Cervical squamous intraepithelial lesions grades 1 and 2: value of an alternative morphological classification and of p16 immunohistochemistry in the prediction of clinical outcome

Lesões intraepiteliais escamosas cervicais graus 1 e 2: valor de uma classificação morfológica alternativa e do estudo imuno-histoquímico com p16 na predição do desfecho clínico

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ABSTRACT

Introduction: Diagnostic reproducibility and determination of prognostic factors in cervical intraepithelial neoplasias grades 1 and 2 are still relevant problems in the daily practice of gynecological histopathology. **Objective:** To correlate the value of morphological reclassification and of p16 immunoexpression in cervical intraepithelial neoplasias grades 1 and 2 with clinical outcome. **Materials and methods:** Sixty-six patients were included (34 with cervical intraepithelial neoplasia grade 1, and 32 with grade 2); an immunohistochemical study with p16 and reclassification according to the Lower Anogenital Squamous Terminology (LAST) Consensus and by the alternative proposal of Herfs and Crum were done; unfavorable outcome was defined as a subsequent histologic diagnosis of cervical intraepithelial neoplasia grade 3 or invasive squamous cell carcinoma. **Results:** We observed superior performance of the alternative morphological classification ($p = 0.002$) to determine unfavorable outcome. We also detected superior performance of p16 in the same determination ($p = 0.002$). **Conclusion:** The use of an alternative morphological classification is promising; in the context of the use of immunohistochemical antibodies as biomarkers, p16 showed good sensitivity and negative predictive value in the determination of cases in which the outcome was unfavorable.

Key words: cervical intraepithelial neoplasia; cyclin-dependent kinase inhibitor p16; outcome assessment (health care).

INTRODUCTION

Cervical cancer is a neoplasia with a high incidence worldwide, with 528,000 new cases/year, 85% of which occur in developing countries, being responsible for 266,000 deaths[1]. In Brazil, for the year 2016, 16,340 new cases were estimated, with a rate of 15.85/100,000 women[2].

Multiple nomenclature and criteria are still used for diagnostic interpretation of preinvasive squamous lesions in cervical biopsies, contributing to the great intraobserver and interobserver variability. The lowest indices of diagnostic agreement are observed in the

categories of cervical intraepithelial neoplasia grade 2 (CIN 2) and grade 1 (CIN 1). At a study using samples originally interpreted by pathologists from Costa Rica, two independent reviewing pathologists of the National Cancer Institute agreed with only 13% and 31% of the CIN 2 diagnoses, and only 24% and 20% of CIN 1 diagnoses. There was no agreement between them for any cases diagnosed as CIN 2[3]. Additionally, at a more recent North-American study, agreement of independent reviewing gynecologic pathologists with the initial diagnosis made by pathologists of the state of New Mexico was 38.2% for CIN 1 and 38% for CIN 2[4]. At a study using the diagnostic categories benign, low-grade squamous intraepithelial lesion (LSIL) (CIN 1), and high-grade squamous

intraepithelial lesion (HSIL) (including CIN 2 and CIN 3) in 56 analyzed histological slides by 22 pathologists of four continents, only 20% of the cases received a unanimous diagnosis from all pathologists; and 27%, diagnoses that ranged from benign to HSIL[5].

In the transforming infections by human papillomavirus (HPV) of high oncogenic risk, there is overexpression of p16, which can be detected by immunohistochemistry. This happens due to oncoprotein E7-mediated E2F displacement from its binding site on pRb. Increase in free E2F levels stimulates nuclear and cytoplasmic p16 overexpression[6].

