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ARTÍCULO ORIGINAL

Standardization of P16/KI-67 immunocytochemistry in conventional cytology for detection of high-grade cervical squamous intraepithelial lesion

Estandarización de la doble biomarcación inmunocitoquímica P16/KI-67 en citología convencional para la detección de lesión intraepitelial escamosa de alto grado del cuello uterino

Alejandra Pineda Castilla[1], Ines Benedetti Padrón[2] & Lía Barrios García[2]

[1] Servicio de Patología, Clinica IMAT Oncomedica Auna. Montería, Colombia.
[2] Grupo de investigación Histopatología. Facultad de Medicina. Universidad de Cartagena, Cartagena, Colombia.

ACCESO ABIERTO

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Autor de correspondencia:
Ines Benedetti Padrón
ebenedettip1@unicartagena.edu.co

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ABSTRACT

Introduction: the p16/Ki-67 immunocytochemical assay reveals the simultaneous expression of these two biomarkers which appears only during neoplastic transformation and increases the performance of cervical cytology to detect carcinoma or high-grade squamous intraepithelial lesion (HISL) of the cervix. Very few studies have evaluated this dual staining in conventional cytology.

Objective: to standardize the use of p16/Ki-67 dual staining in conventional cervical cytology smears with HSIL or a more advanced cervical intraepithelial neoplasia (CIN2+), with a corresponding confirmatory biopsy study.

Methods: cytology smears processed by conventional technique were used. A re-evaluation was performed to verify the presence of well-preserved abnormal cells. Samples were treated to remove the coverslip, discoloration, rehydration, and antigen retrieval, and CINtec®PLUS Cytology staining protocol was applied in previously demarcated areas containing morphologically altered cells, with different incubation times of the primary antibodies. Bivariate analysis was performed using Chi-square test and Student's t-test, to determine the relationships between processing and reactivity in the staining process, using Stata v16 software.

Results: were included 40 smears with median storage of 233 days. There were neither significant differences in p16/Ki-67 dual staining in relation to slide storage time, xylene immersion, primary antibodies incubation, nor with the results of conventional cytology or histopathological diagnosis. p16/Ki-67 dual staining was positive in 75% of smears, corresponding to 75% (27/36) of HSIL, 66.6% (2/3) of carcinomas, and 100% (1/1) of adenocarcinomas.

Conclusions: p16/Ki-67 dual staining can be performed on previously stained conventional cervical cytologies with good results. It is possible that the abundant hematic material observed in some smears could interfere with the dual staining. Furthermore, the absence of dual staining in some cases with HSIL/CIN3 may be due to the loss of its antigenicity.

Keywords: Cytology; Immunocytochemistry; Squamous Intraepithelial Lesions of the Cervix; Uterine Cervical Dysplasia.

RESUMEN

Introducción: el ensayo inmunocitoquímico p16/Ki-67 revela la expresión simultánea de estos dos biomarcadores que aparece sólo durante la transformación neoplásica y aumenta el rendimiento de la citología cervical para detectar carcinoma o lesión intraepitelial escamosa de alto grado (LIE-AG) del cérvix. Muy pocos estudios han evaluado esta tinción dual en citología convencional.

Objetivo: estandarizar el uso de la tinción dual p16/Ki-67 en extendidos de citología cervical convencional con LIE-AG o lesión de mayor grado, con estudio de biopsia confirmatoria correspondiente.

Métodos: se utilizaron frotis de citología procesados mediante técnica convencional. Se realizó una reevaluación para verificar la presencia de células anormales bien conservadas. Las muestras fueron tratadas para remoción del cubreobjetos, decoloración, rehidratación y recuperación de antígenos, y se aplicó el protocolo de tinción de Citología CINtec®PLUS en áreas previamente demarcadas que contenían células morfológicamente alteradas, con diferentes tiempos de incubación de los anticuerpos primarios. Se realizó análisis bivariado mediante Chi-cuadrado y t de Student, para determinar las relaciones entre procesamiento y reactividad en el proceso de tinción, utilizando el software Stata v16.

