types of tumors—median and interquartile rank analysis by the Kruskal-Wallis test. Medians of PCNA expression were as follows: adenocarcinoma 1.231; squamous cell carcinoma 2.293; large cell carcinoma 1.746; and small cell carcinoma 2.240. No significant difference was found for $p < 0.05$.

carcinoma, and adenocarcinoma. Even so, no significant statistical difference was found (Figure 3).

3.4. Comparison between the Human Genes' Expression and HPV Status.
In FFPE and FFPE+FF samples, we compared the human gene expression according to HPV status. No statistical significance was found between PCNA HPV+ and PCNA HPV-, p21 HPV+ and p21 HPV-, and p16 HPV+ and p16 HPV-. Further, even analyzing both groups together (FFPE+FF), no significant difference was observed.

3.5. Correlation Analysis.
The correlation between p21, p16, and PCNA genes evaluated in FFPE samples showed positive correlations with statistical significance (Figure 4).

The correlation between PCNA, p21, and p16 was also observed in HPV+ and HPV- FFPE samples and was found a strong positive correlation between PCNA HPV+ and p21 HPV+, PCNA HPV+ and p16 HPV+, PCNA HPV- and p16 HPV-, PCNA HPV- and p21 HPV-, p21 HPV+ and p16 HPV+, and p21 HPV- and p16 HPV-. Besides, a strong negative correlation between p21 HPV- and p16 HPV+ and a

light negative correlation between PCNA HPV+ and PCNA HPV-, PCNA HPV+ and p21 HPV-, PCNA HPV+ and p16 HPV+, PCNA HPV- and p21 HPV+, PCNA HPV- and p16 HPV+, p21 HPV+ and p21 HPV-, p21 HPV+ and p16 HPV-, and p16 HPV+ and p16 HPV- were also found.

The statistically significant correlation in FFPE samples were PCNA HPV+ and p21 HPV+, p21 HPV+ and p16 HPV+, PCNA HPV- and p16 HPV+, p21 HPV- and p16 HPV+, and PCNA HPV+ and p16 HPV+ (Figure 5).

In addition, we evaluated the correlation between p16, p21, and PCNA in HPV+ and HPV- in all samples (FFPE and FF). The result was similar to the analysis with only FFPE samples: a strong positive correlation between PCNA HPV- and p16 HPV-. Light positive correlation between PCNA HPV+ and p21 HPV+, PCNA HPV- and p21 HPV-, p21 HPV+ and p16 HPV+, p21 HPV- and p16 HPV-, and p16 HPV+ and p16 HPV-. There is a strong negative correlation between PCNA HPV+ and p16 HPV+ and p21 HPV- and p16 HPV+. There is a light negative correlation between PCNA HPV+ and PCNA HPV-, PCNA HPV+ and p21 HPV-, PCNA HPV+ and p16 HPV-, PCNA HPV-
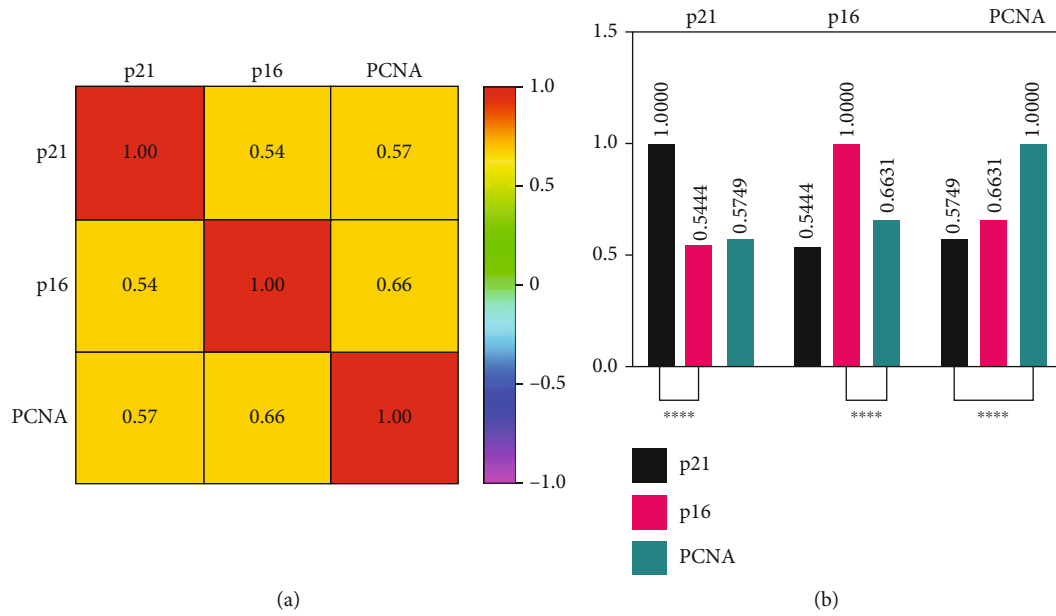
(a)

