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Hierarchical evaluation of histology and p16-labeling can improve the risk assessment on cervical intraepithelial neoplasia progression

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ABSTRACT

Objective: High-grade cervical lesions (HSIL) are associated with the presence of high-risk HPV types, tissue expression of p16, and increased chance of malignant progression, requiring surgical intervention. To improve risk evaluation, we assessed the discriminatory power of the histological findings associated with p16 immunohistochemistry (IHC) staining to classify the low-grade cervical lesion (LSIL) and HSIL.

Methods: We collected cervical biopsies from colposcopy-visible lesions and non-affected tissue (adjacent to the lesions) of 62 Brazilian women and labeled them with anti-p16 antibodies. In addition to the observational pattern and labeling to define the latent classes (affected vs. non-affected), a computational tool was used for semi-quantitative analysis of p16 expression. The intensity of staining of the nucleus or cytoplasm was captured using the Gimp 2.10 software. ROC curves were used to determine cutoff values for p16 expression in patients classified as LSIL and HSIL by latent class statistics for each labeling stratum.

Results: p16 nuclear labeling showed the best sensitivity and specificity to discriminate LSIL with low p16 expression (62%) and HSIL with high p16 expression (37%). Many patients whose lesions had intermediate levels of p16 nuclear staining were subsequently stratified according to the expression of p16 in the cytoplasm, indicating that five of 21 LSIL were at risk of progression, and 13 of 41 HSIL at risk of regression.

Conclusions: We suggest a hierarchical analysis, with histology at the first level, followed by a labeling analysis in the nucleus and then in the cytoplasm to increase the accuracy of the HPV cervical lesion stratification.

List of Abbreviations

AUC	Area under the curve
BL	Benign lesions
CC	Cervical cancer
CDKN2A	Cyclin dependent kinase inhibitor 2A
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia (1,2,3)
CISAM	Integrated Health Center Amaury de Medeiros

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DNA	Deoxyribonucleic acid
DL	Degree of labeling
GAPDH	Glyceraldehyde phosphate dehydrogenase
H&E	Hematoxylin and eosin
HPV	Human papillomavirus
HrHPV	High-risk human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
IHC	Iimmunohistochemistry
IMIP	Instituto de Medicina Integral Professor Fernando Figueira
INKs	kinase inhibitors
LP	Labeling pattern
LrHPV	Low-risk human papillomavirus
LSIL	Low-grade squamous intraepithelial lesion
NT	Normal tissue
OR	Odds ratio
P	*P* value
PBMC	Peripheral blood mononucleated cells
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein
ROC	Receiver Operating Characteristic

1. Introduction

Cervical cancer (CC) is the fourth most common cancer in women, accounting for approximately 311,365 deaths worldwide (Cohen et al., 2019). In Brazil, CC has an incidence of 16.35 per 100,000 and mortality of 6.17 per100,000 women and is considered a public health problem (Nacional De Câncer, I., and Gomes Da Silva, 2020). Persistent high-risk human papillomavirus (HrHPV) infection is an important etiological factor for the progression of precancerous lesions to CC, mainly when the integration of the virus into the host genome occurs (Doorbar et al., 2015; Hu et al., 2019). Low-grade squamous intraepithelial lesion (LSIL) includes the cervical intraepithelial neoplasia (CIN) 1, and the high-grade squamous intraepithelial lesion (HSIL) encompasses CIN 2 or CIN 3 subsets. The discrimination between LSIL and HSIL is crucial for the indication of surgical intervention, which may have a potentially negative impact on young sexually active women (Cohen et al., 2019; Balasubramaniam et al., 2019). According to the literature, there is high inter-observer variability in histological diagnostics of cervical lesions based on hematoxylin and eosin (H&E) stained biopsies. The use of biomarkers (p16, HPV-L1 capsid protein, importin-β, PD-L1, Ki-67, amplification of 5p15 telomerase chromosomal region) contributes to improving the differentiation between LSIL and HSIL and early detection of CC (Koo et al., 2009; Kudela et al., 2014; Ghosh et al., 2019; Li et al., 2019; Nuovo et al., 2019; Nuovo, 2020). Though the evaluation of biomarkers may generate a more significant economic burden, the assessment of p16 immunohistochemistry (IHC), which is already routinely performed in many centers, does not substantially elevate the final cost of cervical lesion therapy (Mendaza et al., 2020).

The p16 protein inhibits the CDK4 and CDK6 kinase-dependent phosphorylation of pRb protein, leading to cell cycle arrest at the G1 phase (Singh et al., 2010; Sherr et al., 2016), a natural mechanism to control the growth of unnecessary or defective cells. However, the oncoprotein HrHPV E7 binds and activates pRB, regardless of the presence of high levels of p16, contributing to the loss of cycle control and initiation of tumor progression (Wentzensen and Von Knebel Doeberitz, 2007; Romagosa et al., 2011; Balasubramaniam et al., 2019).

Although high expression of p16 in cervical biopsies has been associated with HSIL and CC (Murphy et al., 2005; Lesnikova et al., 2009; Darragh et al., 2013; Hu et al., 2019; Li et al., 2019; Nuovo, 2020), the guidelines for interpreting the p16 expression are still in progress (Singh et al., 2018). An increased number of studies have reported p16 expression in the nucleus and cytoplasm, in transformed and normal cells, in the epithelial layers or stroma, and in non-affected areas adjacent to cervical lesions, emphasizing the limitation of the procedure and the interpretation of the p16 IHC analysis (Sano et al., 1998; Romagosa et al., 2011; Murphy et al., 2005; Lesnikova et al., 2009; Mendaza et al., 2020).

