Original article

Potential diagnostic value of P16 expression in premalignant and malignant cervical lesions

Narges Izadi-Mood[1], Kourosh Asadi[2], Hadi Shojaei[3], Soheila Sarmadi4, Seyed Ali Ahmadi[5], Sanaz Sani[6], Leila Hashemi Chelavi[3]

[1]Professor, Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. [2]Resident, Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. [3]Research Center of Woman Hospital, Tehran University of Medical Sciences , Tehran, Iran. [4]Associate Professor, Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. [5]Associate Professor, Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. [6]Resident, Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

Background: The goal of this study was to evaluate the results of the expression of p16INK4a in normal uterine cervical epithelium, low-grade cervical intraepithelial neoplasia (CIN), high-grade CIN, squamous cell carcinoma (SCC), and adenocarcinoma of the cervix, in order to help draw a distinction between low risk and high risk patients with cervical lesions. **Materials ans Methods**: P16INK4a expression was evaluated by immunohistochemistry in 78 paraffin-embedded tissue samples including 39 normal cervical tissues, 11 low-grade CINs, 11 high-grade CINs, 22 cervical SCCs and 8 cervical adenocarcinomas. Two parameters in immunohistochemical p16 expression were evaluated: percentage of p16-positive cells, and reaction intensity. **Results:** The p16INK4a expression rate was 81.8% in low-grade CINs, 91% in high-grade CINs, 90% in SCCs and 75% in cervical adenocarcinomas. 10% of normal cervical samples expressed p16. Moreover, there was a significant relationship between the histological diagnoses and percentage of positive cells and reaction intensity of p16 ($p < 0.005$). The intensity of the reaction was the best parameter to evaluate the positivity of p16. **Conclusions:** Over-expression of the p16INK4a was typical for dysplastic and neoplastic epithelia of the uterine cervix. However, p16INK4a-negative CINs and carcinomas did exist. Although negative p16INK4a expression does not definitely exclude the patient with cervical lesion from the high-risk group, immunohistochemical study for p16INK4a may be used as a supplementary test for an early diagnosis of cervical cancers.

Key words: P16INK4a, Cervical intraepithelial neoplasia, Immunohistochemistry, Human papilloma virus.

INTRODUCTION

Cervical cancer is one of the most common cancers affecting women worldwide. Since the implementation of Pap smear screening, cervical cancer morbidity and mortality have declined drastically. Nevertheless, the number of newly diagnosed cases worldwide is still significant, reaching about 400000 cases each year.[1] Epidemiologic and laboratory data supports the conclusion that human papillomavirus (HPV) is the etiologic agent for the vast majority of premalignant and malignant epithelial lesions of the cervical mucosa, as HPV DNA can be detected in 95% to 100% of all cases.[2-5]

Papillomavirus is a double-stranded DNA virus encased in a 72-sided icosahedral protein capsid. More than 120 types of HPV have been identified, which can be divided into high-risk, intermediate-risk, and low-risk types. The persistent high-risk type HPV infection of the cervical epithelium appears to trigger neoplastic progression.[2,6]

The protein p16INK4a (henceforth referred to as p16) is a cellular protein involved in cell cycle regulation, and its expression is tightly controlled in normal cells. In normal nondysplastic cells, p16 protein is expressed at a very low level and is almost undetectable by immunohistochemistry (p16 can be expressed physiologically in a few cells, especially those undergoing squamous metaplasia during this transdifferentiation process). On the contrary, due to the transforming activity of the E7 oncogene of all high-risk human papillomavirus (HR-HPV) types, p16 is strongly overexpressed in dysplastic cervical cells and may be easily detected by immunohistochemistry (IHC).[7,8] Therefore, p16 may be considered a surrogate marker for the activated oncogene expression of HR-HPV in dysplastic cervical cells.[9, 10]

P16 is a cyclin-dependent kinase (CDK)-4inhibitor. It is the product of the INK4a gene on chromosome 9 and specifically binds to cyclin D–CDK4/6 complexes to control the cell cycle at the G1-S interphase. P16 is integral to p-retinoblastoma (p-Rb) mediated control of the G1-S phase transition of the cell cycle; it puts a brake on the cell cycle by inacti-

Address for correspondence: Narges Izadi-Mood, [1]Professor, Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran. Email: nizadi@sina.tums.ac.ir
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vating the CDKs that phosphorylate Rb protein. In preneoplastic and neoplastic cervical lesions associated with high risk HPV infection, there is functional inactivation of Rb by HPV E7 protein. This results in an accumulation of p16 protein, because normally Rb inhibits transcription of p16.[8] P16 expression can also be regarded as a marker of E7 gene activity.