The use of adjunct p16 immunohistochemistry increases inter- and intraobserver agreement in the evaluation of histopathological cervical specimens[6, 7], being recommended by the Lower Anogenital Squamous Terminology (LAST) Consensus – that officially introduced complementary methods to the anatomopathologic exam in the diagnostic flowchart of cervical tissue samples – in the following contexts: 1st) samples in which there is doubt between precancerous lesion and benign mimics; 2nd) cases in which the pathologist is considering (or wants to confirm) the diagnostic interpretation of CIN 2[8]; and 3rd) diagnostic disagreement between two professionals analyzing the specimen, and whose differential diagnosis includes precancerous lesion. The use of p16 in samples of non-neoplastic cervical tissue or presenting CIN 1 is contraindicated – except in situations of high risk for misdiagnosis of high-grade lesion (defined by previous colpocytologic exam as HSIL, atypical squamous cells – one cannot rule out a high grade lesion (ASC-H) –, atypical squamous cells of uncertain significance (ASC-US)/HPV-16+ or atypical glandular cells (AGC) or not otherwise specified (NOS) (4th context)[9].

The application of p16, despite its high negative predictive value (NPV)[10, 11], is considered limited in predicting evolution or stratifying the risk of patients with CIN 1, probably not altering management protocols currently recommended[12, 13]. By contrast, the use of p16 in samples with diagnosis of CIN 2 is associated with downgrading – that is from -IN 2 to LSIL, when p16 is negative – in up to around one third of the cases and, therefore, potentially reduces the frequency of unnecessary excisional procedures in patients with lesions of lower biological risk[14, 15]. However, it is important to remember that some cases of HSIL can exhibit negative or doubtful results for p16, that the marker is not also 100% specific, and interpretation is made difficult in small or scanty samples, tangential histological sections or unoriented tissue[16].

More recently, taking into account the limitations of morphological analysis and also immunohistochemical complementary exam with p16, another group of researchers proposed a pragmatic subdivision of histopathological diagnoses of cervical biopsies into LSIL, HSIL, and questionable grade squamous intraepithelial lesion (QSIL), suggesting that the patients with lesions classified as QSIL should initially undergo a more rigorous follow-up[17].

Considering the persisting problems in the histopathological diagnosis of CINs (especially CIN 2, a category of low diagnostic reproducibility, variable/uncertain biology, which can generate unnecessary interventions, costs and psychological stress), our study aimed to assess the performance of different systems of morphological classification and also of p16 immunohistochemistry in predicting an unfavorable outcome in cases of CIN 1 and CIN 2.

MATERIALS AND METHODS

Cases and clinical outcome

This is a retrospective longitudinal study, which used initial biopsies with histological diagnosis of CIN 1 or CIN 2 of patients treated at Núcleo de Prevenção de Doenças Ginecológicas (NUPREV) of Universidade Federal de São Paulo (UNIFESP) between January 2008 and March 2015. We evaluated sections obtained from paraffin blocks with tissue microarrays (TMA) – previously prepared with samples from NUPREV for use in studies about biomarkers in CINs – and also sections obtained from conventional paraffin blocks: using samples of TMA blocks previously constructed, 17 CIN 1 and 13 CIN 2 cases were included; using conventional paraffin blocks, 17 CIN 1 and 19 CIN 2 additional cases were included.

Cases were selected for this study based on suitability of samples (that is, presence of the area of interest in the sections for p16 immunohistochemical evaluation) and also on the presence of clinical follow-up with anatomopathological examination. The outcome was obtained through the active search of subsequent diagnoses for all tested cases in the files of the Department of Pathology of UNIFESP: "unfavorable clinical outcome" was defined by subsequent CIN 3 or more severe histological diagnosis. The following were excluded: patients with immunosuppression of any type or intensity; women who were pregnant or breastfeeding at diagnosis; patients clinically followed-up at another service and/or lost to follow-up in this service; patients with favorable clinical outcome, but with clinical follow-up shorter than six months; patients with clinical outcome possibly unfavorable, but based only on results of cytopathological tests with no histopathological confirmation in this Department.

TMA construction

The technique was performed as described by Kononen *et al*. (1998)[18], using a device of fixed base for withdrawal of 1-mm tissue cylinders. According to availability and size of material, one to three cylinders were obtained per paraffin block. From the new slides obtained after TMA confection, and when necessary, from the whole histological sections, we carried out a review of initial diagnosis by Richart's classification with division of cases into CIN 1 and CIN 2, according to classical criteria previously established[19], performed in consensus by two pathologists.