(b)

FIGURE 4: Correlation analysis in FFPE samples. (a) Heatmap of Spearman's correlation test between p21, p16, and PCNA in FFPE samples. r coefficient is placed inside the cells with a variation from 1 (strongest positive correlation) to -1 (strongest negative correlation). (b) Correlation between p16, p21, and PCNA in FFPE samples. Each gene section shows the positive ($r = 1$) or negative ($r = -1$) correlation coefficient in the bar notation of the analyzed target. p value: ****<0.0001.
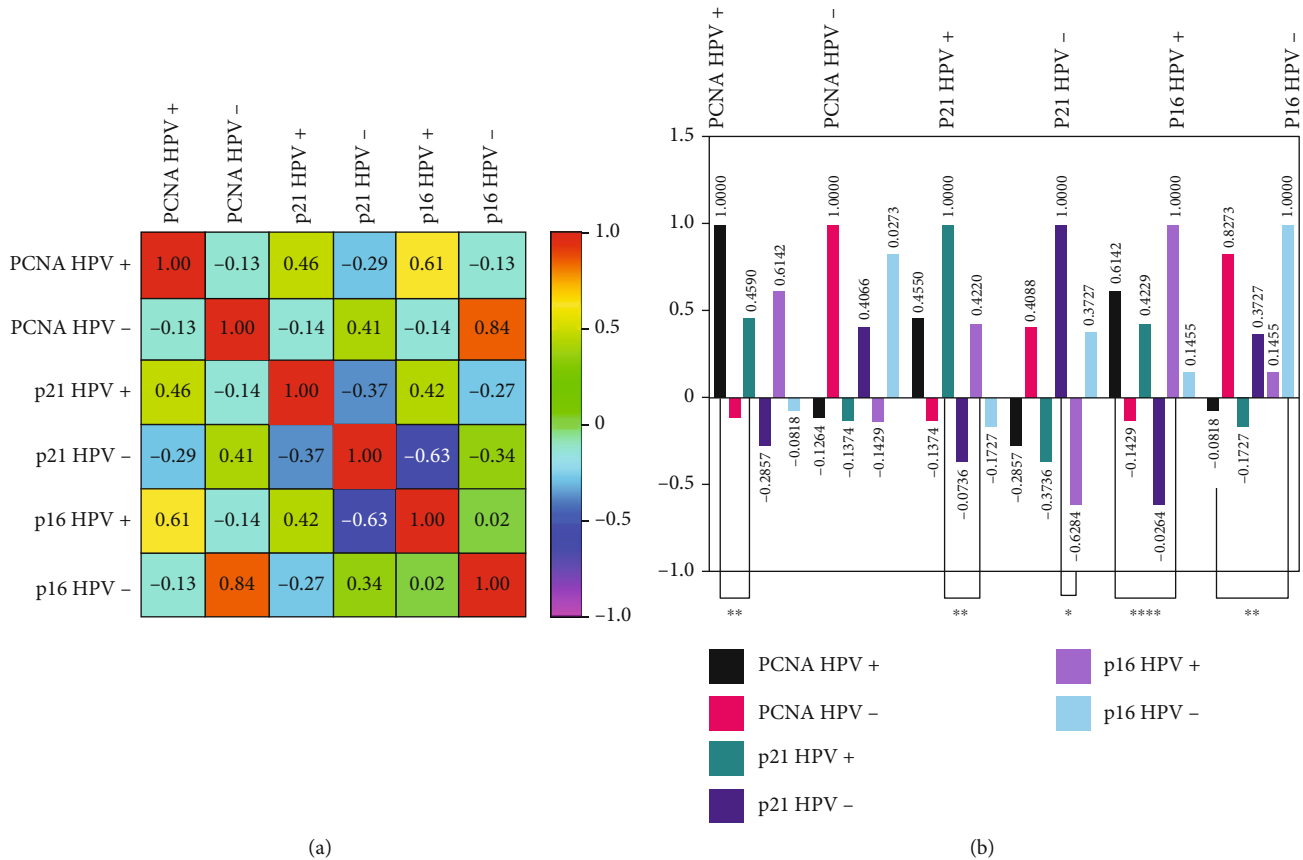