However, it is also clear that focal, or even diffuse, p16 expression in the cervix and other tissues may occur as a result of non-HPV related mechanisms. Despite the p16 overexpression in association with high risk HPV, there is no slowing effect on the cell cycle because Rb has already been blocked by the E7 oncoprotein.[11] The role of p16 immunohistochemistry as a diagnostic aid in gynecological pathology has recently been reviewed.[12]

Our objective was to investigate, through IHC, the expression of p16INK4a in biopsies of normal uterine cervical tissue as well as pre-cancerous and cancerous lesions. The goal of this study was to evaluate the results of the expression of p16INK4a in normal uterine cervical epithelium, low-grade cervical intraepithelial neoplasia (CIN), high-grade CIN, squamous cell carcinoma (SCC), and adenocarcinoma of the cervix, in order to help draw a distinction between low risk and high risk patients with cervical lesions.

MATERIALS AND METHODS

Study design

Formalin-fixed and paraffin-embedded cervical biopsy samples were selected from the pathology files of women who referred to Mirza Koochak Khan Hospital, Tehran, Iran. Haematoxylin and eosin-stained slides of all biopsy samples were reviewed by two pathologists and classified according to criteria outlined by the World Health Organization. Ethical approval for use of all specimens was obtained from the research ethics committee of the Tehran University of Medical Sciences. Overall, 39 normal cervical biopsies, 11 low-grade CINs, 11 high-grade CINs, 20 invasive squamous cell carcinomas and 8 adenocarcinomas were selected.

Antibodies and Immunohistochemistry

Commercially available mouse monoclonal F-12 anti-p16INK4a (Santa Cruz, CA, USA) and CIN-tec™ p16 Cytology kit (Dako Cytomation, now Dako AS, Glostrup, Denmark) were used. Serial sections (4 μm-thicks) of formalin-fixed and paraffin-embedded biopsy samples were prepared, one of which was stained with H&E to confirm the histopathologic diagnosis. Consecutive sections were processed for IHC analysis. Sections were immersed in xylene to remove paraffin and then rehydrated through graded alcohol. Epitope retrieval was performed by heating at 110°C for 10 min in 10 mM citrate buffer (pH 6.0) in an autoclave. Endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide in PBS for 5 min. After blockage of nonspecific binding by preincubation with 1.5% normal horse serum (Vectastain ABC Kit; Vector, Burlingame, CA) for 30 min, primary antibodies were added for 45 min at room temperature. For detection, biotinylated horse anti-mouse or antirabbit sera (Vectastain) were applied as secondary antibodies for 30 min, followed by incubation with the avidin-biotin complex (Vectastain) for 30 min. The reaction was developed using the chromogen aminoethylcarbazole (AEC; Sigma, Deisenhofen, Germany) mixed with hydrogen peroxide in acetate buffer, and the sections were counterstained with hematoxylin. The specificity for each staining run was evaluated on cytospins of positive control cell lines, on which the IHC procedure started with blockage of the endogenous peroxidase activity, and a positive reaction required the addition of specific primary antibodies.[8]

The reaction was considered positive when a chestnut-brown color was seen in the nucleus and cytoplasm. Two parameters were evaluated, percentage of p16-positive cells and reaction intensity. The percentage of positive cells was evaluated in the highest expression area (''hot spot'') and graded as follows, negative (grade 0) when no cells stained, positive cells > 0-10 % (grade 1), positive cells >10-50% (grade 2), positive cells > 50-80% (grade 3) and positive cells > 80% (grade 4). The intensity of the reaction was scored as negative (0), weak (1), moderate (2), and strong (3).

The usefulness of the p16 (INK4a) expression in biopsies for distinction between normal uterine cervical tissue, pre-cancerous and cancerous lesions was evaluated by Statistical Package for Social Sciences version 18.0 (SPSS Inc., Chicago, IL, USA) software for Windows using chi-square and Fisher's exact test. P value less than 0.05 was considered statistically significant.

RESULTS

According to histopathological examination, 89 cases were classified as follows: 39 patients as normal/ negative for neoplasia (including squamous metaplasia), 11 cases as low-grade CIN, 11 high-grade CINs, 22 cervical SCCs and 8 cervical adenocarcinomas.

