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Prognostic role of immunohistochemical overexpression of the p16 protein in women under the age of 35 and diagnosed with HSIL (CIN2) subjected to "cervix sparing" excision

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Abstract. – OBJECTIVE: To evaluate the role of immunohistochemical staining overexpression of p16 protein (p16 IHC) as a prognostic factor of persistence or recurrence of intraepithelial disease after excision procedure in young women diagnosed with HSIL (CIN2).

PATIENTS AND METHODS: 62 women with a histological diagnosis of HSIL (CIN2) subjected to "cervix sparing" excisional procedure were included in this retrospective study. All had age less than or equal to 35 years, negative history of immunosuppression, available follow-up, and assessment of the resection margins state. Immunohistochemical staining for the p16 protein was evaluated on reviewed and confirmed HSIL (CIN2) histological specimens with negative resection margins. The post-treatment follow-up, including cytology, colposcopy, and histology, ranged from a minimum of 6 months to a maximum of 60 months. The persistence or recurrence of SIL during the follow-up period was based on histologic referral and defined as "the presence of SIL", "the presence of HSIL" and "progression to HSIL (CIN3)".

RESULTS: 31/62 patients were positive for immunostaining (p16 IHC+), and 31/62 were negative (p16 IHC-). Persistence or recurrence after excision occurred more frequently within the p16 IHC+ than in p16 IHC- group, both as SIL (29% p16 IHC- *vs.* 32.3% p16 IHC+, $p = 0.783$) and HSIL (6.5% p16 IHC- *vs.* 12.9% p16 IHC+, $p = 0.671$). None of the patients in the p16 IHC- group showed progression to CIN3 for the entire observation period, whereas 9.7% of p16 IHC+ women progressed to CIN3 lesion ($p = 0.042$). The p16 IHC positivity showed a significant association with progression to CIN3 in 5 years of follow-up ($p = 0.029$) and with the presence of SIL after two years of follow-up ($p = 0.031$). The differences between the two groups increased after two years post-treatment: the p16 IHC- patients still had SIL only in 3.2% of cases and no longer had HSIL, while the p16 IHC+ women still showed SIL in 19.4% and HSIL in 6.5% of cases. The negative predictive value (NPV) of p16 IHC in predicting SIL's presence after treatment increased with the severity of the lesion (NPV for SIL 70.97%, for HSIL 93.55%, for CIN3 100%).

CONCLUSIONS: The study suggests that young patients with p16 IHC- HSIL (CIN2) have a better post-excisional course of the cervical intraepithelial disease compared to p16 IHC+ women and that p16 IHC could have prognostic utility during the long-term follow-up, especially in forecasting progression to CIN3 in consideration of the high NPV (up to 100%). The efficacy of the adjuvant HPV vaccination in the management of HSIL (CIN2) p16+ young women is to be evaluated as part of the fertility-sparing treatment.

Key Words:
HSIL (CIN2), High grade cervical intraepithelial neoplasia, p16, Immunohistochemistry, Young patients, HR HPV, Prognosis, Fertility-sparing.

Introduction

The uterine cervix's carcinoma constitutes a significant health problem worldwide, representing one of the most common neoplasia. In women, it is the fourth malignancy by incidence and mortality after breast, colorectal, and lung cancer[1,2]. Morbidity and mortality have significantly decreased in recent decades, thanks to the introduction of screening programs that aim to identify precancerous lesions early, contrasting the

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onset of invasive forms[2-5]. It is well known that the HR HPV persistence represents the necessary but not sufficient cause of cervical cancer and that it must be associated with other epigenetic cofactors (cigarette smoking, prolonged use of oral contraceptives, immunosuppression, etc.) for development and progression of the cervical squamous intraepithelial lesion form low grade versus high grade and invasive neoplasia[6]. The current classification recognizes only two classes of precancerous lesions: low grade squamous intraepithelial lesion (LSIL), which includes grade 1 cervical intraepithelial neoplasia (CIN1), and high grade intraepithelial lesion (HSIL), which includes cervical intraepithelial neoplasms grade 2 and 3 (CIN2 and CIN3)[7,8]. These two categories require different clinical management, per all national and international guidelines[9-13], which unanimously recommend excisional treatment in patients with HSIL and management through long-term follow-up programs. The excisional procedure has both diagnostic and therapeutic purposes. It aims to remove the clinically visible and potentially progressive lesion and identify invasive occult, micro-invasive, or frankly invasive lesions, which are observed respectively in 6-12% and 2% of cases of HSIL[14,15]. Conization of the uterine cervix is an effective treatment. However, it may lead to adverse events in reproductive performance such as preterm birth, low birth weight, premature rupture of membranes, and second trimester abortion[16-23]. The risk increases significantly in patients undergoing large excisions and repeated treatments[24,25]. This is relevant in young women of childbearing age, in whom therapeutic planning should follow an approach as conservative as possible, aimed at maintaining fertility, as recommended by national and international guidelines[9-13].

