Expression of the Immunohistochemical Markers p16 and Ki-67 and Their Usefulness in the Diagnosis of Cervical Intraepithelial Neoplasms

Reprodutibilidade do diagnóstico das neoplasias intraepiteliais cervicais e a influência dos marcadores imuno-histoquímicos p16 e Ki-67 como ferramentas auxiliares

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Abstract

Objective   The aim of this study was to determine the expression of the immunohistochemical markers p16 and K_i-67 in cervical intraepithelial neoplasms and their influence on the level of agreement among different observers and for the same observer.

Methods   The study included 184 patients with cervical intraepithelial neoplasms previously confirmed through biopsies performed between 2005 and 2006. Three pathologists reviewed the biopsies by using hematoxylin-eosin staining to reach a consensus on the diagnosis. Subsequently, an immunohistochemical study analyzed the expression of p16 and K_i-67 in such cases.

Results   The comparison among the reviewing pathologists revealed only moderate agreement (kappa = 0.44). The agreement improved when the differentiation of high-grade lesions (cervical intraepithelial neoplasm – CIN – 3) was analyzed (kappa = 0.59). p16 staining exhibited a high negative predictive value and sensitivity; however, the specificity was low. Overall, both qualitative and quantitative analyses of p16 and a quantitative analysis K_i-67 exhibited low accuracy. The agreement among diagnoses before immunohistochemistry was 0.47. The use of immunohistochemistry increased the agreement to 0.68.

Keywords
► cervical intraepithelial neoplasia
► HPV
► p16
► K_i-67
► kappa

Conclusion   Our study showed that the agreement among observers using traditional diagnostic criteria of cervical intraepithelial lesions can improve with the use of immunohistochemistry.

Resumo

Objetivo   Observar a expressão dos marcadores imuno-histoquímicos p16 e K_i-67 em neoplasias intraepiteliais cervicais e sua influência na concordância entre observadores diferentes e, entre o mesmo observador.

Métodos   Foram incluídas no estudo 184 pacientes com neoplasias cervicais intraepiteliais confirmadas por biópsia realizadas durante os anos de 2005 e 2006. As biópsias foram revistas, primeiramente, por três patologistas utilizando-se apenas a coloração de Hematoxilina-Eosina. Foi realizado um consenso acerca do diagnóstico. Posteriormente, foi realizado o estudo imuno-histoquímico e analisada a expressão de p16 e K_i-67 nesses casos.

Resultados   A comparação entre os patologistas revisores mostrou uma concordância apenas regular (k = 0,44. A concordância foi melhor quando analisada apenas a capacidade de diferenciar lesões de alto grau (NIC 3) (k = 0,59). A marcação de p16 mostrou alto valor preditivo negativo e sensibilidade, porém baixa especificidade. Em geral, tanto p16 qualitativo, quanto p16 quantitativo e K_i-67 quantitativo mostraram baixa acurácia geral. A concordância entre os diagnósticos antes da imuno-histoquímica obteve k = 0,47, e após o auxílio da imuno-histoquímica houve um aumento do Kappa para 0,68. A marcação de p16 mostrou alto valor preditivo negativo e sensibilidade, porém baixa especificidade. Em geral, tanto p16 qualitativo, quanto p16 quantitativo e K_i-67 quantitativo mostraram baixa acurácia geral.

Conclusão   Nosso estudo mostrou que a concordância no diagnóstico tradicional de lesões intraepiteliais cervicais é regular e que pode ser melhorada como o auxílio da imuno-histoquímica.

Palavras -chave
► neoplasia intraepitelial cervical
► HPV
► p16
► K_i-67
► kappa

Introduction

Every year, there are ~500,000 new cases of uterine cervical cancers in women worldwide. In 2012, 265,000 deaths were reported. In Brazil, uterine cervical cancer is the third most frequent cancer among women (not considering non-melanoma skin cancer).[1] Approximately 80% of deaths could be prevented by screening for precursor lesions in women 25 to 65 years of age.[2]

Histopathological examination is the gold standard for a proper intraepithelial neoplasm diagnosis, and this technique is used to determine the best treatment for uterine cervical cancer patients. The reproducibility of the diagnosis is crucial. However, clinical studies have shown that the reproducibility of cervical biopsy interpretations is, at most, moderate.[3,4] Multiple factors not related to the human papilloma virus (HPV), such as atrophy, immature metaplasia, and reactive/inflammatory atypia can change cervical mucus. Indeed, these conditions can simulate cervical squamous intraepithelial neoplasms and cause discrepancies even among experienced pathologists.[5–7]

The literature suggests that the regular use of immunohistochemical markers, such as p16 and K_i-7, can improve diagnostic reproducibility.[7–12]

Thus, the present study investigated the relationship between the expression of the immunohistochemical markers p16 and K_i-67 and the grade of cervical intraepithelial neoplasms. In addition, we determined the usefulness of these markers as auxiliary pathologist tools to detect high-risk cases with an improved degree of agreement.