Morphological and immunohistochemical reclassification

According to sample availability, we conducted reclassification according to: a) alternative morphological evaluation as proposed by Herfs and Crum[17], distributing cases into LSIL, QSIL and HSIL; and b) reclassification of CIN 2 cases with the use of p16 immunohistochemistry (see the following criteria), distributing the cases into LSIL and HSIL.
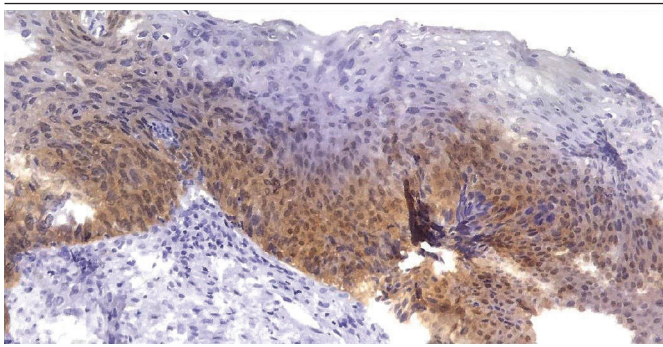

FIGURE – *Photomicrograph of p16 immunohistochemical staining in cervical squamous intraepithelial lesion, demonstrating minimum criteria for positivity determination according to recommendation of LAST Consensus: strong and diffuse block staining, continuous from the basal layer, nuclear (or nuclear and cytoplasmic), affecting at least the lower third of epithelium (and preferably reaching the middle third) (100×)*

LAST: Lower Anogenital Squamous Terminology.

Immunohistochemical reactions and interpretation

Slides went through special immunohistochemical stains and polarization, received histological sections of 4 micrometers, obtained through a standardized conventional microtome, and underwent deparaffinization in incubator – temperature set at 57ºC for 60 min. After that, the automated protocol was applied according to the manufacturer's guidelines – DAKO Autostainer 48 tm – with the steps described next: antigen retrieval in buffer solution PT Link (Dako), washing in buffer solution for 5 min and

application of primary antibody (p16[INK4a] clone G175-405, Zeta Corporation, in titers standardized by the service in 1/50); reaction amplification by the use of polymer EnVision Flex (Dako) for 20 min, washing in buffer solution for 5 min, color development of reaction in diamenobenzidine [(DAB); Sigma-Aldrich Chemical] Flex for 5 min and counterstaining with hematoxylin Flex for 10 min. Interpretation was made according to LAST[9] recommendations into: negative – absence of immunoexpression or focal and/or discontinuous immunoexpression; and positive – diffuse block-positivity (nuclear, with or without associated cytoplasmic positivity) affecting at least the lower third of the epithelium (Figure). The cervical sample with CIN 3 was used as positive external control. The typical endocervical columnar epithelium was used as external negative control; and plasmocytes, used as positive internal control.

Statistical analysis

The categorical variables were described as graphs and tables. The only discrete quantitative variable (age in completed years) was described using mean and standard deviation. If applicable, Fisher's exact test or its extension was conducted for evaluation of significance probability. Value of $p < 0.05$ was considered statistically significant.

RESULTS

Sample description

When the inclusion and exclusion criteria were applied, 66 patients were included, of which 34 presented initial diagnoses of CIN 1, and 32, of CIN 2, with mean age of 32 ± 9 years. After a minimum follow-up period, we observed nine (13.6%) cases in which there was subsequent histological diagnosis of lesion of higher histological severity (CIN 3 or more severe): two occurred in patients with biopsies initially classified as CIN 1; the other seven, in patients with biopsies initially classified as CIN 2 (**Table 1**).