(a)

(b)

FIGURE 5: Correlation analysis based in the HPV status in FFPE samples. (a) Heatmap of Spearman's correlation test between p21, p16, and PCNA in HPV+ and HPV- in FFPE samples. The r coefficient is placed inside the cells with a variation from 1 (strongest positive correlation) to -1 (strongest negative correlation). (b) Correlation between p16, p21, and PCNA in HPV+ and HPV- in FFPE samples. Each gene section shows the positive ($r = 1$) or negative ($r = -1$) correlation coefficient in the bar notation of the analyzed target. p value: *<0.05; **<0.01; ***<0.001; ****<0.0001.
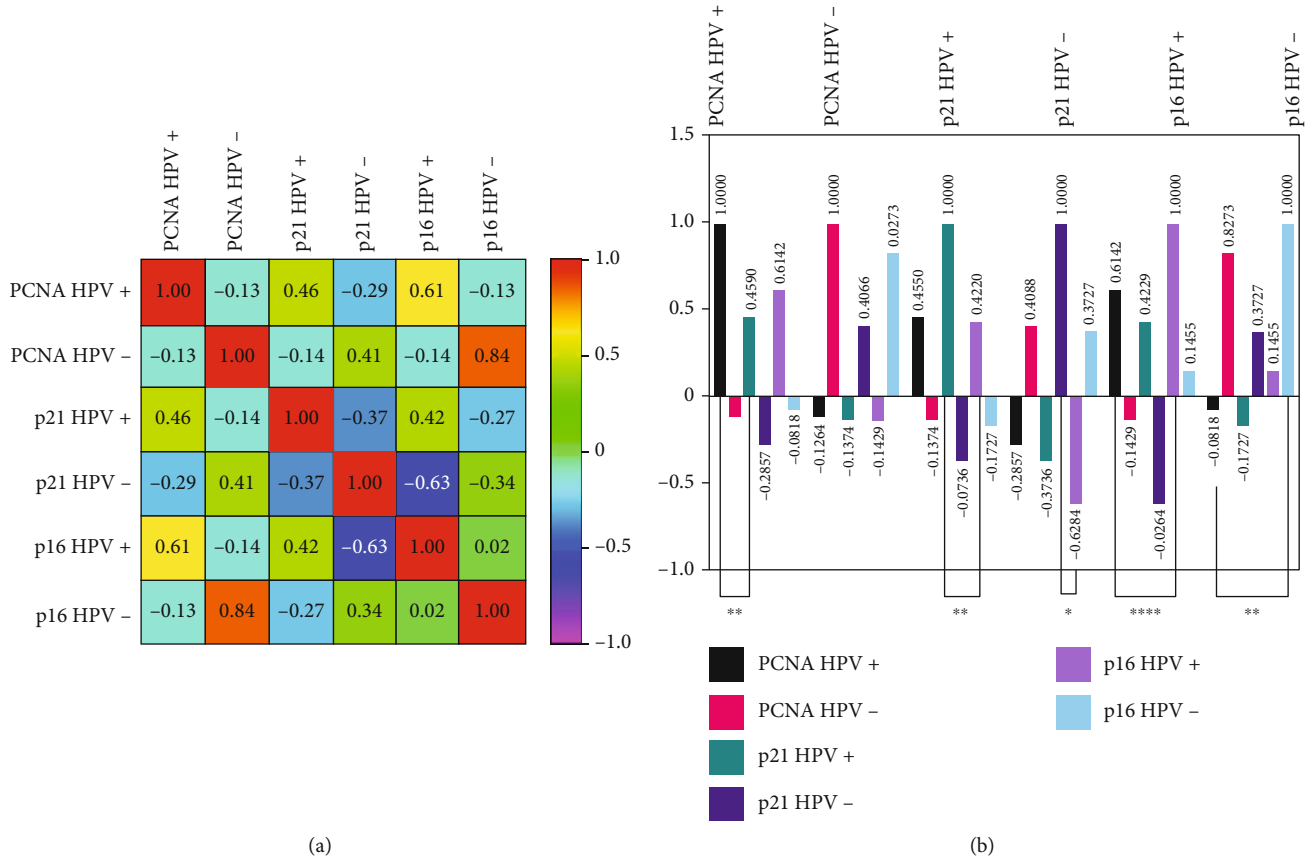
(a)