The expression and cellular location of p16 can provide helpful information about the severity of cervical lesions when associated with histology findings (Mendaza et al., 2020); however, the qualitative evaluation of p16 expression is subjective, and there is no consensus on its interpretation. In this study, we evaluated: i) the degree of p16 labeling (no labeling, low, moderate, and strong), ii) the p16 labeling pattern (stroma, epithelium, and glands), and iii) the semi-quantitative p16 expression (in pixels), using a computational tool. The ensemble of three variables determined two cutoff points, distinguishing low or high p16 expression, as detected in the nucleus, cytoplasm, or both. Based on these values, we discussed the role of p16 labeling on the discrimination of the risk for cervical precancerous lesion progression.

2. Materials and methods

2.1. Patients and samples

This study was approved by The Ethics Committee of the IMIP Hospital, CISAM Health Center and of the Oswaldo Cruz Foundation (CAEE 51111115.9.0000.5190) and included 62 women with a median age of 34 years (20 to 62 years), who gave written consent to participate in the study. Samples of cervical cells were collected using Cytobrushes® during the colposcopic examination and were used for molecular diagnosis and HPV typing. All patients exhibited visible atypical lesions in colposcopy, according to the nomenclature of the International Federation for Cervical Pathology and Colposcopy - IFCPC (Bornstein et al., 2012). Cervical biopsies were collected from the lesions ($n = 62$) and adjacent areas ($n = 62$) for histological and IHC analyses. Thirty (24.2%) biopsies were excluded from the analysis. The technical excluding criteria included small sample size, making it impossible to stratify CIN, presence of only stromal tissue, biopsy slice detachment during tissue processing, and presence of cracked fragments. After histology, four of the 30 samples were excluded, two samples diagnosed as adenocarcinoma and two samples biopsied in the non-affected adjacent area of the lesion, but they were classified as CIN 1 and CIN 3 in histology (Supplementary Fig. S1).

Among the 94 H&E-stained biopsies, 46 were from non-affected tissue and 48 from lesions. Two pathologists blindly classified lesions as benign (7 cases), CIN 1 (12 cases), CIN 2 (9 cases), and CIN 3 (20 cases) and evaluated the p16 expression. Squamous metaplasia, simple hyperplasia, and acute or chronic cervicitis were all included in the benign lesion group.

2.2. HPV diagnosis

Genomic DNA was extracted from the cervical samples using the Illustra Blood Kit (Healthcare®, Little Chalfont, Buckinghamshire, UK) and submitted to the Polymerase Chain Reaction (PCR) amplification using specific primers for the glyceraldehyde phosphate dehydrogenase human (GAPDH) constitutive gene. Positive samples were submitted to new PCR using the MY09 and MY11degenerate primers, which amplify the 450-base pair fragment of the HPV L1 gene (Manos, 1989). The viral amplicon sequences were determined by the Genetic Analyzer ABI 3500 equipment (Applied Biosystems, Foster City, CA), using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems), and the chromatograms were submitted to the BLASTn algorithm of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) for HPV type identification. The study population encompassed 22 (35.5%) women negative for HPV infection, 36 (58.0%) classified as HrHPV (16, 18, 31, 33, 51, 52, 58, 59 and 66), and in four samples (6.5%), HPV genotyping was not possible due to unsuccessful sequencing possibly related to infection by multiple HPV types.

2.3. Histology and immunohistochemistry

Biopsies were fixed in 10% buffered formalin for 24 h and embedded in paraffin. The tissue embedded-paraffin blocks were cut in 4-μm-thick slices, submitted to the H&E staining, and classified as a non-affected tissue, benign lesion (BL), CIN 1 (LSIL), and CIN 2 and CIN 3 (HSIL) (Richart, 1973).

For IHC analyses, tissue slices were processed with the Dako EnVision ™ FLEX+ Kit (K8002; Dako Laboratories, Carpinteria, CA) prior to incubation with the primary rabbit polyclonal anti-p16 antibody (ab108349; Abcam, Cambridge, UK) diluted 1:100, and the negative control was prepared with Dako Antibody Diluent (S0809). After labeling, the slices were counterstained with hematoxylin. The p16 labeling was confirmed by brownish staining in the nucleus and cytoplasm; and epithelium or gland cells. We also evaluated the p16 labeling in the stroma, aiming to evaluate the complex interaction of tumor and immune cells in the surrounding stromal tissue that modulate the local inflammatory and cellular immune response (Reeves and James, 2017).

The p16 expression was analyzed in three random fields in each slide using a magnification of x400. First, the degree of labeling (DL) was evaluated by observational analysis, categorizing the labeling as no staining (0), weak staining (1), moderate staining (2), and intense staining (3), according to Koo et al. (2009). Second, the labeling pattern (LP) was classified as no labeling (0), exclusive labeling in the stroma (1), exclusive labeling in the epithelium or gland (2), and labeling in epithelium or gland plus stroma (3). The same pathologist scored all slides, blinded to clinical and epidemiological data. Third, the intensity of p16 labeling was estimated by the logarithmic histogram in the color range 254 using the Gimp 2.10 software (GNU Image Manipulation Program, UNIX platforms, www.gimp.org), and the p16 staining was quantified according to the colored pixels in the image. The absolute values in pixels were obtained according to the exclusive labeling in the nucleus (N) or cytoplasm (C) and by combined nucleus and cytoplasm (N + C) labeling.

