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Expression of P16 and Ki-67 in Cervical High-Grade Squamous Intraepithelial Lesion in Patients under 30 Years Old in South of Brazil

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Abstract

Background: Immunohistochemistry is a technique that evaluates the association of biomarkers with morphological changes, offering higher level of reproducibility, sensitivity, and specificity in the diagnosis of various neoplasms, with p16, associated with high-risk HPV and Ki-67 with cell multiplication.

Objective: To determine the expression of p16 and Ki67 in high-grade intraepithelial lesions in patients aged 30 years or younger and correlate with clinical variables and relapse or progression.

Methods: Retrospective cross- sectional study, in women diagnosed with HSIL, treated at the gynecology outpatient clinic of Hospital de Clínicas de Porto Alegre, from April 2004 to April 2016.

Results: We evaluated 68 patients. The median (md) age was 27 years old, and 47 (69.1%) of them were non-smokers. Only 21 (30.9%) of them had finished High School and 5 (7.4%) had Higher Education. Considering the histological characteristics, among the patients, 53 (77.9%) had the diagnosis of cervical intraepithelial neoplasia (CIN) 3, and 15 (22.1%), cervical intraepithelial neoplasia (CIN) 2. Almost all were positive for p16INK4a and Ki67 (97.1% and 98.5%, respectively). Only 1 (1.5%) had a recurrence of the lesion during follow-up. P16INK4a and Ki67 were positively related (Spearman, $\rho=0.702$, $p\leq0.001$).The analyses showed a high expression of these two biomarkers in the young population of the study but neither correlation between p16 and Ki67 with the variables of interest (age, parity, smoking and immunosuppression) nor with clinical outcome (recurrence or progression).

Conclusion: P16 and Ki67 biomarkers had a strong positivity in HSIL in women aged up to 30 years old, butwe could not find correlation considering the prognosis of precursor lesions.

Keywords: High-grade cervical intraepithelial lesion; Cervical cancer; HPV;Biomarkers; p16, Ki-67

Introduction

High grade Squamous Intraepithelial Lesions (HSIL) are associated with persistent infection by Human Papillomavirus (HPV) of oncogenic subtype (in 70%of the cases HPV 16 and 18) and are the precursor lesions of cervical cancer **[1,2].** Approximately 5% of CIN 2 and 15% of CIN 3 will progress to an invasive lesion **[3,4].** Treating CIN when necessary and in a timely manner, the cure rate is approximately 100% **[5].** Only by HPV vaccination programs, organized screening, and appropriate treatment of pre-invasive lesions can a positive impact on cervical cancer incidence and mortality be achieved **[6-9].** Cervical cancer is the fourth most common malignancy among women worldwide. The global incidence rate is 15.6/100,000 women, ranging from 7.2 in Australia to 51/100.00 in Mozambique **[7,10-13].** In Brazil, the estimative ofnew cases was 16,710, and 6,596 deaths in 2020 **[14].** Immunohistochemistry is a technique that evaluates the association of biomarkers with morphological changes, offering greater reproducibility, sensitivity, and specificity in the diagnosis **[15].** The p16 biomarker is used by many pathologists to make the diagnosis of HSIL more efficient, and its staining is overexpressed in infections caused by a high-risk of HPV. Ki-67 is expressed in all phases of the active cell cycle, except for the initial G0 and G1 **[16].** Its exacerbation is closely associated with the rate of cell multiplication **[17,18].** According to the criteria defined by the LAST project (The Lower Anogenital Squamous Terminology) of the American Society for Colposcopy and Cervical Pathology (ASCCP) and The College of American Pathologists (CAP), an HPV- associated lesion is to be reclassified from HSIL to LSIL if immunohistochemistryfor p16 is negative, favoring clinical follow-up and thus avoiding unnecessary surgeries, especially in young patients, whose follow-up might influence their reproductive future **[19-22].** The objective of this is study was to determine the rate of positivity of Ki67and P16 in patients up to 30 years old treated for HSIL in Hospital de Clínicas dePorto Alegre. Additionally, we tried to associate the expression of these markers to the prognosis of the patients.