Therefore, the HSIL (CIN2) entity's debate is still in progress, especially in young childbearing age patients. Indeed, these intermediate lesions are characterized by a high inter- and intra-observer variability and by intermediate regression and progression percentages concerning the upper and lower grade categories[26-31]. This is especially true in young women who have higher spontaneous regression rates, as in this age group CIN2 have a natural history much more similar to CIN1 than CIN3[29,32-35]. Accordingly, the guidelines recommend a better characterization of HSIL (CIN2) in young women to avoid as much as possible extensive and repeated excisional treatments. In this regard, the LAST project in 2012[8] proposed using the immunohistochemical staining of p16 (p16 IHC) in CIN2 to determine whether it belongs to the LSIL or HSIL category. This recommendation results from numerous studies that have shown the role of p16 overexpression as a diagnostic biomarker in HSIL (CIN2) intermediate-grade precancerous lesions, as it reduces interobserver variability and significantly increases diagnostic accuracy[36-47]. In particular, the p16 protein is a tumor suppressor belonging to the INK4a family, that inhibits cyclin-dependent protein kinases (CDK4, CDK6) and therefore the activation of the cell cycle through the maintenance of the pRb-E2F complex in the bound form. In the carcinogenesis of the cervical neoplasia, the expression of p16 protein is consequent to HPV infection, especially of high-risk genotypes, since the synthesis of the viral oncoprotein E7, by degrading the pRb-E2F complex, activates the progression of the cell cycle from the G1 to the S phase and consequently, with positive feedback, the synthesis of p16[48,49]. This is the reason why the overexpression of the p16 protein, besides being an indirect indicator of the persistence of HPV HR infection, also correlates very well with the degree of cervical dysplasia. Currently, the immunohistochemical staining of p16 (p16 IHC) has become a standardized diagnostic marker in clinical practice. A CIN2 p16 IHC negative lesions can be classified as LSIL and a CIN2 p16 IHC positive as HSIL.

This study aims to evaluate whether the overexpression of p16 IHC has a prognostic role after tailored excisional "cervix sparing" treatment in young women diagnosed with HSIL (CIN2). Indeed, expected results could represent useful information for the clinician, in the management of the long-term follow-up, and for the patient herself because of reproductive performance planning.

Patients And Methods

This retrospective study included patients referred to the Colposcopy and Pathology of the Lower Genital Tract outpatient clinic, Sant'Andrea University Hospital, Sapienza University of Rome, between November 2005 and November 2019. It was carried out in collaboration with the Unit of Histopathology and Pathological Anatomy of the same Institution.

The selection of patients to be included in the study was carried out considering a total of 396 cases of HSIL (CIN2) treated in our Unit within the same time interval. The conventional Papanicolaou's test was used for cytologic evaluation