A total of 162 images from 48 biopsies were captured in areas of cervical lesions, encompassing 67 CIN 3 (41.4%), 31 CIN 2 (19.1%), 43 CIN 1 (26.5%), and 21 BL (13.0%). Additionally, 134 images were captured from 46 slides of non-affected tissue, biopsied adjacent to the cervical lesion, and showing normal histology.

2.4. Statistical analysis

To refine the interpretation of p16 expression, we used the latent class analyses to evaluate the combination of qualitative and quantitative analyses, permitting the generation of cutoff values to discriminate low- from high-grade lesions. To characterize the p16 expression in areas exhibiting or not the cervical lesion, we combined the qualitative variables regarding the degree of labeling (DL) (labeling intensity) and the labeling pattern (LP); and the continuous variable of p16 expression in pixels. For the qualitative variables (DL and LP), univariate analysis employing a logistic regression approach was used. The p16 expression in pixels was determined in N, C, and N + C, as a median of three photographed fields. The association analysis was performed using the chi-square test or Fisher's exact test, considered significant at $P \leq 0.05$.

Through the receiver operating characteristics curve (ROC), the highest cutoff for non-affected tissue (low p16 expression) and the lowest cutoff for any tissue damage (high p16 expression) were established to assess the accuracy of p16 expression in pixels in N, C, and N + C, for stratification of risk of progression of cervical injury (Table 1). Sensitivity and specificity were calculated within each latent class (lesioned and adjacent non-affected area) with the respective 95% confidence intervals (CI), positive test likelihood ratio (LH+), and negative test likelihood ratio (LH-). The kappa index was used to assess the agreement between the cutoffs and the latent classes. Pixels between both classes (low and high p16 expression) were defined as a gray zone, exhibiting an intermediate expression of p16. Finally, we evaluated the power of p16 expression defined by the latent classes in discriminating BL, CIN 1, CIN 2, and CIN 3 lesion considering the expression of p16 in the nucleus, cytoplasm, or both (Supplementary Tables 1, 2, and 3).

The proportions of lesions according to the HPV infection (positive or negative) status were also investigated. The variables were previously evaluated for the assumption of homogeneity using the Breslow Day/Woolf test. The Cochran – Mantel – Haenszel test was applied, verifying independence, and confirming or rejecting the hypothesis that the proportions assessed between the strata were equal. All decisions were made at a significance level of 5%, and the software used was the R Core Team (R Core Team, 2018).

3. Results

3.1. Labeling pattern and degree of p16 staining

In areas exhibiting precancerous lesions (CIN 1, CIN 2, and |CIN 3), IHC revealed intense and diffuse p16 labeling in N, C, or N + C of epithelial cells; and weak labeling in fibroblasts, endothelial cells, and some inflammatory cells in the stroma. Poor p16 labeling in the nucleus and cytoplasm in epithelial cells was observed in non-affected tissue areas and the benign lesion. Overall, the intensity of the p16 staining increased with the severity of the lesion (Fig. 1).

The ROC curve for the p16 expression in pixels in non-affected tissue area provided values of the area under the curve (AUC) of 0.936 (labeling in N), 1.000 (in C), and 0.939 (N + C labeling). The labeling intensity in cervical lesions showed similar AUC values of 0.999, 0.966, and 0.972, respectively, for nuclear, cytoplasmic, and N + C labeling, indicating the high quality of the test prediction (Table 1). According to the parameters of the ROC curve presenting high sensitivity and specificity, different cutoff values were generated for evaluation of p16 expression in presence or absence of cervical lesion for each strategy of labeling evaluation, whether exclusively in nucleus or cytoplasm or both cellular compartments. These cutoff values showed substantial concordance with the probability of risk to injury defined in the latent class study (Kappa Index >0.705) (data not shown).

3.2. Expression of p16 and cervical lesion discrimination

Initially, we assessed the accuracy of p16 labeling in discriminating HSIL from LSIL, using multiple labeling quantification on different slide fields to maximize the study power. When we used the nucleus labeling exclusively to evaluate p16 expression, we considered cutoff values of ≤97,974 and > 414,696 pixels for the lowest and the highest probability for cervical lesion development, which allowed us to separate high-grade CIN samples (including CIN 2 and CIN 3) from non-affected tissue ($P < 0.0001$ and $P < 0.0001$), benign lesions ($P = 0.0005$ and $P < 0.0001$) and from CIN 1 samples ($P = 0.0142$ and $P < 0.0001$). These cutoff values did not discriminate non-affected tissue ($P = 0.0521$) or benign lesion ($P = 0.1556$) from CIN 1 (Supplementary Table 1).

When we used the cytoplasm labeling exclusively to evaluate p16 expression, the range of the lowest and highest probability for cervical lesions development was set at the cutoff ≤42.704 and > 387.156 pixels, respectively. These cutoffs discriminated non-affected tissue from CIN 1 ($P < 0.0001$), CIN 2 ($P < 0.0002$), or CIN 3 ($P < 0.0001$) samples and discriminated benign lesion from CIN 1 ($P = 0.0362$) samples. However, it did not discriminate CIN 1 from CIN 2 ($P = 0.4624$), CIN 1 from CIN 3 ($P = 0.4015$), and CIN 2 from CIN 3 ($P = 0.7145$) samples (Supplementary Table 2).