(b)

Figure 6: Correlation analysis FFPE+FF samples. (a) Heatmap of Spearman's correlation test between p21, p16, and PCNA in HPV+ and HPV- in FFPE and FF samples. r coefficient is placed inside the cells with a variation from 1 (strongest positive correlation) to -1 (strongest negative correlation). (b) Correlation between p16, p21, and PCNA in HPV+ and HPV- in FFPE and FF samples. Each gene section shows the positive ($r = 1$) or negative ($r = -1$) correlation coefficient in the bar notation of the analyzed target. p value: [*]<0.05; [**]<0.01; [***]<0.001; [****]<0.0001.

and p21 HPV+, PCNA HPV- and p16 HPV+, p21 HPV+ and p21 HPV-, and p21 HPV+ and p16 HPV- (Figure 6).

Among the results above, PCNA HPV+ and p21 HPV+, p21 HPV+ and p16 HPV+, PCNA HPV+ and p16 HPV+, p21 HPV- and p16 HPV+, and PCNA HPV- and p16 HPV- showed statistical significance.

In FF biopsies, it was possible to see the correlation between the viral oncogenes and human gene expression. A strong positive relation was found between PCNA and E5, p21 and p16, and E5 and E7. Also, a weak positive relation between PCNA and E6, PCNA and E7, PCNA and p16, E5 and E6, E5 and p16, and E7 and p16 was found. Between E6 and p21, a lightly negative correlation and a weak negative correlation between PCNA and p21, p21 and E5, p21 and E7, and E6 and E7 were found (very weak negative correlation).

Statistically significant correlations were shown between p21 and E5 and E5 and E7 (Figure 7).

3.6. p16 Immunohistochemistry. From the 63 FFPE slides analyzed, 11 (17.46%) were positive for p16 immunohisto-chemistry. Interestingly, only 1 sample negative for HPV was positive for p16 with a moderate signal in the nucleus; the other 10 samples were p16-positive and HPV-positive samples (Figure 8).