Methods

It was a retrospective cross-sectional study, carried out with women with histological diagnosis of HSIL treated at the gynecology outpatient clinic of the Hospital de Clínicas de Porto Alegre, a tertiary university hospital in South of Brazil, from April 2004 to April 2016. Patients aged 30 years old or under with histological diagnosis of HSIL in a conization specimen were selected. The immunohistochemical reaction was performed on the Benchmark ULTRA® automation platform (Ventana Medical Systems, Tucson, Arizona): Antibodyp16 INK4a (G175-405) Zeta Corporation: As a sample, they were cut into a paraffin block on a microtome set to 3µm, and each slide additionally received a positive control. Dewaxing was performed on the instrument using theEZ PREP reagent. Antigen retrieval was performed with the conditioner CC1 (CellConditioning 1), pH 9.0, at 100ºC for 64 minutes. The primary anti p16 antibody, clone G175-405, from Zeta Corporation, was incubated for 40 minutes at 38°C. After the primer incubation, the reaction was detected by the OptiView DAB IHC Detection Kit detection system (multimers+ linker), using the chromogen Diaminobenzidine (DAB). The slides, then, with differentiated Mayer differentiation, with Mayer bluing reagents (LiCO3+ Na2) and examinations with the

slides after separation were assembled. Antibody: anti Ki67 (30-9) Roche: The samples, in paraffin block, were cut in a microtome set to 3µm, and each slide additionally received a positive control. Deparaffinization was performed onthe instrument using the EZ PREP reagent. Antigen retrieval was performed withCC1 buffer (Cell Conditioning 1), pH 9.0, at 95ºC for 64 minutes, followed by peroxidase blocking, with the Ultra View Universal DAB Inhibitor reagent (3% H2O2), present in the detection system. The primary anti Ki-67 antibody, clone (30-9), Roche brand, was incubated for 16 minutes at 37ºC. After the incubationof the primary antibody, the reaction was detected by the Ultra View Universal DAB detection kit (multimer) detection system, using the chromogen Diaminobenzidine (DAB), present in the kit. The slides were then counterstained with Mayer's hematoxylin, differentiated with the bluing reagent (Li2CO3+ Na2CO3) and examined after dehydration and mounting. All immunohistochemistry slides were reanalyzed by two pathologists from the Department of Pathology of Hospital de Clínicas de Porto Alegre. The study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre. Demographic and clinical data (age, diagnosis, education, parity, smoking, comorbidities, and immunosuppression) and follow-up for 12 and 24 months after treatment were collected from the hospital records. Patients with LSIL, atrophy, metaplasia and 3 patients with squamous cell carcinoma were excluded.

Data were input in the SPSS program, version 18.0 [SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.]. Descriptive analyses were expressed by measures of central tendency and dispersion: for quantitative variables by means ± standard errors of means (±SME) or by medians (md) and interquartile ranges (IQR, 25th and 75th percentiles), according to the Shapiro-Wilk normality test, for qualitative variables by absolute (n) and relative (%) frequencies. To identify possible associations between qualitative variables of interest, the Qui-squared test was used, with adjusted residual analyses. Besides, Spearman correlations were conducted between the variables of interest in the study. In all analyses, the significance level was set at 5%.

Results

We analyzed 110 patients. Of these, 21 (19.1%) of the histological slides were unavailable for analysis. In addition, another 21 (19.1%) were excluded because they did not meet all the inclusion criteria: 2, due to wear of the histological slides; 1, presence of artifact or pre-analytical defect of fixation; 1, atrophy; 1, low-grade lesion; 3, squamous cell carcinoma; and 13, metaplasia. Therefore, the final sample analyzed consisted of 68 patients **(Figure 1).**