Methods

We retrospectively analyzed surgical uterine cervical samples obtained at the Pathological Anatomy Service of Santa Casa de Misericórdia de São Paulo, São Paulo, Brazil, between 2005 and 2006. These samples were previously diagnosed as positive for cervical intraepithelial neoplasia. The material used was derived from tissue removed by incisional and excisional biopsies (loop electrosurgical excision procedure (LEEP) and hysterectomies), fixed in 10% formalin and embedded in paraffin. Three pathologists reviewed the cases first independently and then jointly by using only hematoxylin-eosin staining. We used the independent diagnoses from each pathologist to analyze inter-observer agreement. The result of the joint analysis by the three pathologists was defined as the consensus diagnosis and considered to be the gold standard. Subsequently, samples stained with

hematoxylin-eosin and samples that underwent immuno-histochemistry were analyzed together.

The paraffin blocks were manually cut using a microtome, and sections were stained with hematoxylin-eosin. Histological sections were placed on silanized slides for immunohistochemistry with the markers p16 (p16 INK4a, clone G175–405, Zeta) and K_i-67 (clone MIB-1, Dako). Immunohistochemistry was performed using the streptavidin-biotin-peroxidase technique following the manufacturer's instructions and routine immunohistochemistry protocols of the Pathological Anatomy Service of Santa Casa de São Paulo.

We assessed p16 staining using two methods: qualitative and quantitative. The qualitative method was based on Lesnikova et al.[8] The sample was considered positive if at least 10% of the epithelial cells surrounding the lesion showed nuclear and/or cytoplasmic expression (starting from the basal/parabasal layer and variably extending to the intermediate and superficial layers). The sample was defined as negative if the p16 expression was less than 10%. The quantitative method was based on Nam et al.[13] Samples were defined as Grade 0 when less than 1% of the epithelial cells surrounding the lesion were positive for p16, Grade 1 when 1 to 5% of the epithelial cells were positive for p16, Grade 2 when 5 to 25% of the cells were positive for p16, and Grade 3 when over 25% of the cells surrounding the lesion were positive.

K_i-67 staining was evaluated quantitatively according to Nam et al.[13] The samples were defined as Grade 1 when less than 5% of the epithelial cell nuclei stained positive for K_i-67, Grade 2 when 5 to 30% of the epithelial cell nuclei stained positive for K_i-67, and Grade 3 when the nuclear positivity was greater than 30%.

Statistical Analysis

To determine the rates of agreement (original diagnosis, reviewing pathologists, and consensus), we used the Kappa test to examine the results of the same diagnostic test across different individuals and diagnoses provided by the same individual at different times. The data are described as a simple agreement rate (the percentage of diagnoses that were similar), which we evaluated using the kappa statistic and its confidence interval and p-value. The confidence interval was 95%, and p-values < 0.05 were considered statistically significant.

The consensus diagnosis was considered the gold standard for all analyses. The Kappa test requires symmetric contingency tables; thus, the cases diagnosed as metaplasia were excluded from the analysis between the original diagnosis and that of each reviewing pathologist or the consensus.

To evaluate the agreement among observers, comparisons between cervical intraepithelial neoplasia (CIN) groups included metaplasia versus CIN 1, CIN 2, or CIN 3 and CIN 3 versus the other diagnoses. There were no asymmetry problems with the contingency tables after grouping.

We assessed the value of immunohistochemical markers as independent diagnostic criteria by calculating the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of each marker to properly diagnose CIN 3 when compared with the consensus diagnosis.

The influence of the immunohistochemical markers on the rate of agreement between each pathologist and the consensus was calculated by directly comparing the kappa agreement rates. We then compared the diagnoses reported by the pathologist with and without the use of immunohistochemistry. The cases without immunohistochemical data or previous and consensus diagnoses were not included in the final analysis. The calculations were performed using SPSS 15.0 for Windows.

The Ethics Committee of the Hospital Santa Casa de Misericórdia de São Paulo approved this study.

Results

A total of 184 cases of cervical intraepithelial neoplasms were included in the study. According to the original diagnosis, 41 cases were CIN 1, 59 cases were CIN 2, and 84 cases were CIN 3. The age of patients ranged from 16 to 81 years, and the mean and median ages were 36 and 38 years, respectively. The age distribution of the group consisted of 17% of patients between 16 and 25 years of age, 47% of patients between 26 to 40 years of age, 28% of patients between 41 to 60 years of age, and 8% of patients over 60 years of age. The analyzed material was a biopsy in 61% of cases, an electrosurgical excision in 37% of cases, and a surgical specimen (cervix and uterus) in 2% of cases. Immunohistochemical reactions were performed in 153 cases. Total 31 cases were excluded from the analysis because the material was insufficient to complete the experiment. Because of these limitations, we only calculated the agreement rate before and after access to the immunohistochemical markers for Pathologist 1.