4. Discussion

In a previous group study, we identified a high prevalence of HPV in lung cancer patients in northeast Brazil [16]. Because of that, we analyzed a total of 63 lung cancer samples to evaluate if the HPV was not only present but also expressing its oncogenes and if it could be correlated with the widely known cancer-related human genes, p21, p16, and PCNA.

In fresh-frozen (FF) lung carcinoma samples, we detected E5, E6, and E7 mRNA even in samples previously identified as HPV- in the initial screening performed by conventional L1 gene PCR amplification. This detection indicates that the applied detection method can generate variation and may be the reason for divergent results in previous studies [3]. In FF samples, using L1 for HPV detection can be a hazard because this genomic site can suffer a partial loss during viral genome integration into host DNA; thus, E6/E7 detection seems to be a more accurate alternative [42, 43]. However, in FFPE samples, longer regions such as E6/E7 are not recommended because of the high level of DNA fragmentation, formalin-induced inhibition of PCR, and DNA cross-linkage. Both detection procedures of minor regions inside L1 and E6/E7, and in our case E5, seem to be
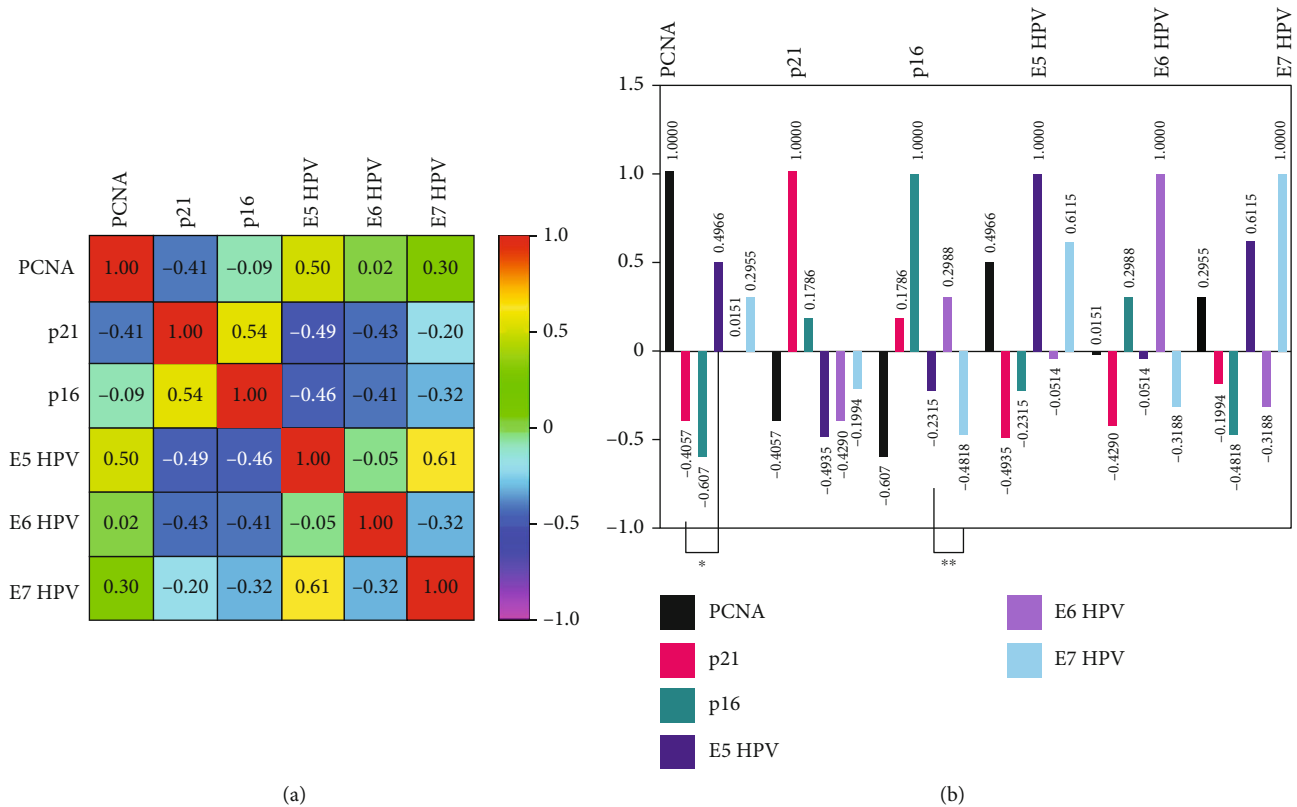
(a)