Lastly, considering the nucleus and cytoplasm (N + C) combined p16 labeling established cutoffs of ≤279,899 and > 597,009 pixels as the lowest and the highest probability for cervical lesions development, it was possible to discriminate HSIL (CIN 2 and CIN 3) from non-affected tissue ($P < 0.0001$ and $P < 0.0001$), and from BL samples ($P = 0.0061$ and $P < 0.0001$). However, it was not discriminative for CIN 1 and CIN 2

Table 1
Analysis of the ROC curve of the p16 labeling (in pixels) according to labeling location for stratification of risk of progression of cervical injury.

Area	Location	Cutoff	Sensitivity (95% CI)	Specificity (95% CI)	AUC	*P*	Cutoff	N	(%)	Minimum	Maximum	Mean	Median	Standard deviation	Standard error
Non-affected tissue	Nucleus	> 97.974	89.7 (79.9–95.7)	86.4 (75.7–93.6)	0.936	0.0001		134	100	0	589.635	120.918	104.924	98.028	8.468
							≤ 97.974	64	47.8	0	97.974	40.775	43.806	30.625	3.828
							> 97.974	70	52.2	101.457	589.635	194.191	180.334	79.133	9.458
	Cytoplasm	> 42.704	98.8 (93.4–99.8)	100.0 (93.0–100.0)	1.000	0.0001		134	100	0	1.179.013	182.625	127.463	215.623	18.627
							≤ 42.704	52	38.8	0	42.704	1.606	0	7.045	977
							> 42.704	82	61.2	62.400	1.179.013	297.418	232.945	204.777	22.614
	N+C	> 279.899	88.0 (75.7–95.4)	84.5 (75.0–91.5)	0.939	0.0001		134	100	2.630	1.462.009	303.543	253.287	258.728	22.351
							≤ 279.899	77	57.5	2.630	279.899	130.704	116.541	86.890	9.902
							> 279.899	57	42.5	283.821	1.462.009	537.028	510.897	228.411	30.254
Lesions	Nucleus	> 414.696	100.0 (87.5–100.0)	98.5 (94.7–99.8)	0.999	0.0001		162	100	0	1.164.708	233.041	149.821	225.693	17.732
							≤ 414.696	132	81.5	0	414.696	148.367	115.317	123.985	10.792
							> 414.696	30	18.5	421.234	1.164.708	605.608	515.223	192.673	35.177
	Cytoplasm	> 387.156	88.8 (80.3–94.5)	98.6 (92.6–99.8)	0.966	0.0001		162	100	0	1.649.262	425.599	384.920	300.642	23.621
							≤ 387.156	82	50.6	0	387.156	186.615	183.631	121.095	13.373
							> 387.156	80	49.4	389.756	1.649.262	670.557	649.079	221.636	24.780
	N+C	> 597.009	86.5 (77.6–92.8)	97.3 (90.4–99.6)	0.972	0.0001		162	100	58.773	2.193.774	658.640	580.867	440.406	34.602
							≤ 597.009	83	51.2	58.773	597.009	306.569	288.261	157.998	17.343
							> 597.009	79	48.8	598.252	2.193.774	1.028.536	960.811	321.912	36.218

NOTE: The group with cervical lesion comprises the BL, CIN 1, CIN 2 and CIN 3. Abbreviations: number of different quantifications per analyzed area (N). Area under the curve (AUC).