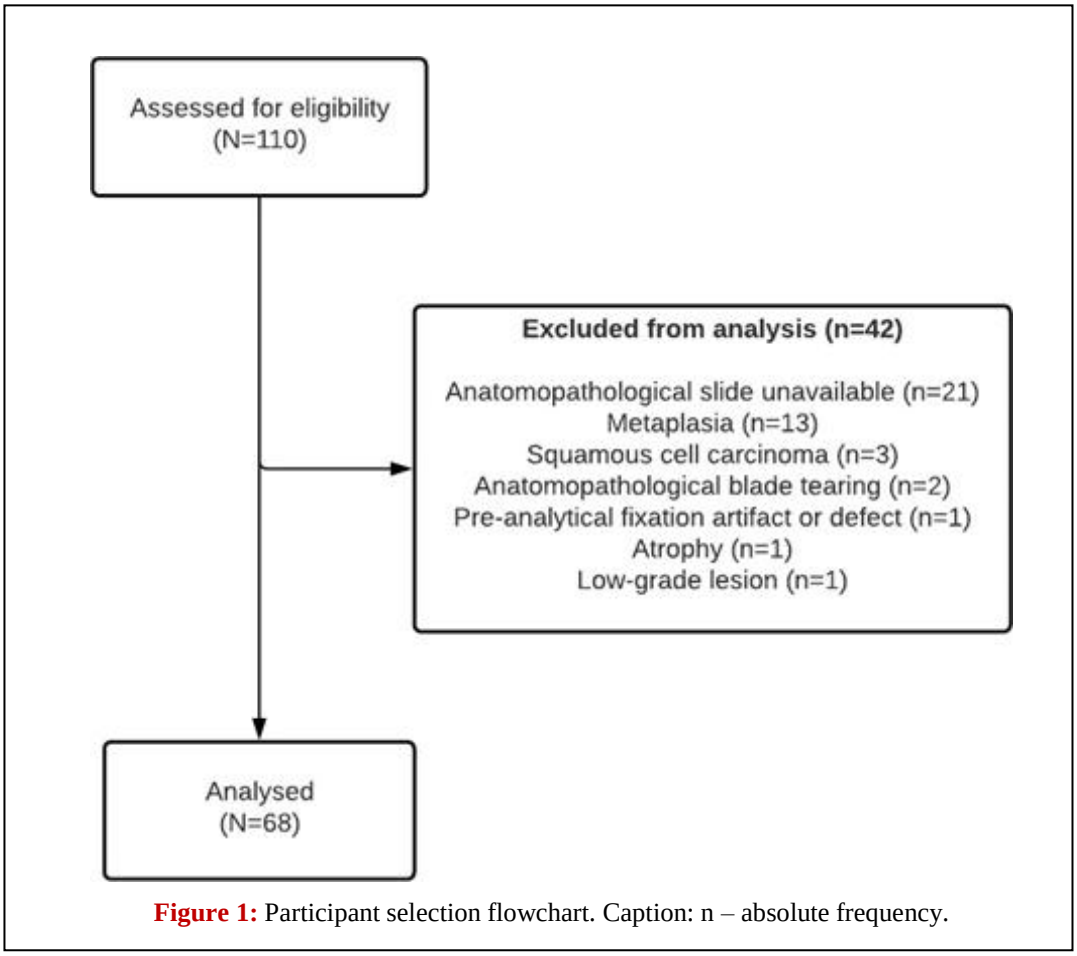

Figure 1: Participant selection flowchart. Caption: n – absolute frequency.

The median (IQR) age at diagnosis was 27.0 (18.0 – 29.0) years old, ranging from 18 to 30 years old. Most patients were self-declared non-smokers (69.1%), multiparous (35.3%) or primiparous (39.7%), with incomplete Elementary School (35.3%) or complete High School (30.9%) in relation to educational level. Considering the total, 17 (23.9%) women had some comorbidity, namely: 10 (14.7%), HIV/AIDS; 3 (4.4%), hepatitis C; 2 (2.9%), systemic arterial hypertension; 2 (2.9%), epilepsy; 1 (1.5%), diabetes mellitus; 1(1.5%), Crohn's disease. Of the total, 11 (16.2%) had immunosuppression, namely: 10 (14.7%), HIV/AIDS; and 1 (1.5%), Crohn's disease **(Table 1).**

Table 1: Sociodemographic characterization of the analyzed patients.