(b)

FIGURE 7: Correlation analysis in FF samples. (a) Heatmap of Spearman's correlation test between E5, E6, E7, p21, p16, and PCNA in FF samples. r coefficient is placed inside the cells with a variation from 1 (strongest positive correlation) to -1 (strongest negative correlation). (b) Correlation between all targets in FF samples. Each gene section shows the positive ($r = 1$) or negative ($r = -1$) correlation coefficient in the bar notation of the analyzed target. p value: *<0.05; **<0.01.

successful even with the above features when applied together [44]. In the literature, HPV prevalence and activity still are controversial [3, 45]. The difference between HPV detection in lung samples may vary due to increased sensitivity of qPCR compared to the conventional PCR screening, DNA extraction method, and geographic location [43, 46–49]. Only one more work has been done in Brazilian patients and found no HPV in southeast lung cancer samples; however, 14.3% of the samples were positive for p16 staining. Two questions arise from this: (a) Is p16 staining a golden standard for HPV-related lung cancer and HPV-related cervical cancer? (b) Is this difference in HPV incidence due to geographic location?

Brazil has a vast territory and comprises habitats with different ethnicities, climate, and socioeconomic features. This characteristic impacts HPV infection rates in other types of tissues and might influence HPV infection in lung cancer as well, with a lack of studies and data to analyze HPV and lung cancer demographics in all parts of Brazil [50–52].

Interestingly, E5 oncogene was the most prevalent expressed oncogene among the samples. This oncogene was pointed out as more expressed at the beginning of infection and normally lost at later stages during the viral genome integration [53]. This expression can suggest that perhaps the HPV's DNA is not integrated into the host genome, which is an established step for cancer formation [54]. Even

so, E5 was also found in Brazilian patients' cervical cancer and precancerous cervical lesions, suggesting this oncoprotein activity even at the late stages of cervical lesions [55].

Due to the relation between HPV and p16 expression in cervical cancer and its use as a biomarker, we evaluated if the same relation occurs in lung cancer tissue [56]. The inactivation of p16 function in several tumors is well described in the literature, but in cervical cancer, the p16 protein expression is augmented and accumulated in HPV-infected cells. This accumulation is associated with E7 activity on pRb protein and E2F transcription factor release, which stimulates p16 expression. A weak positive correlation was found between E7 and p16, however not statistically significant, perhaps due to the number of samples. In the meantime, p16 levels were higher in FF HPV-positive samples and FFPE+FF HPV-positive samples. In FFPE, a negative correlation between the expression of p16 in HPV-positive and p16 in HPV-negative samples was not statistically significant, though.