We observed a significant relationship between the

histological diagnoses and percentage of positive cells and reaction intensity of p16 ($p < 0.005$) (Fig 1-5). Table 1 depicts the percentage of cells positive for p16 according to histologic diagnosis. Four out of 39 cases diagnosed as normal cervical tissue were positive for p16, whereas 85.7% of the cases with invasive carcinoma were p16-positive.

Table 2 shows the increasing reaction intensity of p16 from normal cases to invasive carcinoma, in which we observed moderate to strong reaction intensity. Univariate logistic analysis showed that the intensity of the reaction was the best parameter to evaluate the positivity of p16.

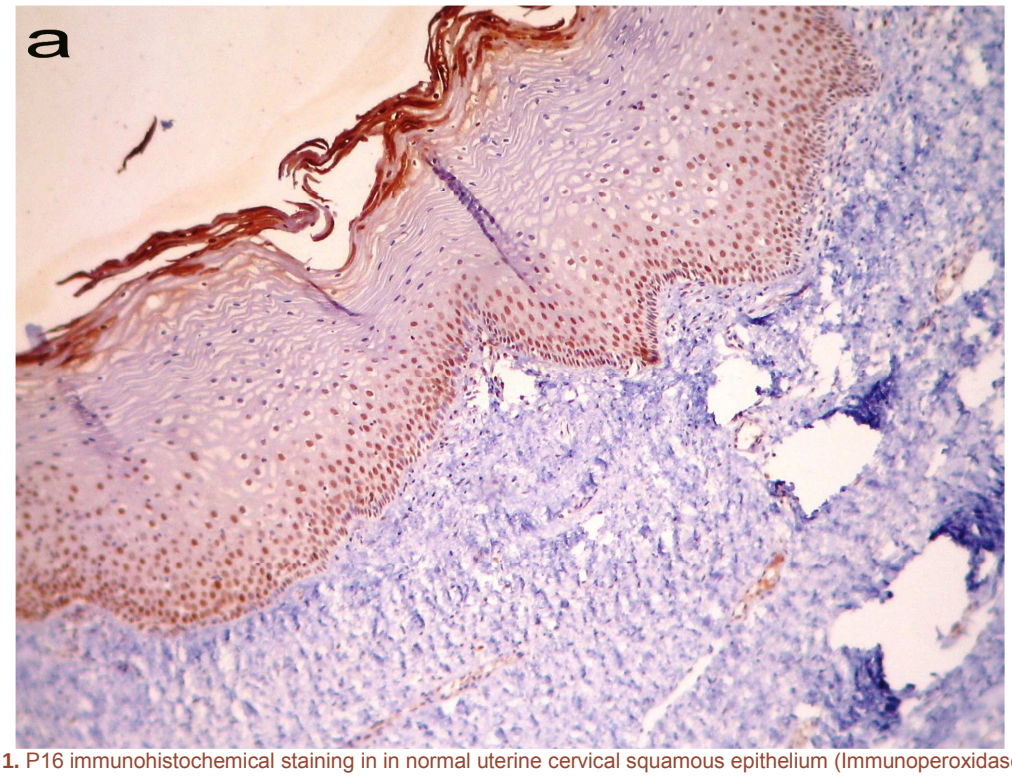

Figure 1. P16 immunohistochemical staining in in normal uterine cervical squamous epithelium (Immunoperoxidase, ×100)