Resultados: se incluyeron 40 extendidos con mediana de almacenamiento de 233 días. No hubo diferencias significativas en la tinción dual p16/Ki-67 en relación con los tiempos de, almacenamiento de la lámina, inmersión en xilol o incubación de anticuerpos primarios, tampoco con el resultado de la citología convencional o el diagnóstico histopatológico. La tinción dual p16/Ki-67 fue positiva en 75% de los extendidos, correspondientes al 75% (27/36) de LIE-AG, 66.6% (2/3) de carcinomas y 100% (1/1) de adenocarcinomas.

Conclusión: la tinción dual p16/Ki-67 se puede realizar en citologías cervicales convencionales previamente coloreadas con buenos resultados. Es posible que el abundante material hemático observado en algunos frotis pueda interferir con la doble tinción. Además, la ausencia de doble tinción en algunos casos con LIE-AG/NIC-3 puede deberse a la pérdida de su antigenicidad.

Palabras Clave: Citología; inmunocitoquímica; Lesiones Intraepiteliales Escamosas de Cuello Uterino; Displasia del cuello uterino.

INTRODUCTION

Cervical cancer is the fourth most common malignant neoplasia in women worldwide, with 604,127 new cases reported in 2020. It constitutes a major public health issue in countries with low human development index, ranking second in incidence (18.8 per 100,000) and mortality (12.4 per 100,000) among cancers in women (1). In Latin America, despite screening programs implementation, cervical cancer still has a significant impact (2). Conventional cervical cytology has been a useful tool for the early detection of this neoplasia, (3), however, both, this and liquid-based cytology (LBC) have limited sensitivity and specificity in premalignant lesions with a high number of false negative results (4).

Cervical cancer is associated to infection by high-risk genotypes of the Human Papillomavirus (HR-HPV) (5). The HR-HPV test improves the sensitivity to identify high-grade squamous intraepithelial lesions (HSIL: grade 2 or more advanced cervical intraepithelial neoplasia, CIN2+) (6), however, due to the high prevalence of transient infections, it has a low specificity (7). These transient infections generate intraepithelial morphological abnormalities, which can remit spontaneously, although a small percentage of patients develop a persistent infection, that leads to the development of premalignant lesions and invasive cancer (8).

In the case of persistent infection, high levels of HPV oncoproteins E6 and E7 are synthesized which alter the cell cycle regulatory mechanisms. The tumor suppressor protein p16 (p16INK4a for its acronym in English: INhibitors of CDK) has an antiproliferative effect, but the degradation of retinoblastoma protein (pRb) by E7 oncoprotein will cause an entry into the S phase of the cell cycle (9). As a result, p16 overexpression occurs in affected cells to counteract cycle dysregulation, an action that is useless due to E7-mediated inactivation of pRb, leading to its accumulation in the nucleus and cytoplasm of transformed cells (10). This accumulation of p16 can be evidenced by immunostaining and it is considered a biomarker of HR-HPV transforming infections (11-12). Ki-67 is a nuclear antigen associated with cell proliferation, present in every phase of the cell cycle, except the Go phase, whose immunostaining is used to determine cell proliferation (13). Its expression is observed in epithelial basal layer cells, benign proliferative lesions, HSIL, and cervical carcinomas (14).

p16/Ki-67 dual immunocytochemical staining is able to detect both biomarkers on a cervical cytology. Their simultaneous expression only appears during neoplastic transformation since their functions are mutually exclusive; hence its usefulness for increasing the accuracy of cervical cytology in detecting cervical malignant lesions or HSIL (15). It allows for the identification of cells that, in addition to presenting proliferative activity, show overexpression of p16; that is, those that have integration of HR-HPV genetic material, therefore, representing high-grade lesions capable of progressing (16). p16/Ki-67 dual staining can be implemented in LBC and conventional cytology (17), where its use in LBC has shown high concordance for the detection of HSIL (18-19), however, very few studies have evaluated it in conventional cytology.

In many Latin American countries, conventional cervical cytology is recommended as a screening test in women under 30 years of age, while in those over 30 years the HR-HPV testing is used; however, when it is not available, they use conventional cervical cytology (3, 20-23). Thus, if p16/Ki-67 dual staining in this type of previously stained smears behaves similarly to that reported in CBL, it could be used as a method for confirming the presence of a cervical lesion with real risk of progression, in contrast to the HR-HPV testing, which confirms the infection, but not the existence of a clinically significant lesion (24). Such knowledge is necessary and useful in our region, where most routine cervical cytologies are conventional, and very few studies have evaluated the use of these two biomarkers in this type of sample (25). This study aimed to standardize the use of p16/Ki-67 dual staining in conventional cervical cytology and to determine its performance in the detection of HSIL.