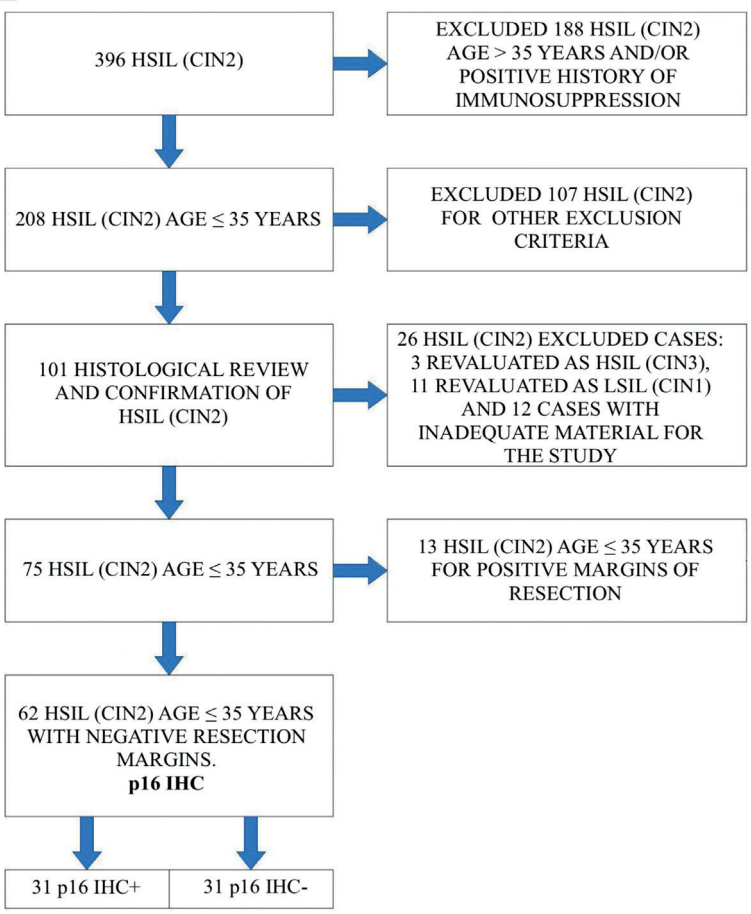

Figure 1. Flow-chart of patients' selection.

according to the "Bethesda System for reporting cervical cytology 2014"[50]. The colposcopic examinations were performed by experienced colposcopists and the abnormal colposcopic findings reported as abnormal transformation zone grade 1 or minor (ATZ G 1: thin aceto-white epithelium, fine mosaic, fine punctuation) or abnormal transformation zone grade 2 or major (ATZ G 2: dense aceto-white epithelium, coarse mosaic, coarse punctuation, cuffed gland openings), in accordance with the 2011 IFCPC (International Federation of Cervical Pathology and Colposcopy) nomenclature[51]. All patients presenting with an abnormal transformation zone underwent targeted biopsy under colposcopic guidance. Cases with histological diagnosis LSIL and HSIL (CIN3) were excluded from the series. In our outpatient clinic, the first choice of treatment of HSIL (CIN2) in young women, adequately informed and with signed written informed consent, is the "cervix sparing" excision of the lesions. In all study cases this procedure was carried out using a pure cut radiofrequency loop (LEEP) in an outpatient setting under colposcopic guidance by senior colposcopists (AL, AF). The aim was to optimize the cost-benefit ratio, minimize the removal of healthy cervical tissue and the resulting damage to the cervix, and thus prevent possible reproductive morbidity. The inclusion criteria were histological diagnosis of HSIL (CIN2), age ≤ 35 years, negative history of immunosuppression and previous SIL treatment, negative surgical resection margins, and available follow-up. Therefore, we excluded cases with a diagnosis of HSIL (CIN2-3) or HSIL (CIN3), age over 35 years, positive history of immunosuppression, previous SIL treatment, inadequate material for the study purposes, and positive margins of resection. First of all, 188 patients with age over 35 years and/or previous SIL treatments and/or positive history of immunosuppression or previous SIL treatment were excluded (Figure 1).

Of the remaining 208 young women, only those adherents to follow-up after excision were included, for a total of 101 selected cases, in which a histological review was carried out by coauthor

AV to confirm the diagnosis of CIN2, reduce inter-operator variability and verify that the tissue sample was adequate for p16 IHC immunohistochemical staining. A further 26 cases have been excluded: 3 revealed as HSIL CIN3, 11 as LSIL CIN1, and 12 with material considered inadequate for immunohistochemistry.

After the revaluation of the state of the resection margins on 75 confirmed HSIL (CIN2) cases, the other 13 cases (17.3%) were excluded because they had positive endocervical, ectocervical, or both margins for SIL. The final study group was, therefore, composed of 62 enrolled patients. Immunohistochemical staining for the p16 protein was carried out on 62 histological excisional specimens following the recommendations issued by the LAST 2012 project[8].

The characteristics of the patients included in the study are shown in Table I: the average age of 29.5 years (21-35y); cytological examination: LSIL/ASCUS in 35 (56.5%) cases and HSIL/ASCH in 27 (43.5%) cases; abnormal colposcopic findings: ATZ G1 in 26 (41.9%) cases and ATZ G2 in 36 (58.1%) cases. The HPV DNA test (HC2) in the pretreatment phase was available using HC2 in 36 cases; of these, HR HPV was positive in 35 and LR HPV in 1 case while it was not available or not obtained by the standardized test in the remaining 26 cases related to the older series.