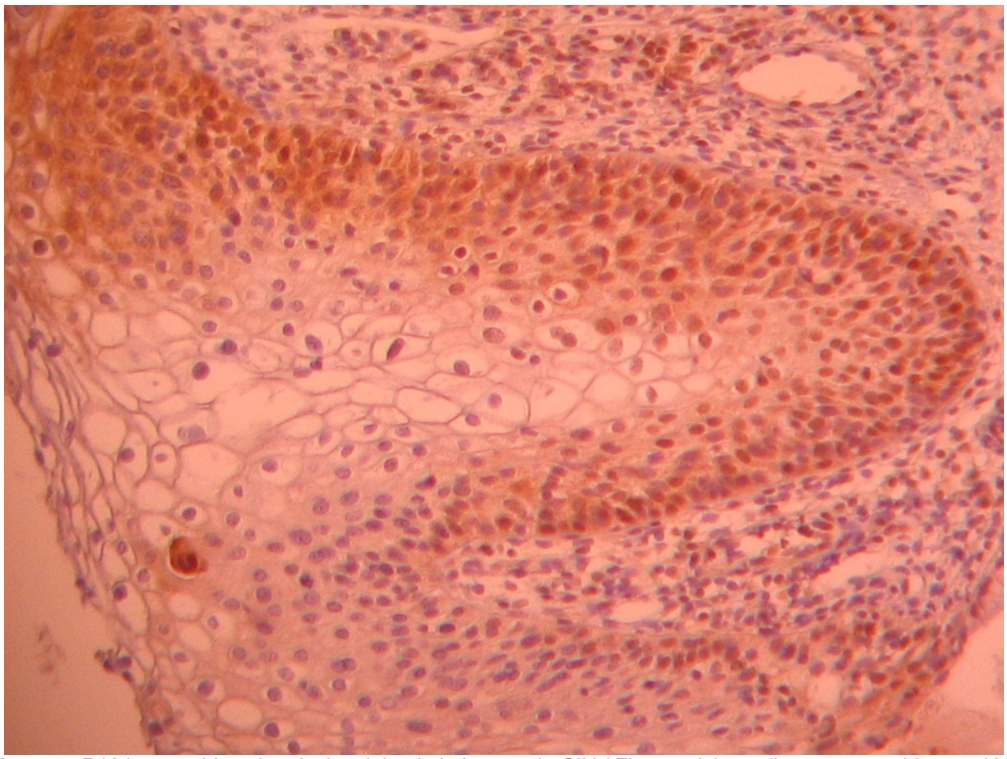

Figure 2. P16 immunohistochemical staining in in low grade CIN / Flat condyloma (Immunoperoxidase, ×100)

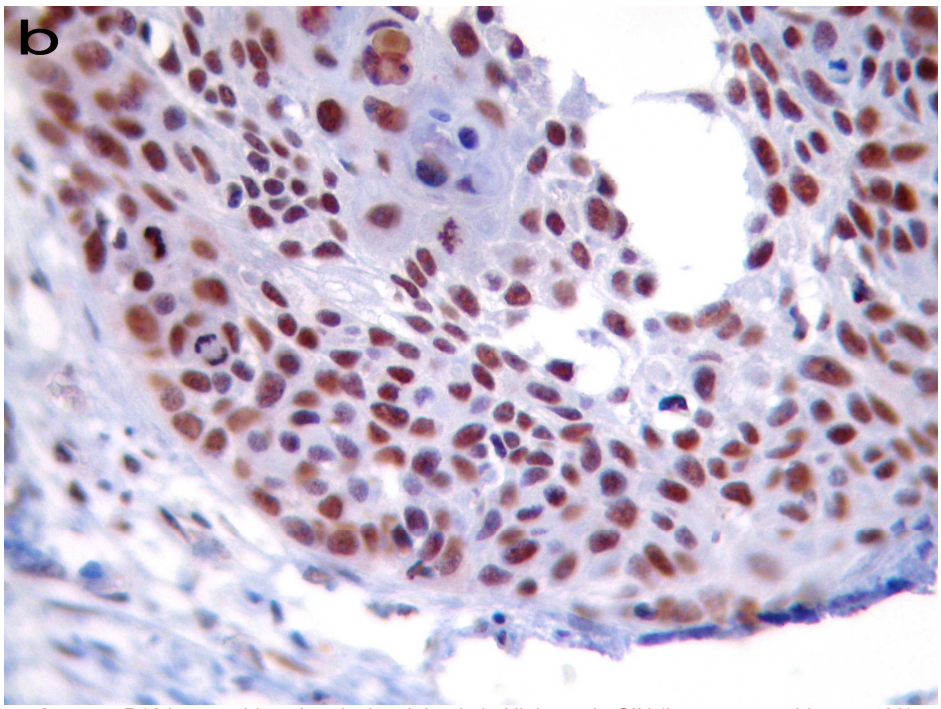

Figure 3. P16 immunohistochemical staining in in High-grade CIN (Immunoperoxidase, ×400)

Table 1. Correlation between histological diagnosis and percentage of cells positive for p16INK4a staining

Histological diagnosis	Percentage of cells positive for p16INK4a				
	0%*	0-10%*	10-50%*	50-80%*	80-100%*
Normal	35(89.7%)	2(5.1%)	2(5.1%)	0	0
CIN (Low grade)	2(18.2%)	0	3(27.2%)	6(54.6%)	0
CIN (High grade)	1(9.09%)	0	1(9.09%)	1(9.09%)	8(72.7%)
SCC	2(10%)	1(5%)	2(10%)	2(10%)	13(65%)
Adenocarcinoma	2(25%)	1(12.5%)	2(25%)	1(12.5%)	2(25%)