We must highlight that seven of the nine patients with an unfavorable outcome had presented a concomitant cytology of HSIL; one, previous recent cytology of HSIL; and one, previous concomitant cytology of ASC-H. Besides, all later histopathology diagnoses of more severe histology [eight cases with CIN 3 and one case with squamous-cell carcinoma (SCC) stage IB1] occurred in the first six months of follow-up, indicating that the most severe lesions were probably already present, but were not sampled in the biopsies used in this study.

TABLE 1 – General data on the cases (n, age group, and outcome)*

	n	Age (mean ± SD)	Unfavorable outcome
CIN 1	34	32.46 ± 8.47	2
CIN 2	32	32.06 ± 8.82	7
Total	66	32.27 ± 8.58	9

CIN 1: cervical intraepithelial neoplasia grade 1; CIN 2: cervical intraepithelial neoplasia grade 2; SD: standard deviation.

*See text of "Results" (in the section "Sample description") for more details regarding cases that presented unfavorable outcome.

P16 immunohistochemistry

Positivity for p16 was observed in 26 (39.4%) of the cases – five (14.7%) of the 34 CIN 1 and 21 (65.6%) of the 32 CIN 2. Comparison of the marker frequency distribution in the alternative classification demonstrated increasing positivity indices in the three proposed tiers. In the cases initially classified as CIN 1, positive p16 was more frequent in the patients reclassified as QSIL than in those reclassified as LSIL ($p = 0.019$) (**Table 2**). The p16 marker alone – even independently of any classification – obtained statistical significance

in outcome prediction, besides expressive values of sensitivity and NPV for this determination (**Table 3**).

Classification according to LAST consensus, alternative morphological classification and correlation with outcome

After p16 immunohistochemistry, following recommendations of LAST Consensus, among 32 CIN 2 cases, 11 (34.4%) would have gone downgrading, that is, would be reclassified as LSIL (Table 2). Comparison of group outcomes according to Richart's classifications and after conduction of adjunct p16 immunohistochemistry in CIN 2 cases (according to recommendations of the LAST Consensus) did not show any statistically significant difference. Conversely, according to criteria of the alternative system proposed by Herfs and Crum, we would reclassify 33 (53.2%) of 62 cases as LSIL; 18 (29%) as QSIL and 11 (17.8%) as HSIL. The comparison among these three last subgroups regarding outcome demonstrated a statistically significant difference (**Table 4**).

TABLE 2 – General frequency of p16 immunoexpression in Richart's and the alternative classifications, and specific frequency of p16 immunoexpression (correlating both different classifications)

General frequency of p16 immunoexpression							
							Positive p16
Richart's classification (n = 66)							
CIN 1							5/34
CIN 2							21/32
Total							26/66
Alternative classification (n = 62)							
LSIL							4/33
QSIL							10/18
HSIL							9/11
Total							23/62

Specific frequency of p16 immunoexpression								
	LSIL		QSIL		HSIL		Total	p
CIN 1								
P16								
Negative	28	90.3%	-	-	-	-	28	84.8%	0.019ᵃ
Positive	3	9.7%	2	100%	-	-	5	15.2%
Total	31	100%	2	100%	-	-	33	100%
CIN 2								
P16								
Negative	1	50%	8	50%	2	18.2%	11	37.9%	0.195ᵃ
Positive	1	50%	8	50%	9	81.8%	18	62.1%
Total	2	100%	16	100%	11	100%	29	100%

CIN 1: cervical intraepithelial neoplasia grade 1; CIN 2: cervical intraepithelial neoplasia grade 2; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; QSIL: questionable grade squamous intraepithelial lesion.
ᵃ: Fisher's exact test or its extension.

TABLE 3 – Correlation between p16 immunoexpression and outcome

		Outcome								
		Favorable		Unfavorable		Total		p-value	S	Sp	PPV	NPV
	Negative	39	68.4%	1	11.1%	40	60.6%	0.002ᵃ	0.889	0.684	0.308	0.975
p16	Positive	18	31.6%	8	88.9%	26	39.4%
	Total	57	100%	9	100%	66	100%

S: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value.
ᵃ: Fisher's exact test.