Follow-up was performed in all patients with cytology, colposcopy and, when indicated, biopsy sampling. HR HPV follow-up testing was not performed in all cases and in all control visits by different physicians.

The first follow-up visit was performed no earlier than six months after LEEP procedure and subsequently at 6-month intervals for a maximum period of 5 years and a median of 30.8 months (min-max 6-60 months, DS 18.3). Retrospectively we considered the following intervals of clinical observation: T0 (time of diagnosis of HSIL CIN2), T1 (from 6 to 12 months), T2 (from 13 to 24 months), T3 (from 25 to 36 months), T4 (from 37 to 48 months), T5 (49 to 60 months). In addition to these intervals, two observation intervals have been added [T<2 (from 6 to 24 months) and T>2 (from 25 to 60 months)], which refer to the relative follow-up periods considering them in a single time span.

For the assessment of the clinical course of the disease in the various follow-up intervals, the following defined criteria based on the histological examination were considered: "presence of SIL" corresponding to LSIL or HSIL, "presence of HSIL" corresponding to HSIL (CIN2+) and "progression to CIN3" intended as the presence of a lesion of a greater degree than that of initial diagnosis. All definitions have been used, including both disease persistence and recurrence. An occult microinvasive or invasive squamous or glandular lesion was never diagnosed.

Immunohistochemical Technique

For the immunohistochemical staining of p16, the indirect enzyme immunoassay technique was used through the primary monoclonal antibody of the murine type P16INK4a dilution 1:100 (UCS Diagnostic, Rome, Italy, and EnVisionTM FLEX + MOUSE) as per the supplier's instructions.

It was started by providing the histological sample's antigenic deparaffinization and unmasking, which was subsequently treated with hydrogen peroxide. After a washing phase with PBS-T buffer solution, the primary murine type monoclonal antibody (p16INK4a dilution 1:100 (UCS Diagnostic Srl, Rome, Italy) was added in quantities varying according to the size of the lesion. The process was incubated at room temperature for 60 minutes. At the end of the incubation period, a further washing in PBS-T was carried out. The enhancement (EnVisionTM FLEX + MOUSE) was subsequently added for the amplification of the signal and then the secondary antibody. At this point, after a new washing step in a buffer solution, the chromogenic substrate DAB (Diaminobenzidine) was added. Finally, a counter-coloration with Hematoxylin was carried out. The procedure ended with the dehydration and preparation of the slide.

The positivity index for p16 was expressed on the basis of a binary system made up of "positive" or "negative" assessments, in accordance with the guidelines issued by WHO[7] and by the LAST project[8]. A "positive" evaluation was then assigned to the samples that showed continuous staining of cells in the basal and parabasal layer of the epithelium, extended to more than a third of the entire epithelial thickness, with or without staining of surface layer cells ("diffuse staining method") and exclusively intense nuclear or nuclear and cytoplasmic localization. Height limitation is recommended as it adds specificity. A "negative" evaluation was assigned to the samples showing the absence of staining in the epithelium ("negative staining method"), staining of isolated cells or small, slender cell clusters with discontinuity characteristics, an extension less than one-third of the thickness epithelial ("focal staining

Table I. Characteristics of patients and homogeneity test of groups.

		Total	p16 IHC-	p16 IHC+	Homogeneity test
Age	Mean (SD)	29.5 (3.86)	29.9 (4.15)	29 (3.57)	0.362
	Min-Max	21-35	21-35	23-35	
Cytology	LSIL/ASCUS	35 (56.5%)	21 (67.7%)	14 (45.2%)	0.075
	HSIL/ASCH	27 (43.5%)	10 (32.3%)	17 (54.8%)	
Colposcopy	ATZ G 1	26 (41.9%)	16 (51.6%)	10 (32.3%)	0.127
	ATZ G 2	36 (58.1%)	15 (48.4%)	21 (67.7%)	
Total	n.	62	31	31	

method") and staining patterns described as cytoplasmic only.

Informed Consent

Data were obtained from the electronic and written medical records. This retrospective study was performed in accordance with the Declaration of Helsinki and approved by the Internal Institutional Ethics Board. All patients gave written informed consent for research purposes.