METHODS

This study was carried out to standardize the use of p16/Ki-67 dual staining in conventional cervical cytology smears with a result of HSIL or worse (CIN2+), classified according to the Bethesda System 2014 (26). The cases were obtained from a gynecological pathology reference laboratory. Smears processed by conventional techniques were used and confirmatory biopsies were

available. A re-evaluation was performed to verify the presence of well-preserved abnormal cells. Samples were processed, CINtec® PLUS Cytology staining protocol was applied, and the presence of transformed cells was evaluated. Statistical analysis was performed to determine the relationships between processing and reactivity in the staining process. The study was approved by the Ethics Committees of the participating institutions.

Sample collection

A search was made among the cases registered in the laboratory database between January and July 2020, by means of a successive non-probabilistic sampling, using the initial processing date. A re-evaluation of both, the conventional cytology smears and the corresponding histopathological study was performed to confirm the reported diagnosis and to verify the presence of well-preserved abnormal cells in the smears for the registered cases. Under microscopic vision, the areas of interest in the smears were delimited with a permanent marker and microphotographs of these representative areas were obtained.

Sample processing and dual staining evaluation

For the standardization of dual staining, the following steps were followed: pretreatment of the slides in xylol in order to remove the coverslip, during two to three days depending on the required time for their spontaneous detachment. Subsequently, the smears were decolorized and rehydrated in four consecutive ethilic alcohol baths with decreasing concentrations of 100%, 96%, 70%, and 50%, for a total of 5 minutes in each solution. They were immersed in distilled water for 5 minutes, and then in an epitope retrieval solution inside a pressure chamber (DakoCytomation® Pascal model S2800) at 99 °C for 10 minutes, and afterwards they were left at room temperature for 20 minutes. Subsequently, the area of interest (previously marked in the microscopic evaluation) was delimited using a liquid isolating pencil designed for staining processes (Liquid Blocker Super PAP Pen®, Cancer Diagnostics Inc., Birmingham, Michigan, USA). The smears were placed in a humid chamber at room temperature and the staining protocol was followed on the delimited area, using the commercial CINtec® PLUS Cytology kit (Roche, AG, Mannheim, Germany) for p16INK4a/Ki-67 dual immunocytochemical staining, which contains a cocktail of primary antibodies: a mouse monoclonal antibody (clone E6H4TM) against the human protein p16INK4a and a rabbit monoclonal antibody (clone 274-11 AC3) against the human protein Ki-67, in addition to the ready-for-use visualization reagents, which are a Diaminobenzidine (DAB) chromogen and a Fast Red chromogen, whose transformation mediated by horseradish peroxidase and alkaline phosphatase, respectively, reveal the reaction products at the antigen site.

The staining was manually performed following the manufacturer's instructions, using 100 µl of the reagents on each smear, briefly: the cytological smears were incubated with the mouse monoclonal antibody anti-human p16INK4a (clone E6H4TM), and the anti-rabbit monoclonal antibody anti-human Ki67 (clone 274-11AC3V1) during 60 minutes for the first group, and during 75 minutes for the remaining groups, at room temperature. Then, the smears were incubated during 15 minutes with each of the secondary antibodies, followed by the DAB chromogen for 10 minutes, and the Fast-Red chromogen for 15 minutes. The contrast was carried out with alcohol-free hematoxylin. Immunostaining was independently interpreted by two pathologists. Cells were considered positive when brown staining was observed in the cytoplasm and when the nucleus stained red (Figure 1).