*percentage of cells stained.
CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma

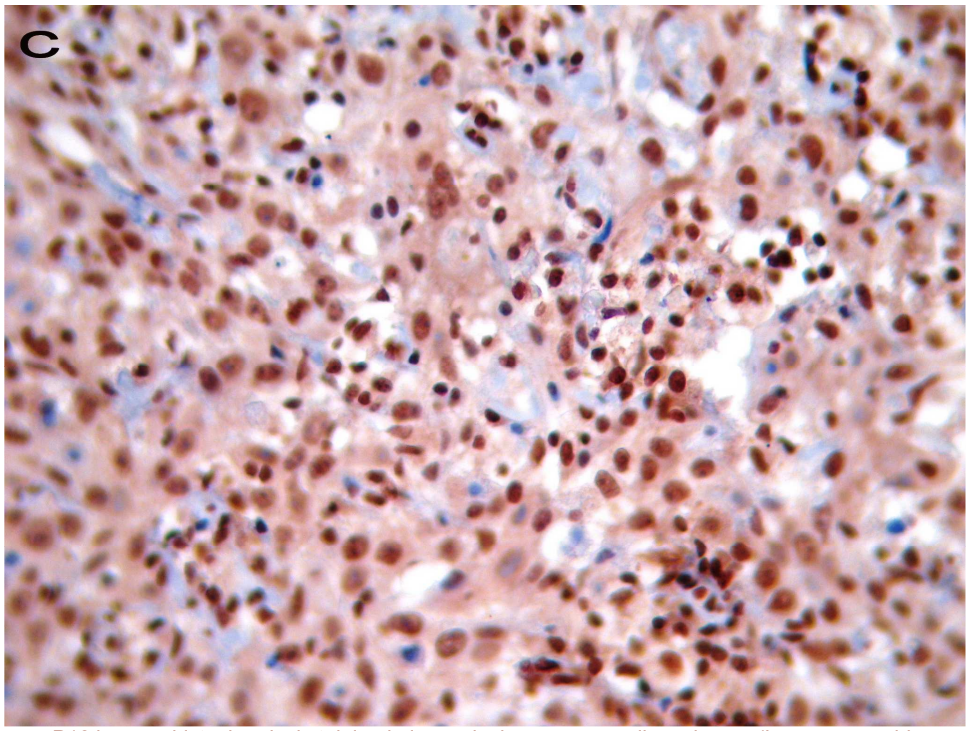

Figure 4. P16 immunohistochemical staining in in cervical squamous cell carcinoma (Immunoperoxidase, ×400)