Statistical Analysis

The sample was analyzed using descriptive statistics, using mean median and standard deviation for the quantitative variables, absolute frequencies, and percentages for the categorical ones. The results have been summarized in figures and tables. The groups' homogeneity was assessed by *t*-test for the difference between means or proportions, the association between p16 IHC, and the presence of SIL by Chi-square test and Fisher Exact test for tables with sizes <5. The percentage of progression-free patients in the two groups was estimated with the Kaplan-Meier curves compared using the Log-rank test. The histological examination was considered the gold standard to calculate the accuracy parameters of p16 IHC (sensitivity, specificity, positive predictive value, and negative predictive value). The p <0.05 was considered statistically significant. The analyzes were conducted with the STATA software version 13 (StataCorp Release 13. College Station, TX, USA).

Results

The cervical tissue samples belonging to the 62 selected young patients with confirmed histological diagnosis of HSIL (CIN2) and negative resection margins obtained after LEEP excision underwent immunohistochemical staining for the p16 IHC protein. According to the positivity or negativity of the p16 IHC, this group was further divided into two subgroups (Table I). Both subgroups were made up of 31 patients. Clinical parameters were examined, and the *t*-test confirmed that the two subgroups were homogeneous for each of the variables tested: age (p-value = 0.362), cytology (p-value = 0.075) and colposcopy (p-value = 0.127). In the p16 IHC negative subgroup, the initial cytological examination was, in most cases, LSIL/ASCUS (21/31, 67.7%) and less frequently HSIL/ASCH (10/31, 32.3%). Regarding colposcopy, unlike the similar distribution between abnormal colposcopic findings ATZ G1 and ATZ G2 in the p16 IHC negative patients, the p16 IHC positive presented mostly ATZ G2 patterns (21/31, 67.7%).

Table II shows the course of cervical intraepithelial disease in the entire series of patients subjected to follow-up after LEEP. Comparing the two subgroups relating to p16 expression, HSIL was mainly found in the positive p16 IHC subgroup (66.7%), which also represented all the cases of HSIL (CIN3).

We observed a significant correlation between p16 IHC and the presence of disease after excision only for SIL and only after the two years of follow-up (Table III). Indeed, during the first 24 months, the two subgroups did not differ in clinical course, with a percentage of SIL in p16 positive only slightly higher than that found in p16 negative (32.3% *vs.* 29%, p=0.783). Against a different trend was observed at T>2 period where the percentage of SIL detected in positive p16 IHC subjects was much more significant (about six times) than that in negative p16 (19.4% *vs.* 3.2%, p = 0.031).

The rate of HSIL in the first two years of follow-up in the p16 IHC negative persons was about half the positive (6,5% *vs.* 12,9%). This difference further increased after two years of follow-up, so that during the 25 to 60 months period, none of the patients in the p16 IHC negative subgroup

Table II. Follow-up after excision in the entire series.

	Total n (%)		p16- n (%)	p16+ n (%)
Total SIL	19 (30.6)	p16- 9/19 (47.4) p16+ 10/19 (52.6)	9/31 (29)	10/31 (32.3)
LSIL	13 (21)	p16- 7/13 (53.8) p16+ 6/13 (46.2)	7/31 (22.6)	6/31 (19.4)
HSIL (CIN2+3)	6 (9.7)	p16- 2/6 (33.3) p16+ 4/6 (66.7)	2/31 (6.5)	4/31 (12.9)
HSIL (CIN3)	3 (4.8)	p16- 0/3 (0) p16+ 3/3 (100)	0/31 (0)	3/31 (9.7)
Total	19/62		9/31	10/31

showed high-grade lesions anymore, as opposed to the positive ones that instead continued to show HSIL in 6.5% of cases (Table III).

A different clinical course during the follow-up over 24 months of women who recurred within the first two years is shown in Table IV. During the T<2 follow-up, 19 patients recurred, 13 as LSIL and six as HSIL. Of them, nine were p16 IHC negative and ten p16 IHC positive. In the p16 IHC negative subgroup, seven recurred as LSIL at T<2, of which only one still revealed LSIL at T>2 (25-60 m) while two recurred as HSIL (CIN2) at T<2 and subjected to further excision than resulted negative to subsequent follow-up. At T>2, only one p16 negative case had low grade cervical intraepithelial neoplasia.