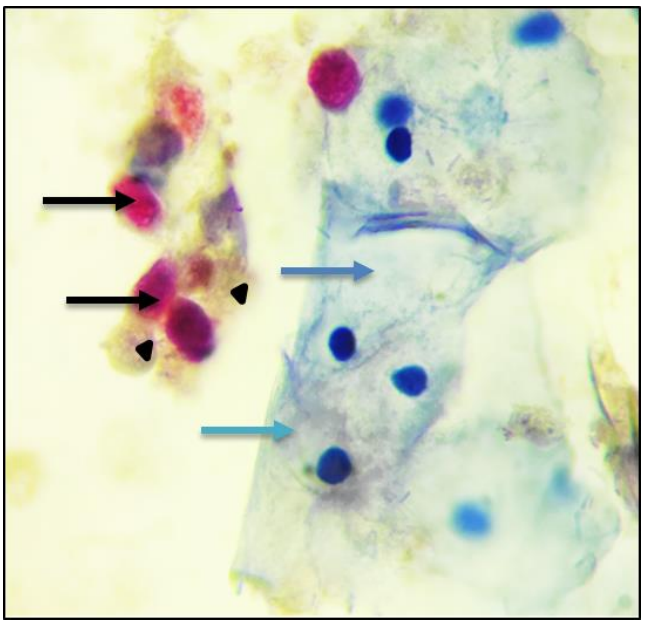


Figure 1. Conventional cervical cytology smear with positive squamous cell carcinoma for p16/Ki-67 dual immunostaining. Positive nucleus for Ki-67 (red staining, black arrows) and positive cytoplasm for p16 (brown staining, arrowheads), normal cells negative for dual staining (blue arrows). IHC p16/Ki-67, 400X, (Source: original photomicrographs).

Statistical analysis

Cytological results, biopsy diagnoses, storage times, number of days in xylol, incubation times for primary antibodies, and presence of p16/Ki-67 dual staining were recorded in a Microsoft Excel® database. Bivariate analysis was performed using the Chi-square test and Student's t-test, to determine the relationships between processing and reactivity in the staining process, p16/Ki-67 dual staining was established as a dependent variable. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using Stata v16 software (Stata Corp, Texas, USA).

RESULTS

Between January and July 2020, 127 conventional cervical cytologies with HSIL were registered, where 42 of them had a confirmatory biopsy, two of which were excluded because the smears did not show well-preserved cells. Forty cases were included, with a median cytological smear storage time of 233 days (IQR=219.5-248.5), all were stored under the same conditions, at room temperature. Most cases had a cytological result of HSIL and few cases had a more advanced lesion, while the histopathological diagnoses were distributed more evenly between HSIL/CIN2, HSIL/CIN3, and carcinomas (Table 1). Seven of the cases with a diagnosis of carcinoma confirmed by biopsy had been initially reported as HSIL on cytology.

Smears processing results

The smears were processed in four sessions of laboratory assays. In relation to the time required for removing coverslips, 80% (n = 32) of the slides required a time in xylene of two days, 15% (n = 6) needed three days, whereas two slides only required two minutes in xylene because they had not been mounted with coverslip. The smears processed in the first assay (n=17) were incubated during 60 minutes with the primary antibodies, following the manufacturer's recommendation; however, as no staining was evidenced in some smears, the incubation time was increased to 75 minutes in the following assays (n=23). Dual staining for p16/Ki-67 was observed in 75% (n = 30)

of the smears, (Figure 2). In three cases (7.5%), only staining for p16 was observed, while there was one case that only showed staining for Ki-67. In 6 of the first group smears incubated during 60 minutes, no staining was observed. Very few inflammatory cells sometimes showed immunoreactivity for p16.

Table 1. Distribution of diagnosis by cytology and biopsy

Diagnosis	n	%
Conventional cytology		
HSIL	36	90%
Adenocarcinoma	3	7.5%
Squamous cell carcinoma	1	2.5%
Biopsy		
HSIL/CIN 2	13	32.5%
HSIL/CIN 3	16	40%
Squamous cell carcinoma	9	22.5%
Adenocarcinoma	1	2.5%
Undifferentiated carcinoma	1	2.5%

Source: original data

When comparing dual staining with cytological results, 27 (75%) of the cytologies reported HSIL, 2 (66.6%) of those were reported as squamous cell carcinoma and the only one reported as adenocarcinoma was p16/Ki-67 positive.