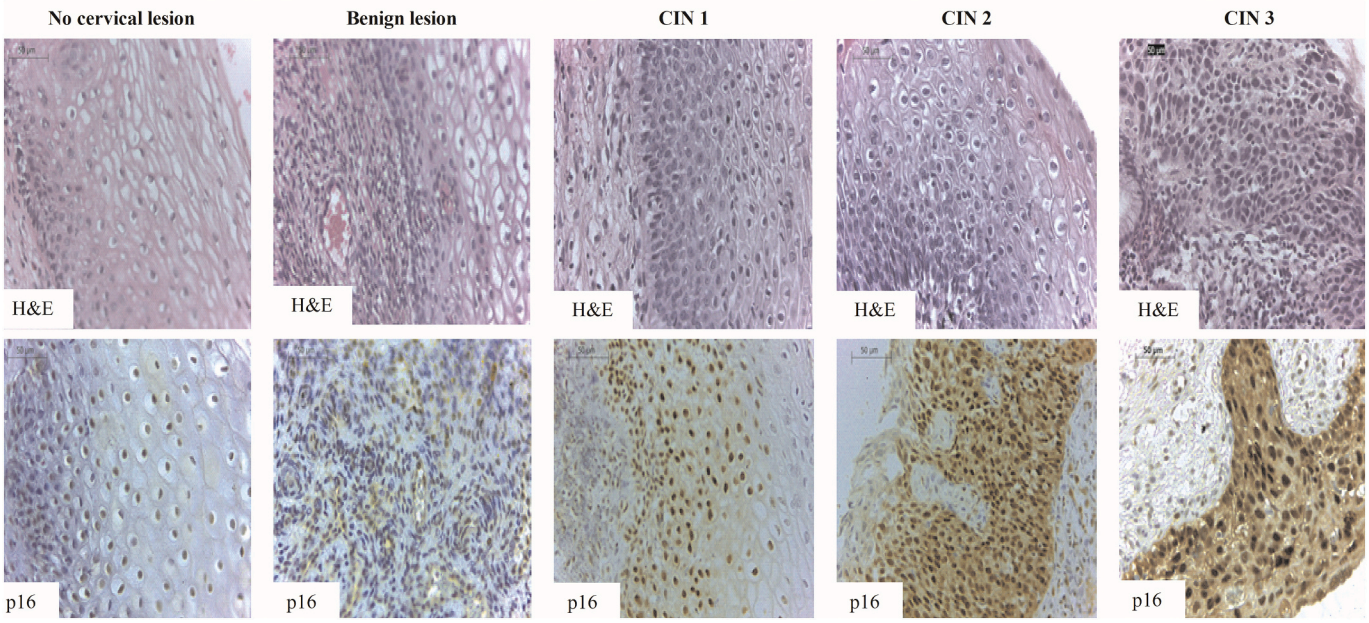


Fig. 1. Hematoxylin and Eosin (upper images) staining and immunohistochemistry staining for p16 (lower images) in cervical biopsy samples. The non-affected tissue area of a uterine cervix biopsy shows preserved stratified cervical epithelial tissue and weak p16 marking predominantly in the nucleus. Benign lesion (BL) shows epithelium with preserved structure and the inflammatory infiltrate in the stroma region with weak expression of p16 in the stromal immune cells. Biopsies with CIN lesion showing changes in epithelium structure corresponding to increased expression of p16 with diffuse staining restricted to the lower third of the epithelium seen in CIN 1. Staining restricted to the lower two-thirds of the epithelium for CIN 2. Staining encompassing all epithelium, seen in CIN 3. Magnification of x400.

samples ($P = 0.1744$) (Supplementary Table 3).

To clarify the relationship between the level of p16 expression and the severity of cervical lesions, we evaluated the proportion of cervical samples of different grades of lesion that were identified in the range of low and high p16 expression, whether using p16 labeling analyses in N, C or N + C (Fig. 2 A, B, C). We observed that low p16 nuclear labeling was more discriminative to distinguish low- from high-grade lesion; i. e., 76% of women exhibiting this pattern exhibited benign lesion, and 51% of women exhibited CIN 1. Although high p16 nuclear labeling identified only 29% of the CIN 2 and 27% of CIN 3 samples, a lesser proportion of CIN 1 lesions presenting high p16 expression (false positive) was observed compared to the cytoplasm or both N + C labeling. However, half of the specimens exhibited intermediate levels of p16 expression (42% of the CIN 1, 45% of the CIN 2, and 54% of CIN 3 of samples) (Fig. 2A).

Considering that HPV is a predictor marker for cervical neoplasia, we assessed whether HrHPV infection differentially affected the p16 expression in the nucleus or cytoplasm. Several specimens from HrHPV positive samples were stratified in 50 CIN 3 (46.3%); 28 CIN 2 (25.9%); 27 CIN 1 (25.0%) and 3 BL (2.8%); and specimens obtained from HrHPV negative samples included 6 CIN 3 (14.0%); 3 CIN 2 (7.0%); 16 CIN 1 (37.2%); and 18 BL (41.9%). In HPV positive samples, the best discrimination was also obtained with assessing nuclear p16 expression, which separated CIN 1 from CIN 2 ($P = 0.0058$) and CIN 1 from CIN 3 (P = 0.0058) samples. In HrHPV negative samples, the pattern of p16 expression was not discriminative for the lesion severity, and the statistical power was low in some comparisons due to the limited number of women without proved HrHPV infection carrying high-grade cervical lesions (data not shown).

3.3. Histology and p16 labeling combined features for clinical follow-up

The patients were grouped according to LSIL or HSIL histological classification to assess the impact of p16 nuclear labeling for clinical interpretation of cervical lesion severity. In the LSIL group, 62% of patients showed low p16 expression, exhibiting two conditions associated