TABLE 4 – Outcome of cases according to the different classifications

	Outcomes						
	Favorable		Unfavorable		Total		p-value
Richart's classification							
CIN 1	32	94.1%	2	5.9%	34	100%	0.079ᵃ
CIN 2	25	78.1%	7	21.9%	32	100%
Total	57	86.4%	9	13.6%	66	100%
LAST Consensus							
LSIL	41	93.2%	3	6.8%	44	100%	0.051ᵃ
HSIL	16	72.7%	6	27.3%	22	100%
Total	57	86.4%	9	13.6%	66	100%
Alternative classification							
LSIL	32	97%	1	3%	33	100%	0.002ᵃ
QSIL	15	83.3%	3	16.7%	18	100%
HSIL	6	54.5%	5	45.5%	11	100%
Total	53	85.5%	9	14.5%	62	100%

CIN 1: cervical intraepithelial neoplasia grade 1; CIN 2: cervical intraepithelial neoplasia grade 2; LAST: Lower Anogenital Squamous Terminology; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; QSIL: questionable grade squamous intraepithelial lesion.
ᵃ: Fisher's exact test or its extension.

DISCUSSION AND CONCLUSION

It is necessary to give a name to everything that is discovered in order to make communication and understanding easy. However, for this to happen, criteria that define that name must be widely known and also applied in a uniform and objective manner – a process that can take decades. Richart (1967)[19] formulated a nomenclature that still remains in use in gynecology and pathology clinical practice in many centers, even after it has been the subject of criticism from its own author[20], despite its limitations[3-5] and the publication of LAST[9], which introduced new terminology (revised) as gold standard for classification of non-invasive cervical squamous lesions. That is, CIN still plays an important role in everyday clinical communication due to its propagation and sedimentation in the scientific setting; many times the role and limitations – even more important – of biopsy as diagnostic test, endowed with sensitivity, specificity, predictive values, diagnostic reproducibility, among other characteristics, remaining in second plan. Once medical science always searches for better diagnostic tests, we consider the act of finding new forms of classifying familiar lesions necessary and healthy for progress.

In order to conduct this study, the fundamental classifications for comparison of any new proposal were included: the most widespread and the departure point of the study, Richart's original (three grades: CIN 1, CIN 2, and CIN 3), and the currently adopted by the World Health Organization (WHO) – that of the LAST Consensus (two grades: LSIL and HSIL). We also tried to evaluate an alternative system that permits a more practical approach: the model proposed by Herfs and Crum, of pure morphological character, without the stringency of the criteria proposed by Richart and admitting the existence of a subgroup of lesions in which one cannot determine the grade, the QSIL (besides LSIL and HSIL).

Reclassifications impacted only the group of cases more subject to diagnostic variability[3-5]. The cases belonging to group CIN 1, in most situations, remained in analogous categories or categories of equivalent meaning; the same cannot be affirmed for group CIN 2: a significant proportion of the cases would be reclassified in categories of potentially divergent management in the other two analyzed classification systems. For example, in our case analysis, only 65% of the CIN 2 would be classified as HSIL according to LAST[8, 9] recommendations, and 40% would be kept in the same category if we applied the alternative system proposed by Herfs and Crum[17].

The -IN terminology, regardless of site, is prone to subjectivity of criteria application by many diverse reasons, such as intra- and interobserver variability[3-5], limitations intrinsic to the material

received for analysis[21] – the latter with potential selection bias of this and several studies on the theme – and also the basic question of these diagnostic terms being applied to lesions with variable morphologic spectra – and possibly different clinical behaviours. The presented data confirm this already evidenced limitation of Richart's classification.

Although the present study has not demonstrated statistically significant difference between groups determined by LAST Consensus and outcome, we can point there is a non-confirmed tendency possibly because of lack of statistical power ($p = 0.051$).