Regarding the histopathology of cases, 61.5% (n=8) of the cases with a biopsy diagnosis of HSIL/CIN2, 81.2% (n=13) of HSIL/CIN3 cases, and 81.8% (n = 9) of carcinoma cases, showed p16/Ki-67 dual staining in the corresponding cytology. The p16/Ki-67 dual staining was considered negative in 38.5% of the cases with a histological diagnosis of HSIL/CIN2, and in 18.8%, and 18.2% of those a with HSIL/CIN3 and carcinoma diagnosis, respectively (Table 2). The two smears of carcinoma cases, negative for p16/Ki-67 dual staining, presented abundant hematic material.

There were no statistically significant differences in p16/Ki-67 dual staining in relation to the result of conventional cytology (p=0.098), nor with the histopathological diagnosis (p=0.087) (Table 2). Likewise, no statistically significant differences were observed when comparing slide storage time (p=0.950), time required in xylene (p= 0.853) and primary antibodies incubation time (p=0.641), (Table 3).

DISCUSSION

The immunocytochemical dual staining p16/Ki-67 has shown high sensitivity and specificity for detecting HSIL in cervical cytologies classified as mild abnormalities (ASC-US, LSIL), in patients with LSIL with risk of progression to a more severe cervical intraepithelial neoplasia (CIN2+), as well as in patients with a positive HR-HPV testing and a negative cytology (15-27). Most studies evidencing the usefulness of this stain have been carried out in CBL, (17) few of them carried out in Latin America (28).

Limited studies have evaluated p16/Ki-67 dual staining in conventional cytology, most of them with favorable results. The prospective PALMS study evaluated dual staining as a primary screening test for cervical cancer in a sample of 27,349 women from various European countries, on smears obtained simultaneously with those for Papanicolaou staining, concluding that p16/Ki-67 dual staining has a higher sensitivity than cytology and a higher specificity than HR-HPV testing in every agree group (19).

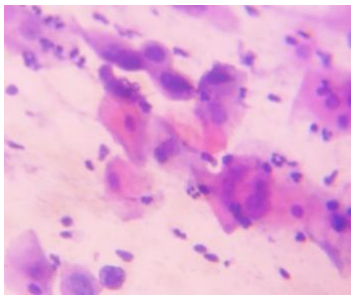
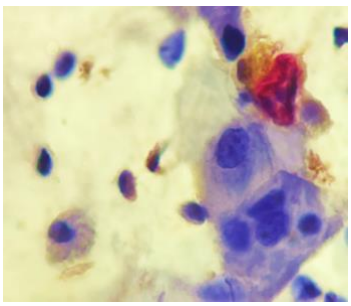
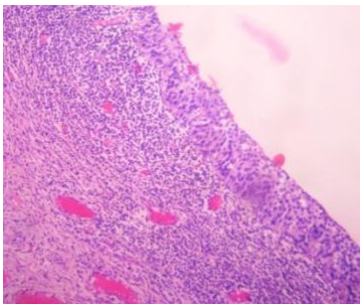
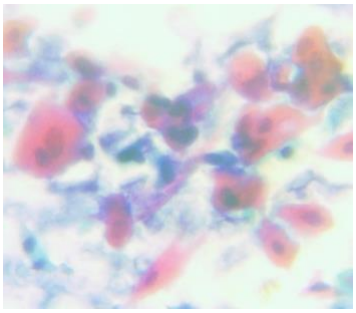
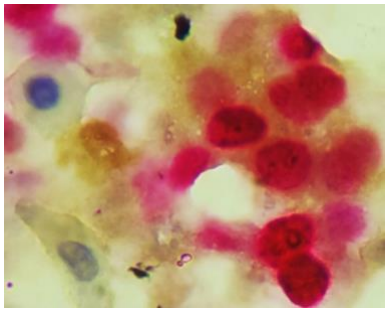
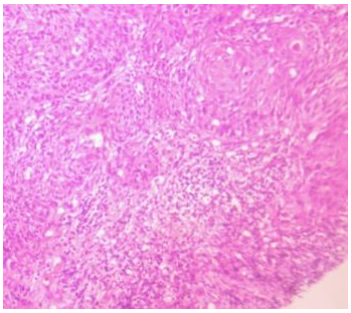
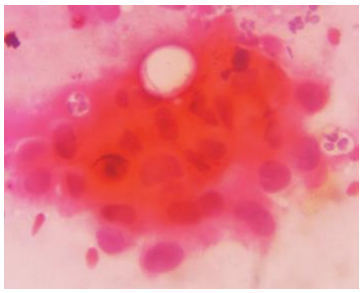
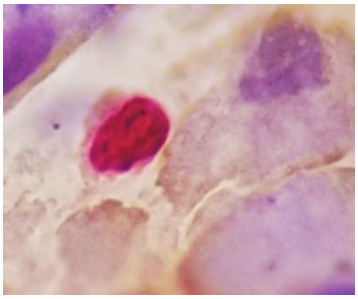
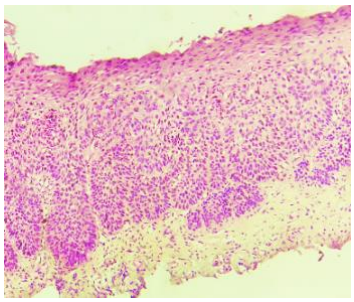
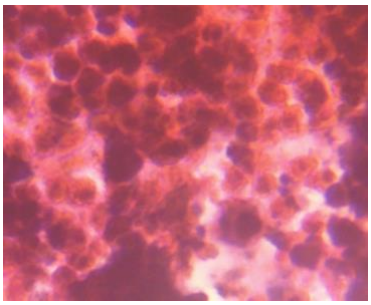
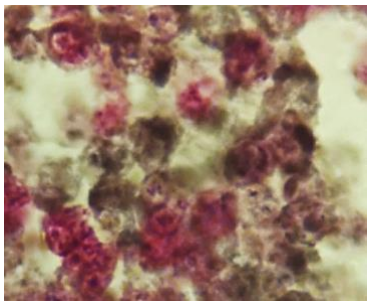
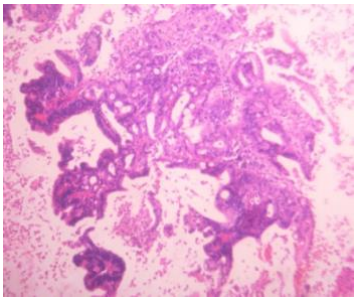
	Conventional cervical cytology Pap staining	Conventional cervical cytology p16/Ki-67 dual immunostaining	Biopsy H&E
Case 1			
Case 2			
Case 3			
Case 4			