p16 also showed higher expression in squamous cell carcinoma, the type of lung cancer with higher HPV prevalence, which is expected since HPV has a tropism for this type of epithelium [57]. Eleven lung cancer samples positive for HPV were also positive for p16 protein expression through immunohistochemistry. This data confirms HPV activity in lung cells. Interestingly, HPV-negative samples showed p16 RNAm expression but not protein expression,
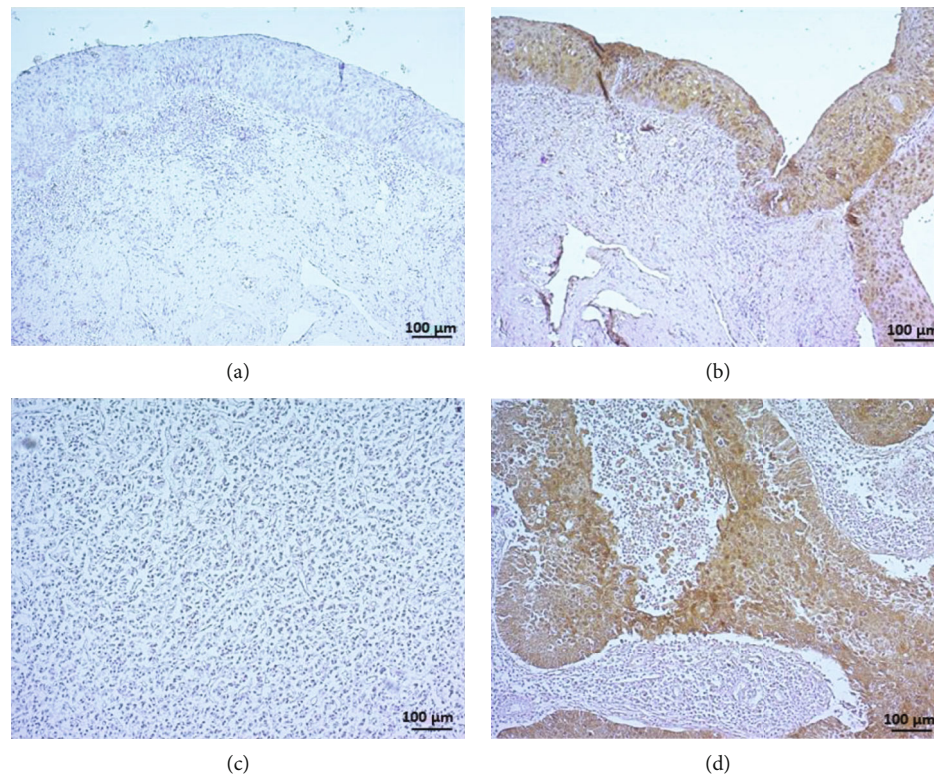
(a)

(b)

(c)

(d)

Figure 8: p16 immunohistochemistry. (a) Cervical cancer tissue as a negative control without primary antibody. (b) Cervical cancer tissue as a positive control for p16 staining. (c) Lung cancer tissue negatives for p16 staining. (d) Lung cancer tissue positive for p16 staining.

which indicates that some type of posttranscriptional regulation of p16 is happening, such as microRNA binding to p16 RNAm [58]. Therefore, our results indicate that p16 may have an important value in distinguishing HPV-infected lung cancer tissues, which differs from other studies where no association was found between p16 and HPV in lung cancer samples [15, 18].

Furthermore, p16-diminished levels in non-small cell lung cancer due to DNA methylation were associated with greater resistance to paclitaxel-based chemotherapy, the first line of treatment for several types of cancer, including lung carcinomas [59]. p16 higher expression in HPV+ patients raises the possibility of higher sensitivity to this chemotherapy approach. However, more analysis needs to be done regarding the p16 role as a marker for HPV-infected lung tumors based on our results from immunohistochemistry.

Like p16, p21 is also a CDK inhibitor responsible for cell cycle arrest [20]. p21 and p16 showed a positive correlation in FFPE+FF samples in the presence of HPV. This result means that p16 and p21 seem to be "walking" in the same direction. Contradictorily, our data showed diminished expression of p21 in HPV+, while p16 is higher in those samples. It is a fact that the activation and inhibition cascade of biological paths have not an exact linear relation but normally a complex concomitant interaction between several components. Perhaps the activity of other human and/or viral proteins could alter the expression profile of both p21 and p16 paths. These variables are not considered in the correlation test.