The search for information able to determine the presence of a disease or risk of a disease to progress/regress has been the recent aim of numerous studies in the varied fields of medicine. We search what we could call ideal biomarker: cheap, reproducible, with high predictive values, high sensitivity and specificity[22]. In the present study, with the use of a biomarker in an attempt to predict outcome in cases of non-invasive squamous cervical lesions, we obtained results that contribute with scientific literature about the theme.

p16 is the most studied marker in the context of non-invasive HPV-related squamous lesions and the only currently officially indicated for routine use as an ancillary method in evaluation of these lesions[9]. The frequencies observed for p16 immunostaining agree with descriptions available in the scientific literature[9]. Data from medical literature widely demonstrate the value of dichotomic classification of non-invasive cervical squamous lesions attached to the routine application of p16[9].

This work corroborates the previously described information, demonstrating the power of p16 of eventually predicting in which cases there is higher risk of association with diagnosis of more severe, concomitant or future lesion, although with low specificity. It was also possible to note the high NPV of this marker, as also already demonstrated in the literature[10, 12].

Due to the small dimensions of residual samples in paraffin blocks after obtainment of sections for p16 immunohistochemistry and also to non-availability of iquid based cytology, it was not possible to perform additional methods such as, for instance, HPV genotyping. However, we must highlight that although the cases of this work did not present better detailing regarding HPV types, the authors believe this case study is the closest to everyday situation in which, frequently, there are no detailed clinical reports, HPV test results, or even previous or concomitant cytological slides for review.

The introduction of a terminology that includes the term "questionable" in one of its categories can meet great resistance from pathologists and gynecologists: QSIL does

not present any definitive criterion for the assisting physician to use it, just uncertainty. This allows such terminology to potentially become a subterfuge of technical quality and also a confounding factor for the gynecologist that will provide the definitive treatment. In spite of the above, in this study, the alternative system was the only that obtained statistical significance ($p = 0.002$), including the highest rate of cases classified as HSIL displaying unfavorable outcome (45.5%). Based on the results of this study, some considerations are relevant: the lines of the used alternative system have been just recently described and not well studied, requiring further studies with more cases to determine their actual applicability; the absence of rigid criteria that define QSIL, despite open to criticism, could on the other hand allow a more complete or comprehensive evaluation of the sample, which should include correlation with results of other inter-related exams[12]; the system is of simple application, and at an initial evaluation, without ethical obstacles for the proposition of its author that cases classified as QSIL be reassessed in six months, taking into consideration the potentially benign natural history of CIN 2[23].

RESUMO

*Introdução: A reproducibilidade diagnóstica e a determinação de fatores prognósticos em neoplasias intraepiteliais cervicais graus 1 e 2 ainda são problemas relevantes na prática diária da histopatologia ginecológica. **Objetivo:** Correlacionar o valor da reclassificação morfológica e da imunoexpressão do marcador p16 em neoplasias intraepiteliais cervicais graus 1 e 2 com o desfecho clínico. **Materiais e métodos:** Incluídas 66 pacientes (34 com neoplasia intraepitelial cervical grau 1 e 32 com grau 2); realizou-se estudo imuno-histoquímico com p16 e reclassificação segundo o Consenso Lower Anogenital Squamous Terminology (LAST) e a proposta alternativa de Herfs e Crum; desfecho desfavorável foi definido como diagnóstico histológico subsequente de neoplasia intraepitelial cervical grau 3 ou carcinoma de células escamosas invasivo. **Resultados:** Observamos performance superior da classificação morfológica alternativa (p = 0,002) para determinação de desfecho desfavorável. Também detectamos performance superior do marcador p16 na mesma determinação (p = 0,002). **Conclusão:** A utilização de uma classificação morfológica alternativa é promissora. No âmbito da utilização dos anticorpos imuno-histoquímicos como biomarcadores, o p16 apresentou boa sensibilidade e valor preditivo negativo na determinação dos casos em que o desfecho foi desfavorável.*

Unitermos: neoplasia intraepitelial cervical; inibidor p16 de quinases dependentes de ciclina; evolução clínica.

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