Figure 2. Cervical smears with Papanicolaou (Pap) staining, positive for p16/Ki-67 dual immunostaining, with the corresponding hematoxylin and eosin staining biopsy. (16200) LIE AG, NIC 3. (28716) Squamous cell carcinoma. (2042) LIE AG, NIC 2. (1409) Adenocarcinoma. (Source: original photomicrographs).

Table 2. Distribution of dual p16/Ki67 staining according to diagnosis

	p16/Ki67 dual staining present n (%)	**p16/Ki67 dual staining absent or incomplete, n (%)**	**P value**
Cytology result			
HSIL	27 (75)	9 (25)	
Adenocarcinoma	1 (100)	0 (0)	0.098*
Squamous cell carcinoma	2 (66.6)	1 (33.4)	
Histopathological diagnosis			
Invasive adenocarcinoma	1 (100)	0 (0)	
Squamous cell carcinoma	7 (77.7)	2 (22.3)	
Undifferentiated carcinoma	1 (100)	0 (0)	0.087*
HSIL/CIN 2	8 (61.5)	5 (38.5)	
HSIL/CIN 3	13 (81.2)	3 (18.8)	

Source: original data, * x^2 test.

Table 3. Distribution of p16/Ki67 dual staining according to technical aspects

	p16/Ki67 dual staining present n (%)	**p16/Ki67 dual staining absent or incomplete n (%)**	**p value**
Storage time	233.5 (219 – 253)	232 (220 – 245)	0.950*
Primary antibody incubation time			
60 minutes	13 (76.4)	4 (23.6)	
75 minutes	17 (73.9)	6 (26.1)	0.853*
Time in xylol			
2 minutes	2 (100)	0	
2 days	24 (75)	8 (25)	0.641*
3 days	4 (66.6)	2 (33.4)	

Source: original data, *t-test

Furthermore, a study conducted in the Netherlands, which included 256 conventional smears with mild abnormalities, showed a significantly higher specificity of p16/Ki-67 dual staining for detecting CIN in comparison to HR-HPV testing, with a similar sensitivity, and demonstrated that, in long-term follow-up, patients with a positive HR-HPV test and a negative p16/Ki-67 dual staining did not progress to CIN3+.(29) In that study, the coverslips were removed with xylol, and a group of slides was discarded due to the presence of background staining caused by a prolonged time in discoloration; other details of the performed processing are not specified (29).