For Pathologist 1, 18% of the cases were CIN 1, 17% of the cases were CIN 2, 38% of the cases were CIN 3, and 27% of the cases were metaplasia. Pathologist 2 defined 11% of the cases as CIN 1, 28% of the cases as CIN 2, and 21% of the cases as CIN 3. Pathologist 2 classified 37% of the cases as metaplasia. Pathologist 3 characterized 15% of the cases as CIN 1, 24% of the cases as CIN 2, 35% of the cases as CIN 3, and 26% of the cases as metaplasia. The mean kappa agreement rate among the pathologists was 0.44 (0.36 - 0.51). This kappa value corresponds to moderate agreement. There was disagreement between the original and consensus diagnoses in 43% of cases and a fair kappa agreement rate of 0.33 (95% CI 0.18 - 0.48, $p < 0.01$).

The general rate of agreement was higher for the cases divided into metaplasia and CIN 1 versus CIN 2 and CIN 3. The agreement between the consensus diagnosis and the original diagnosis was 71% with a kappa of 0.41.

The agreement rate was good for the cases divided into CIN 3 and non-CIN 3 based on the consensus diagnosis. The kappa value for the agreement between the original diagnosis and the consensus diagnosis was 0.37 (95% CI 0.24 - 0.51, $p < 0.01$).

Results Based on Immunohistochemistry

A total of 54.2% of cases were positive for p16. Out of this 54.2%, 38 cases were identified as CIN 3 (►**Fig. 1**), 31 cases
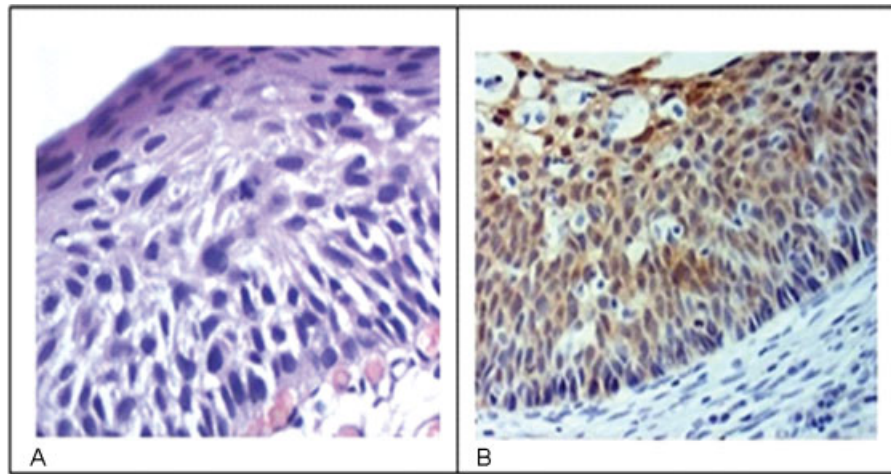
Fig. 1 A uterine cervix exhibited a cervical intraepithelial neoplasm (CIN 3) with hematoxylin-eosin staining (A) and showed diffuse staining with a p16 antibody (B).

were identified as CIN 2, 6 cases were identified as CIN 1, and 8 cases were characterized as metaplasia.

Only two cases of CIN 3 and 6 cases of CIN 2 were characterized by low K_i-67 expression (grade 1). No cases of CIN 1 and one case of metaplasia were characterized by high K_i-67 expression (Grade 3).

Overall, the immunohistochemical markers showed moderate accuracy as independent diagnostic tests for the diagnosis of CIN 3. The accuracy ranged from 70 to 83%. Notably, none of the markers achieved satisfactory rates of both sensitivity and specificity (►Table 1).

The agreement rate before and after immunohistochemistry was low. The pathologist diagnoses before and after immunohistochemistry remained the same in 56% of cases. The kappa agreement rate was 0.39 (95% CI 0.29 - 0.49, $p < 0.01$).

The agreement rate between the pathologist and the consensus increased from 61% (kappa = 0.47, 95% CI 0.38 - 0.56, $p < 0.01$) to 77% (kappa = 0.68, 95% CI 0.60 - 0.77, $p < 0.01$) with the aid of immunohistochemistry (►Tables 2 and 3).