Overall, p21 is underexpressed in lung cancer [24]. In our data, p21 presented lower expression patterns in HPV-positive samples in FF, FFPE samples, and FFPE +FF samples. In previous works, E5 and E7 stimulation on the PI3K-Akt pathway inhibited p21 expression, and E7 activity upon HDAC also led to decreased expression of p21 [22]. Besides these paths, E6 can also block p21 expression by inhibiting p53 [20]. These views make sense that p21 is higher in HPV-negative samples and the significant negative correlation found between p21 and E5. This viral oncoprotein is the least studied, and until now, little is known about E5 and its targets; and our results throw some light on the importance of E5 in an HPV-tumor microenvironment. p21 also showed an inverse correlation to E7, although no statistical significance was found ($p = 0.42$).

Based on the literature, p21 expression was presented as blocking one of the actions of PCNA, which is to initiate cell differentiation and stop proliferation [25]. In the same way, PCNA-augmented expression would exert a feedback reaction and increase p21 expression [60]. In accordance, both FFPE and FFPE+FF analysis presented a statistically significant positive correlation between p21 and PCNA in HPV+ patients.

PCNA plays its role in the repair of DNA damage, chromatin remodeling, cell cycle control, DNA replication, gene expression control, and other processes [31, 61]. Its overexpression in various tumor types was associated with malignancy and tumor aggressiveness, and it has been studied as

a potential therapeutic target and biomarker [26–28, 30]. PCNA presented higher expression levels in HPV-positive FF samples, but the analysis of FFPE and FFPE+FF samples showed higher expression in HPV-negative samples. Besides, we found a trend for a positive correlation between PCNA and E5 ($p = 0.062$) and PCNA and E7 ($p = 0.28$), which is not statistically significant, though. In agreement, Sharma and Munger [62] also did not find a significant correlation between E7 and PCNA. However, previous data in cervical cancer showed E6 and E7 stimulation on cell proliferation through PCNA expression [62, 63], and although no study has evaluated E5 directly, the oncoproteins activity on PCNA could explain the trend in our results regarding E5.

PCNA also showed a significant positive correlation to p16 in the absence of HPV infection (FFPE+FF samples) and a negative correlation in HPV-positive samples (FFPE and FFPE+FF samples). These results agree with the previous data stated above. In a past study, p16 and PCNA protein expression was considered a reliable high-grade cervical lesion diagnostic marker [64]. Also, in other types of non-HPV-related cancer, PCNA overexpression was correlated with an inferior prognosis, tumor invasiveness and aggressiveness, and a reliable marker in the case of colorectal cancer [26, 30]. We may also extrapolate these results for lung cancer positive for HPV infection, and maybe in further studies, PCNA could act as an HPV-associated lung lesion surrogate marker.

The present study is a starting point to show the importance and need to evaluate HPV infection and its action on key human genes and pathways involved in lung carcinogenesis. More extensive analysis with a larger sampling frame is necessary to determine the molecular differences between lung cancer patients in the presence and absence of HPV and improve prognosis and patient's risk stratification and treatment with an individual and personalized approach.

5. Conclusion

The present study showed evidence that HPV is transcriptionally active in lung cancer patients in northeast Brazil. The data is not enough to attribute a causal role to HPV in the carcinogenic process; however, the correlation between viral oncoproteins and the cell targets studied here as well as differences between the target's expression in HPV positive and negative samples is indicative of changes in the tumor microenvironment caused by HPV. Although p21, p16, and PCNA are already expected to be altered in lung tumor, our results show that HPV can aggravate these alterations. This situation should be considered for future research plans focused on evaluating the extension of HPV's effect on this type of cancer and the repercussion for lung cancer patients.

Data Availability

All the data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee (the study protocol was approved by the Federal University of Pernambuco ethics committee, register number 06396812.0.3001.5192) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

B. F. São Marcos and T. H. A. Oliveira contributed equally to the paper.

Acknowledgments

Supplementary Materials

Table S1: sequence of primers used for PCR and qPCR amplification of viral and human target genes. Table S2: annealing temperatures for PCR AND qPCR amplification of viral and human targets genes. (Supplementary Materials)

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