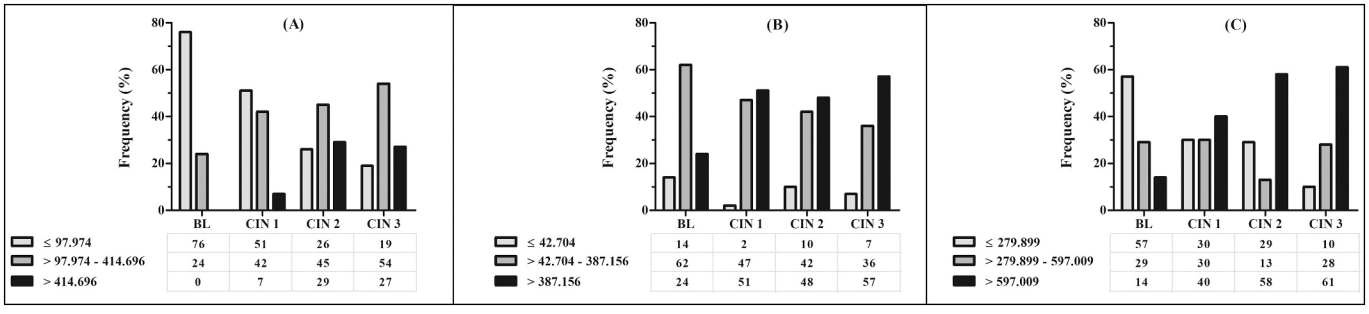


Fig. 2. Frequency of low, intermediate, and high p16 expression in biopsy samples according to the histological grade of cervical lesions. Biopsies from the benign lesion (BL) and cervical intraepithelial neoplasia (CIN) grades 1, 2, or 3 were subject to p16 labeling by immunohistochemistry. P16 expression was determined exclusively in the nucleus (A), in the cytoplasm (B), and both nucleus and cytoplasm compartments (C). The median p16 expression is shown in pixels. The level of expression was defined by previous experiments using the statistical approach of latent classes in low, intermediate, or high levels of expression specific for each nucleus, cytoplasm, or nucleus and cytoplasm labeling values.

with low risk for lesion progression; however, 5% of LSIL patients showed high p16 expression and were at questionable risk for lesion progression. In the HSIL group, 37% of patients showed high p16 expression, exhibiting two conditions for high-risk lesion progression. Therefore, these patients are eligible for surgical intervention. Notably, 26% of patients presenting high-degree lesions showed low p16 expression in the nucleus, suggesting that the impairment of the cell cycle control induced by HPV infection did not take place yet (Fig. 3A).

Regardless of the severity of the histological lesion, specimens exhibiting high p16 nucleus labeling expressed p16 levels 2-folds higher than the upper cutoff point in the cytoplasm. In addition, patients with intermediate expression of p16 in the nucleus, irrespective of the lesion grade, had a similar proportion of patients expressing low (13%), intermediate (27%), or high (60%) levels of p16 in the cytoplasm, which may represent a similar risk for disease progression (Fig. 3B). Although cytoplasmic labeling alone was not discriminative to assess the risk for cervical neoplasia progression, using a hierarchical analysis of the histological and p16 labeling data, it was possible to discriminate: i) patients at very high risk for cervical lesion progression, who exhibited signs of defective cell cycle control as evidenced by the high expression of p16 in the nucleus, or intermediate-expression of p16 in nucleus plus high-expression of p16 in the cytoplasm; ii) patients at middle-risk who presented LSIL but high-expression of p16 in the nucleus or HSIL and low p16-expression levels, and iii) patients at low-risk who presented LSIL and low- or intermediate- levels of p16 in the nucleus (Fig. 3C).

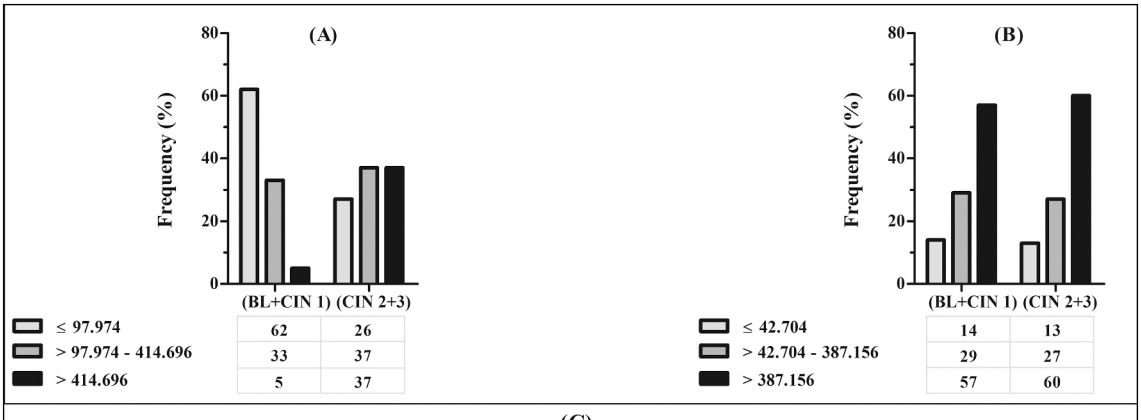
4. Discussion

The discrimination between low-grade (LSIL, CIN 1) and high-grade lesions (HSIL, CIN 2, and CIN 3) is crucial for the indication of surgical intervention (Cohen et al., 2019). According to the Brazilian Guidelines for Cervical Cancer Screening, for patients with histological diagnosis of benign lesion or CIN 1, the recommendation is to monitor the lesions with colposcopy and cytology (PAP smear). At the same time, those diagnosed with CIN 2 or CIN 3 are referred for excision of the cervical transformation zone (Nacional et al., 2016). The p16 IHC has been used as a complement to assist in the diagnosis of cervical lesions of the uterine cervix, according to the Lower Anogenital Squamous Terminology (LAST) project (Darragh et al., 2013). The association of p16 IHC analysis helps discriminate pre-cancer lesions (HSIL) from lesions not related to neoplastic risk as cervicitis and metaplasia. In addition, p16 increases the indices of inter and intra-observer agreement in evaluating histopathological specimens of the uterine cervix, whose differential diagnosis includes precancerous lesion, helping on the decision regarding the diagnosis of CIN 2 (Singh et al., 2018). However, the interpretation of p16 IHC depends on the quality of the specimen labeling and the pathologist experience, challenging the discrimination between LSIL and HSIL (Sano et al., 1998; Liao et al., 2014; Liu et al., 2017; Ghosh et al., 2019). In this regard, the British Association of Gynaecological Pathologists (BAGP) published guidelines to standardize p16 IHC interpretation that has been followed by many pathologists around the world (Singh et al., 2018).