Discussion

Our study confirmed that the reproducibility of traditional pathological examination is not satisfactory for determining the grade of cervical intraepithelial neoplasms. Both the agreement among three pathologists and the agreement between the original and consensus diagnoses ranged from moderate to fair (kappa = 0.44 and 0.33, respectively). The rate of agreement among each reviewing pathologist and the consensus was moderate (kappa = 0.60) and higher than other comparisons. This result may be explained by the use of the same pathologists for individual and consensus diagnoses.

The largest study of the reproducibility of cervical intraepithelial neoplasia diagnoses reviewed 6272 cases that were diagnosed by non-specialist pathologists.[14] Each case was reviewed by one of three teaching gynecopathologists. The agreement rate for that study (0.46) synthesizes the agreement among non-specialists and more experienced pathologists from reference centers in the absence of biases that could affect the results.

Our results confirm the literature data that showed reproducibility rates at the lower limit of moderate agreement.

Many studies show that the primary difficulty is the diagnostic reproducibility in intermediate cases; indeed, the agreement rates for CIN 2 cases are the lowest and negatively affect all statistical parameters.[3,15]

In our study, we found a slightly improved kappa value of 0.33 for the agreement between the original diagnosis and the consensus diagnosis when the cases were divided into two categories rather than into four. When we used CIN 2 as

Table 1 Potential immunohistochemical markers for a CIN 3 diagnosis

	Marker		
	Qualitative p16 (%)	Quantitative p16 (%)	Quantitative K_i-67 (67%)
Sensitivity	97.4	79.5	53.8
Specificity	60.5	84.4	87.7
Positive predictive value	45.8	63.3	60.0
Negative predictive value	98.6	92.3	84.7
Accuracy	70.0	83.0	79.0

The cases with a high degree of staining (grade 3) were used to calculate the quantitative p16 and K_i-67 data.

Table 2 The agreement between pathologists without the use of immunohistochemistry

		Consensus				Total
	Number of cases	CIN 1	CIN 2	CIN 3	Metaplasia	
Pathologist before immuno	CIN 1	12	3	0	17	32
	CIN 2	5	15	2	6	28
	CIN 3	2	20	40	2	64
	Metaplasia	5	3	1	37	46
Total		24	41	43	62	170

Abbreviation: CIN, cervical intraepithelial neoplasm; immuno, immunohistochemistry.
Kappa = 0.47 (95% CI 0.38 - 0.56), $p < 0.01$.

Table 3 The agreement between pathologists using immunohistochemistry for p16 and K_i-67

		Consensus				Total
	Number of cases	CIN 1	CIN 2	CIN 3	Metaplasia	
Pathologist after immuno	CIN 1	19	0	1	8	28
	CIN 2	1	21	3	3	28
	CIN 3	0	14	35	3	52
	Metaplasia	0	2	0	43	45
Total		20	37	39	57	153

Kappa = 0.68 (95% CI 0.60 - 0.77), $p < 0.01$.

the cut-off, the kappa value increased to 0.41, and when we used CIN 3 as the cut-off, the kappa value increased to 0.37. Similarly, the mean kappa value between the pathologist's and the consensus diagnoses was 0.60 for four categories. The kappa value increased to 0.78 with CIN 2 as the cut-off and 0.67 with CIN 3 as the cut-off.

The immunohistochemical analysis revealed an expected staining pattern. The staining percentage of each CIN grade was comparable with the values found in the literature. However, a simple comparison between values should be performed with caution because the criteria used to define positive cases vary greatly among studies. Some studies use the presence of any staining as a positivity criterion, even if the staining is focal and limited.[16] Other studies characterize a positive case by continuous staining throughout the epithelium.[17] Genovés et al[18] and Nishio et al[19] considered both moderate and diffuse staining as a positive marker. K_i-67 values vary greatly in the literature and exhibit the same methodological issues in the definition of positivity, which hinders the ability to directly compare values.

The analysis of immunohistochemical markers has a certain degree of subjectivity; thus, this method does not provide completely objective observations. The level of agreement among observers differs when a specific degree of p16 staining is defined as positive (positive is defined as a strong and diffuse staining in most studies). Galgano et al[5] reported an agreement rate of 0.87 among observers when defining strong and diffuse p16 staining as positive.

Our study shows that p16 and K_i-67 expressions in cervical intraepithelial neoplasms are more common in high-grade lesions. These immunohistochemical markers

do not exhibit adequate accuracy as independent diagnostic markers. However, the negative predictive value of p16 was a useful tool for the identification of cases that required more attention. The kappa agreement rate between the pathologist and the consensus increased from 0.47 to a strong agreement value of 0.68. Our study confirms that the level of reproducibility of the conventional diagnosis of cervical intraepithelial neoplasms is fair; however, the diagnosis can be improved with the use of immunohistochemistry.

Conflict of Interest
The authors declare no conflict of interest in conducting this study.

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