In a previous study of our group, p16/Ki-67 dual staining was evaluated in 42 smears of archived conventional cervical cytologies with different diagnostic categories, following a very similar procedure to the current one, except for a longer incubation time for visualization reagents (25). However, these smears required a much longer time in xylol because they had been stored for a longer time, and p16/Ki-67 dual stain was observed only in 7% of them, where 18% of those reported as HSIL presented double staining, while none of the carcinoma and adenocarcinoma cases showed it (19). In contrast, in this study, most of the previously demarcated abnormal cells in the smears showed positivity for p16/Ki67 dual staining, with a higher rate of positivity in HSIL and

carcinoma. It is possible that the shorter storage time and, consequently, the shorter time in xylol, have been important factors in maintaining cellular immunoreactivity.

The period in which antigenicity begins to be altered in archived smears has been poorly documented. Dorfelt et al., reported that it is possible to perform a reliable detection of cellular antigens in smears with a coverslip as soon as initial staining has been made until immediately before the immunocytochemistry (30); corroborating the previous findings of Aoki et al, which describe that the antigenicity decreases after four weeks in smears without staining and coverslips (31). In the present study, no significant difference was found between the storage time and the immunostaining results, which may be due to the fact that the smears were covered by a coverslip and the storage times were not excessively long, compared with our previous study performed on smears archived for a long time (25).

The positivity for p16/Ki-67 dual staining observed in most of the smears in the present study is considered a good result, although it was expected to be found in all of them. The smears with a HISL/CIN2 report presented dual staining in a great percentage, except for some that could correspond to lesions that probably did not have viral genome integration yet, such as low-grade lesions or neoplasia simulating non-preneoplastic lesions, and therefore would resolve spontaneously. It is possible that the few HSIL/CIN3 smears without biomarkers reactivity simply lost antigenicity. In relation to the two cases of carcinoma that did not show reactivity, abundant blood material was observed in both which could have interfered with staining. Immunostaining results also depend on the preanalytical phase, the storage and the preservation conditions (30).

Unlike p16/Ki67 dual staining, several studies have evaluated p16 immunostaining in archived conventional cervical cytology, reporting it as a very useful tool for cytological diagnosis, allowing for the distinction of HSIL from reactive or inflammatory changes, presenting effective results in this type of smears, that could be a convenient method for retrospective immunocytochemical studies, in addition to being more cost-effective than in LBC.(32-34) Regarding the processing, in the study of Torres et al,(32) the steps in coverslip removal and slide discoloration were the same as those performed in our study, even the same antibody clone was used, but the staining protocol was automated.(37) In the study of Ines et al,(33) p16 staining was evaluated in conventional cytologies with various diagnostic categories, some of which were recently obtained and others archived, without finding differences in staining between discolored and fresh. However, unlike our study, the discoloration was performed using 0.5% HCl and the immunocytochemical staining was automated.(33) In the study of Nieh et al,(34) the smears were discolored with 1% acid-alcohol, and the antigenic recovery was done with citrate buffer and Nonidet P40, which remove the mucus, crucial for preserving the immunostaining quality (34).

A limitation of this study is the number of evaluated smears, requiring it to be continued with studies based on larger samples, where the reagents are applied throughout the slide, and where immunohistochemical staining for p16 in the biopsies is included, for being able to make an adequate correlation of immunocytochemical findings.

In conclusion, p16/Ki-67 dual staining can be performed on conventional cervical cytologies with good results. Techniques for the pretreatment of these smears are diverse but effective, and to achieve an optimal result, the staining protocol should be followed according to the manufacturer's recommendations. This technique facilitates cytological diagnosis by allowing quick and easy identification, even at a lower magnification of

positive cells, offering advantages in terms of reducing the evaluation time of smears. Considering the wide use of conventional cytology in Latin America and the lack of studies regarding the usefulness of p16/Ki-67 dual staining in this type of smear, it is necessary to continue researching in this field.

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