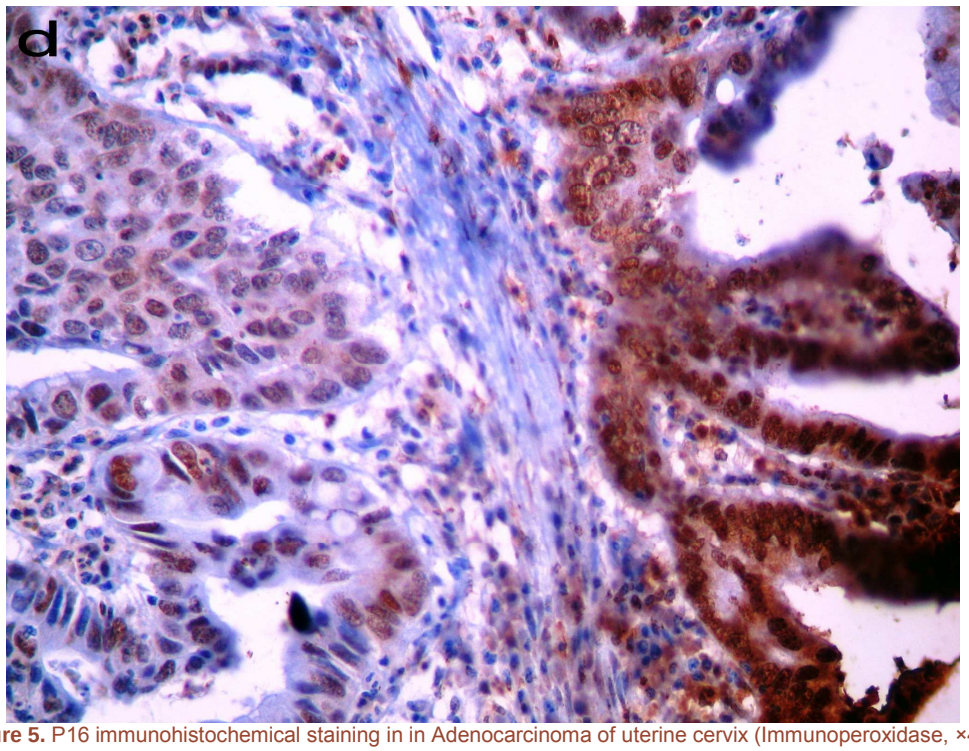

Figure 5. P16 immunohistochemical staining in in Adenocarcinoma of uterine cervix (Immunoperoxidase, ×400)

Table 2. Correlation between histological diagnosis and reaction intensity for p16INK4a staining

Histological Diagnosis	Reaction Intensity (p16 INK4a)			
	Negative	Weak	Moderate	Strong
Normal	35(89.7%)	2(5.1%)	2(5.1%)	___
CIN (Low grade)	2(18.2%)	___	9(81.8%)	___
CIN (High grade)	1(9.09%)	___	2(18.1%)	8(72.7%)
SCC	2(10%)	1(5%)	2(10%)	15(75%)
Adenocarcinoma	2(25%)	1(12.5%)	2(25%)	3(37.5%)

CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma

DISCUSION

Despite extensive studies of cervical cancer precursors, the interobserver variation in the histopathologic interpretation of cervical biopsy specimens still constitutes a dilemma.[13, 14] A search for a specific diagnostic biomarker was launched to solve the problem of lack of interobserver reproducibility in the histological diagnosis of cervical intraepithelial lesions and revealed p16. This is a tumor suppressor protein whose overexpression has been frequently shown in cervical cancers and its precursors. Klaes et al. demonstrated that p16 identifies dysplastic cervical epithelia.[8] In another study, the same group reported that p16 immunostaining allows for a precise identification of small CIN or cervical cancer lesions.[13] It is also very well known that HPV undeniably plays a role in the development of most cervical cancers.[2, 4]

Using IHC analysis, we evaluated the expression of p16INK4a in samples of cervical biopsies from 78 patients with normal cervical epithelium, high-grade CIN, SCC or adenocarcinoma of the cervix, considering the percentage of p16-positive cells and the reaction intensity.

Our findings showed that the expression of p16 increases from normal to invasive squamous carcinoma in the uterine cervix emphasizing that it might be a useful marker for predicting risk of developing cervical cancer in women. Most of the cases of invasive cancer in this study expressed p16, while only one patient among those with high-grade CINs failed to express this marker. Notably, 90% of the patients with high-grade CIN expressed p16; 82% of the cases in this group had high frequency of positive cells, and 72% showed strong expression.

Among our histologically normal cases, four patients showed a weak/moderate positive reaction for p16. The detection of sporadic focal positivity of p16 staining in a small proportion of cases with normal squamous

mucosa had also been noted by other authors.[15-17]

Among p16-positive cases, we verified a direct relationship between lesion severity and reaction intensity. The frequency of positive cells and the reaction intensity were statistically significantly different when compared among different histological groups. Nevertheless, logistic regression model showed that the reaction intensity was superior to any other analyzed parameter, thus being the best indicator of the expression of p16. Taking into account the group pairs of histologically-diagnosed lesions and the evaluation of protein p16 expression by Dunn's and Holmes' tests, we might infer that there is a significant difference between the normal group and the others. Our findings pointed out that p16 can be useful not only in separating normal epithelium from high-grade lesions, but also in suggesting that low-grade lesions are at increased risk of progression to cancer due to possible genomic incorporation of oncogenic HPV.

In recent years, IHC has had a major impact in the field of uterine cervical pathology, as in other areas of diagnostic gynecological pathology. IHC should always be used as an adjunct to morphological examination and the results should not be interpreted in isolation. Of great interest for routine diagnostic use is the fact that IHC testing for p16 to identify positive cells has the potential to recognize those lesions with an increased risk of progression to high-grade lesions. It is likely that in the coming years IHC, along with molecular investigations, will play an increasingly important role in the field of diagnostic uterine cervical pathology. However, further studies of a prospective nature are needed to evaluate the clinical utility of p16 expression as a tumor marker in cervical carcinogenesis.

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