In 1998, the p16 expression was considered negative in cervical epithelium when up to 5% of cells were stained. Positivity was defined for focal/ scattered labeling in fewer than 80% cell staining or diffuse labeling in more than 80% of the cells (Sano et al., 1998). In 2009, the p16 expression was considered positive when moderate or intense staining was observed in more than 10% of epithelial cells and negative in less than 10% of epithelial cells with moderate or intense staining (Lesnikova et al., 2009). Currently, the BAGP guidelines recommend reporting the results as presence or absence of abnormal expression of p16, and when the signal is weak/focal, pathologists generally interpret it as normal/negative expression (Liu et al., 2017).

This study evaluated p16 expression of the cervical lesion area and in non-affected tissue area (adjacent to lesions) to define baseline values for p16 expression in the nucleus and cytoplasm. In addition, we developed a statistical model involving quantitative and qualitative parameters of p16 expression to find appropriate cutoffs of p16 expression in pixels to facilitate discrimination of lesion severity.

We observed p16 labeling in nucleus, cytoplasm, or both in cervical lesions and non-affected adjacent tissue reported that the intensity of labeling raised according to the severity of the cervical lesions,



(C)

Level	Analysis	Risk of disease progression											
1	Histology (H&E)	High-grade (CIN 2+3)						Low-grade (BL+CIN 1)					
		41						21					
2	IHC-nucleus (A)	High (>414.696)		Intermediate (>97.974-414.696)		Low (<=97.974)		High (>414.696)		Intermediate (>97.974-414.696)		Low (<=97.974)	
		15		15		11		1		7		13	
3	IHC-cytoplasm (B)	High (>387.156)		Intermediate (>42.704-387.156)		Low (<=42.704)		High (>387.156)		Intermediate (>42.704-387.156)		Low (<=42.704)	
		9		4		2		4		2		1	

Fig. 3. Relationship between the magnitude of p16 expression in nucleus and the risk assessment of cervical lesions according to histological findings. (A) P16 expression was evaluated in women with LSIL (BL + CIN 1) ($N = 21$) and HSIL (CIN 2 + CIN 3) ($N = 41$). P16 expression was determined exclusively in the nucleus. The level of expression was defined by a previous experiment using the statistical approach of latent classes in low, intermediate, or high expression levels. (B) Women whose biopsies showed intermediate levels of p16 in the nucleus ($N = 22$) were further subjected to evaluation of p16 expression in the cytoplasm. (C) Hierarchical analysis of histological and p16 labeling data to discriminated patients at high and low risk for cervical lesion progression.

corroborating the results of Hu et al., who reported an increase in p16 expression related to the lesion severity, with 33.3% labeling of normal tissue or chronic cervicitis, 75% of LSIL, and 96.3% of HSIL (Hu et al., 2019).

Although we recognized that the in-block p16 expression is a good criterion for evaluating the extent of expression and risk for lesion progression, we did not use the LAST criteria for positivity, which is considered in block labeling, i.e., when a minimum of one-third of the epithelium layer is labeled (Darragh et al., 2013), because it may lead to ambiguous interpretation. This interpretation was reported by Liu et al. in a study of CIN 2 samples from 220 different patients using p16 in block-labeling as criteria of positivity, in which they found only 18% positive cases, 59% negative cases defined by cytoplasmic staining alone, and 23% samples presenting ambiguous labeling with different labeling patterns (Liu et al., 2017). Therefore, we chose to quantify the whole p16-expression using digital tools to select and capture the p16 labeling intensity in the nucleus and cytoplasm in the entire photographed area.

P16 is a constitutive nuclear protein involved in regulating the cell cycle; thus, the cellular compartment exhibiting abnormal expression of p16 seems essential for clinical interpretation of the data. Liu and collaborators considered the p16 expression normal/negative when p16 staining is observed exclusively in cytoplasm (Liu et al., 2017). While Mendaza's group has reported a more intense expression of p16 in the cytoplasm than in the nucleus in uterine cervix carcinoma, raising the hypothesis that p16 binds to the CDK4 and other cytoplasmic proteins, leading to the formation of a sizeable cytoplasmic molecule complex, blocking its traffic through the nuclear membrane pores (Mendaza et al., 2020). In this context, the cytoplasmic location of p16 due to sequestration by CDK4 would be an indirect phenomenon of deregulation in the pRb pathway in cancer cells, which would explain the higher percentage of neoplasia identified by the cytoplasm labeling alone (Romagosa et al., 2011). In our study, the evaluation of precancerous lesions using the cytoplasm labeling exclusively was not discriminative for early diagnosis of LSIL and HSIL, as 51% of CIN 1 positive samples stayed in the upper cutoff range (>387,156 pixels) and 62% of the BL remained in the intermediate range. The zone between cutoffs corresponds to the zone of uncertainty, suggesting that quantification of p16 expression should be performed using nucleus staining (Koo et al., 2009).