Variable	Total (N=68)
Age at diagnosis (in years) – md (IQR)	27.0 (24.0 – 29.0)
(Minimum – Maximum)	(18.0 – 30.0)
Schooling – n (%)	
Incomplete Elementary School	24 (35.3)
Complete Elementary School	9 (13.2)
Incomplete High School	9 (13.2)
Complete High School	21 (30.9)
High Education	5 (7.4)
Parity – n (%)	

Nulliparous	15 (22.1)
Primipara	27 (39.7)
Multipara	24 (35.3)
INO	2 (2.9)
Smoking – n (%)	
NO	47 (69.1)
Ye	19 (27.9)
INO	3 (2.9)
Comorbidities – n (%)	
No	49 (72.1)
HIV/AIDS	10 (14.7)
Hepatitis	3 (4.4)
C Systemic arterial hypertension	2 (2.9)
Diabetes *mellitus*	1 (1.5)
Epilepsy	2 (2.9)
Crohn's disease	1 (1.5)
INO	2 (2.9)
Immunosuppression– n (%)	
NO	55 (80.9)
Yes	11 (16.2)
INO	2 (2.9)

Legend: md – median. IQR – interquartile range (25th – 75th percentiles). n – absolute frequency. n% – relative frequency. INO – information not obtained. HIV – humanimmunodeficiency virus. AIDS – acquired immunodeficiency syndrome.

Regarding histological characteristics, 53 (77.9%) patients had cervical intraepithelial neoplasia CIN 3, and 15 (22.1%), CIN 2. Almost all had positive P16INK4a and Ki-67, that is, 66 (97.1%), and 67 (98.5%), respectively. Eventually, 65 (95.6%) were discharged from the outpatient clinic, and only 1 (1.5%) relapsed during follow-up. Three (4.4%) did not undergo follow-up after diagnosis **(Table 2).**

Table 2: Pathological changes and outcomes.

Variable	**Total (N=68)**
Anatomopathological – n (%)	
NIC II NIC III	15 (22.1)
	53 (77.9)
P16INK4a – n (%)	
Negative	2 (2.9)
Positive	66 (97.1)
Ki67 – n (%)	

Negative	1 (1.5)
Positive	67 (98.5)
Clinical outcome – n (%)	
Outpatient discharge	65 (95.6)
INO	3 (4.4)

Legend: n – absolute frequency. n% – relative frequency. CIN – cervical intraepithelial neoplasia. NSA – not applicable. INO – information not obtained. P16INK4a and Ki67 – immunohistochemical markers.

Patients with CIN 2 were discharged from the outpatient clinic after a follow-up of 12-24 months without recurrence. Only one of the patients with CIN 3 relapsed during follow-up. She had positive immunohistochemistry for both markers, and multiparity was the only risk factor she had. She received additional surgical treatment and, at follow-up, was discharged from the outpatient clinic. The analyses did not show correlations between any of the variables of interest (age, age under or equal to 30 years old, parity, smoking and immunosuppression) with the markers studied (P16INK4a and Ki-67 positive), and there was no correlation between recurrence or progression of disease (Spearman correlations, $p>0.05$ for all analyses) because almost all patients werepositive for the biomarkers and only one had recurrence of the disease in the follow up. Apart from that, P16INK4a and Ki-67 were positively related (Spearman, $\rho=0.702$, $p\leq0.001$) **(Table 3).**

Table 3: Correlations between variables of interest.

Ki67+		**P16+**		
Variable	**P**	**p-value**	**P**	**p-value**
Ki67+	1	-	0.702	≤0.001
P16+	0.702	≤0.001	1	-
Age	0.207	0.09	0.018	0.885
Age ≤25 years	0.165	0.178	0.054	0.664
Parity	0.178	0.153	0.178	0.153
Smoking	0.081	0.51	0.081	0.51
Immunosuppression	0.055	0.658	0.055	0.658

Legends: ρ –Spearman correlation coefficient. p – index of statistical significance. P16INK4a and Ki67 – immunohistochemical markers.

Discussion

Based on the results, 68 women with a median age of 27 years old were evaluated. It was observed that immunohistochemistry for the focused markers was positive in most of the pieces analyzed, as 66 (97.2%) were

positive for p16,and 67 (98.6%), for Ki-67. The staining for p16 was negative in only 2 (2.8%) CIN3, and among these, 1 (1.4%) was also negative for Ki-67. Regarding the number of patients with HSIL, we had a small sample when compared to a study carried out in 2016, in Western Australia, based on the Western Australian Data Linkage System. During a 10-year period, the incidence of HSIL was much higher: 2,692 patients aged 18 to 24 years old had histological diagnosis (CIN 2), but 59.5% had spontaneous resolution of the lesion in 24 months of clinical follow-up without the need for surgery. In our research, only 15patients were diagnosed with CIN 2 and all underwent surgery [4]. Unlike our results, a study carried out in India for the International Agency for Research on Cancer (IARC) from 2010 to 2015 selected patients between 30 and 60 years old, healthy and not pregnant. They underwent screening with hybrid capture (HR-HPV test) and inspection of the cervix with acetic acid; those who tested positive in both screening and inspection underwent colposcopy and biopsy as needed. Women diagnosed with CIN (CIN 1=128; CIN 2=68; CIN 3=52) were selected for the p16 and Ki-67 immunostaining. A small amount of immunohistochemistry was found positive for p16 and Ki-67 (24.4% for CIN 1) and a greater amount was found positive for CIN 2 (47.6%) and for NIC 3 (60.5%). They concluded that immunohistochemistry as an adjuvant technique can improve diagnostic accuracy in routine histopathology and concluded that dual staining can be used as an auxiliary tool to improve diagnostic accuracy [23]. Likewise, in the Certain study (In the Cervical Tissue Adjunctive analysis), 1100biopsies diagnosed with CIN2/3 divided into 275 cases by stratified randomization were evaluated. All HE slides were read by 17 to 18 surgical pathologists; and after carrying out the immunohistochemistry of p16 and rereading by the same specialists, there was an improvement in diagnostic agreement by 4.7%, driven by an increase in sensitivity and specificity, 11.5 and3% respectively, ensuring better treatment without treating more patients [24]. However, during a first phase of this study, where all immunohistochemistry tests were positive, Stoler criticized this system, which prioritized the costs of immunohistochemistry rather than considering the clinical importance for patients, what is best for them and what is needed for them. Such findings and conclusions are compatible with our analyses. In both studies mentioned above, immunohistochemistry improved diagnostic reproducibility, but we could not reach the same finding because immunohistochemistry was positive for most analyses, which has no statistical value for such a statement.

Only 1 patient relapsed during follow-up and, therefore, immunohistochemistry was not found to be a risk factor for relapse or progression. Unlike our analyses, a survey carried out in Italy in 2021 by Lukic and colleagues evaluated 62 patients under the age of 35 with histological diagnosis of CIN 2. In this study all 31patients (50%) had positive immunohistochemistry for P16 and the other half negative. After 2 years of treatment 19.4% had LSIL and 6.5% HSIL on cytology. In 5 years of follow-up: 9.7% of the patients progressed from CIN 2 to CIN 3. Of the patients who presented negative P16, none progressed. And after 2 years of treatment 3.2% had LSIL on cytology. They found a higher risk of persistence, recurrence and greater progression from CIN 2 to CIN 3, when p16 is positive [25]. It is possible that there are higher chances of recurrence or progression when immunohistochemistry is positive for both markers, so surgical treatment ismore appropriate than clinical follow-up, even in young patients. Our study was carried out with a small number of patients, all of whom had a histological diagnosis of CIN 2/3, or which may have been a delimiting fact for our analyses. Nonetheless, the extremely high positivity for P16 in our study, despite different results already published showed before, proves the strong association of HPV with HSIL in young patients.

Conclusion

Most patients had positive immunohistochemistry for both markers, and there was no correlation with clinical outcome. Although the staining was positive in the markers, the routine use of this technique is not justified, considering the additional cost generated to the Health System and low additional benefit in HSIL. This conclusion can dramatically decrease the costs, especially in countries like Brazil. We expected a greater number of negative immunohistochemical tests, as other articles present in this article, but this fact just reinforces the strong association of cervical lesions with HPV, especially in younger patients.

Conflict of Interest: We declare that we have no conflicts of interest.

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