We observed that the p16 expression labeling in nuclei exclusively was a more efficient strategy for separating the low-grade lesions using the lower cutoff range (≤97,974 pixels). Even though a single woman with CIN 1 was identified with high nuclear p16 expression samples (cutoff range > 414,696 pixels), which may represent the proportion of women (5%) that are likely to progress to HSIL (Liao et al., 2014; Da Costa et al., 2015). In this case, the patient follow-up is essential to confirm the sensibility of the nuclear labeling of p16 to discriminate lesions with a high probability to progress to malignancy. Along with this study, it was not possible to observe the progression of this lesion due to the short follow-up period of one year.

We also observed low nuclear expression of p16 in eleven (26%) women with HSIL, whose lesions tend to regress spontaneously by controlling tissue inflammation, reducing the likelihood of developing cervical cancer (Hammes et al., 2007; Martins et al., 2014; Mhatre et al., 2012; Perkins et al., 2020; Trimble et al., 2005). In our previous study, evaluating the DNA index in HIV-HPV co-infected women, we reported that the regression rate of the cervical lesion was higher (77%) than the progression (33%) rate over a follow-up of two years. The low sensitivity and weak power of the DNA index for predicting disease progression were attributed to the presence of chronic cervical inflammation other than HPV infection (Martins et al., 2014) that also increases p16 expression (Hu et al., 2019).

Since the HPV genome is detected in 93–100% of cases of cervical cancer and its precursor lesions (Liao et al., 2014; Hu et al., 2019) and since the presence of HrHPV E6 and E7 oncoproteins are involved in the p16 overexpression (Romagosa et al., 2011; Balasubramaniam et al., 2019), we further evaluated p16 labeling according to the presence or absence of HPV infection. We observed that in the presence of HrHPV infection, high p16 expression in the nucleus (>414,696 pixels) was associated with HSIL, corroborating a previous study that reported the activation of HrHPV replication associated with high expression of p16 in CIN 3 biopsies (Liao et al., 2014; Leeman et al., 2020). Other studies also reported the presence of p16 labeling in CIN 1 and CIN 2 lesions caused by HrHPV, but not by LrHPV-6 or HPV-11 (Nuovo et al., 2019). Since the HrHPV load was reported positively correlated with the severity of the cervical lesion, and p16 labeling score was associated with HrHPV, but not with the Ki-67 level, these findings suggest that p16 levels increase before cell proliferation (Krishnappa et al., 2014; Li et al., 2019). Viral replication was reported positively correlated with p16 labeling after integrating viral DNA in the host genome, leading to the loss of the pRB function and progression of the cervical lesions (Hu et al., 2019). None of our cervical samples was positive for LrHPV, but we showed that cervical lesions in the absence of HPV infection presented less intense p16 labeling and a lower degree of the cervical lesion. Since intense and diffuse immunoreactivity of p16 in nuclei and cytoplasm associated with the presence of HrHPV was observed in cases of HSIL compared to normal tissues, the distinction between HrHPV and LrHPV associated with p16 expression is another important factor for evaluation of viral persistence and risk to developing cervical cancer (Sano et al., 1998; Pauck et al., 2014).

In the first step of the hierarchical analysis, we identified the high-grade lesion based on histology (CIN 2 and CIN 3). At this step, the risk for lesion progression is high, but a percentage of patients may achieve lesion regression by controlling cervical inflammation and secondary infection and improving the mucosal immunity (Martins et al., 2014). When the histology shows a high-grade lesion (CIN 2 and CIN 3) and high expression of p16, it highly indicates high-risk lesion progression. However, every IHC assay has limitations and subjectivity in its interpretation, and we circumvented these limitations by quantifying p16 expression using digital tools. Eleven of 41 patients represent 26% cases with high-degree lesion and low nuclear p16 expression. Due to the close connection between HPV-mediated high p16 expression in cervical cancer, it is fair to speculate that the low p16 expression in the nucleus may represent a condition that the HPV-induced impairment of the cell cycle control has not been ultimately achieved. Based on this biological feasibility, we recognized that these women might be eligible for more conservative treatment. The difficulty remains for patients showing nuclear p16 expression in a gray zone (intermediate expression). In this case, the third level of observation (in the cytoplasm) may help classify high-grade lesions in high-risk lesion progression. Since tumor suppressor pRB deregulation is associated with p16 overexpression and retention in the cytoplasm by target-kinase (Mendaza et al., 2020; Romagosa et al., 2011), the intermediate p16 expression in the nucleus and low level in the cytoplasm (seen in two patients, carrying high-degree lesion) is a challenging issue, deserving further attention. On the other hand, we observed that 1 of 21 patients with low-degree lesions presented high nuclear p16 expression, and the other four patients presented intermediate levels of p16 in the nucleus but high levels in cytoplasm, which may indicate an increased risk for lesion progression. Taken together, we concluded that p16 IHC improved risk assessment of cervical lesions when a hierarchical analysis based on location and intensity of labeling is adopted.

5. Conclusions

In this study, we discussed the limitations of histology and p16 labeling in the characterization of cervical lesions. We reported that the association of the three variables might discriminate the assessing of the risk of cervical lesion progression. We suggested a hierarchical analysis, beginning with first-level histology, followed by labeling analysis in the nucleus and then in the cytoplasm to increase the accuracy of risk

stratification, permitting a more conservative treatment or more assiduous monitoring of the patient. Further studies encompassing more patients for longer follow-ups may answer whether the conservative treatment defined by a hierarchical analysis of histology and immunohistochemistry contributes to the clinical management of women with precancerous cervical lesions.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare that there are no conflicts of interest.

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