On the contrary, among the p16 IHC positive women, 6/10 recurred as LSIL at T<2, and 4 of them still showed LSIL at T>2. The remaining 4/10 that recurred as HSIL at T<2, were retreated with excision and subjected to follow-up, during which two relapsed as CIN3 at T>2. It is worth noting that the majority (60%) of those positive for p16 IHC continued to present the lesion even in the period after two years, contrary to what was observed in negative ones who continued to manifest LSIL only in a significant minority of cases ($p = 0.027$).

When we considered in detail the progression of the intraepithelial neoplasia from CIN2 to CIN3 after excision, it was found that none of the p16 IHC negative patients ever progressed to CIN3 in the whole time observed, contrary to 9.7% positive CIN2 that progressed (Table II). Accordingly, the Kaplan-Meier curves for disease-free interval (Figure 2) showed significant differences between the two subgroups (p-value = 0.042 of the Log-Rank test).

These data were further selectively processed in 17 patients with adherence to follow-up up to T5, 11 p16 IHC negative, and six positives. The association between p16 IHC positivity and pro-

Table III. Correlation between the presence of disease at different T of follow-up and p16 IHC.

	SIL				HSIL			
	T<2 (6-24 months)		T>2 (25-60 months)		T<2 (6-24 months)		T>2 (25-60 months)	
	p16- n/tot (%)	p16+ n/tot (%)	p16- n/tot (%)	p16+ n/tot (%)	p16- n/tot (%)	p16+ n/tot (%)	p16- n/tot (%)	p16+ n/tot (%)
SIL present	9/31 (29)	10/31 (32.3)	1/31 (3.2)	6/31 (19.4)	2/31 (6.5)	4/31 (12.9)	0/31 (0)	2/31 (6.5)
absent	22/31 (71)	21/31 (67.7)	20/31 (64.6)	11/31 (35.5)	29/31 (93.5)	27/31 (87.1)	21/31 (67.8)	15/31 (48.4)
missing	0/31 (0)	0/31 (0)	10/31 (32,2)	14/31 (45.1)	0/31 (0)	0/31 (0)	10/31 (32,2)	14/31 (45.1)
p-value	0.783		0.031		0.671		0.112	

Table IV. Correlation between the presence of SIL after 2 years of follow-up and p16 IHC after recurrence within the first 24 months.

	Presence of SIL at T>2	
	yes	no
p16- IHC	1/9 (11%)	8/9 (89%)
p16+ IHC	6/10 (60%)	4/10 (40%)
	p-value = 0.027	

Table V. Correlation between p16 IHC and progression to CIN3 in patients with full follow-up adherence up to T5 (48-60 months).

p16 IHC	p16-	p16+	*p*-value
Progression to CIN3			
yes	0/11	3/6	0.029
no	11/11	3/6	

gression to CIN3 in 5 years of follow-up was statistically significant (*p*-value = 0.029) (Table V).

To complete our analysis, the accuracy values of the p16 IHC in predicting the presence of intraepithelial disease intended as LSIL, HSIL, and the progression to CIN3 after LEEP excision, were measured on the entire series in different time windows (Table VI). This evaluation showed that the p16 IHC has a higher negative predictive value (NPV) in forecasting the presence of lesions after treatment the greater the lesion's degree: NPV of 70.97% and 93.55% respectively in the predicting of SIL and HSIL. In both cases, the NPV significantly increased in predicting recurrences that occur after two years, so that at T>2, it was 95.24% and 100%, respectively.

Finally, the assessment of p16 IHC as a prognostic factor in predicting the progression of HSIL (CIN2) to HSIL (CIN3) deserved a separate discussion, which in our case study was 100% at all times of follow-up and associated with 100% sensitivity. Protein specificity and positive predictive value (PPV) showed less relevant percentages in all cases and at any time of observation.

Discussion

The efficacy of p16 IHC as a diagnostic biomarker in HSIL intermediate-grade precancerous lesions of the cervix (CIN2) has been widely demonstrated. It reduces interobserver variability and significantly increases diagnostic accuracy[36-47] to be included in the recommendations of the LAST 2012 project[8]. Contrary to this protein's diagnostic role, its efficacy as a prognostic biomarker in HSIL (CIN2) is currently not assessed due to a limited number of studies and the conflicting outcomes of the same, both as regards natural history[52-55], and prediction of recurrence after excisional treatment[56-60].

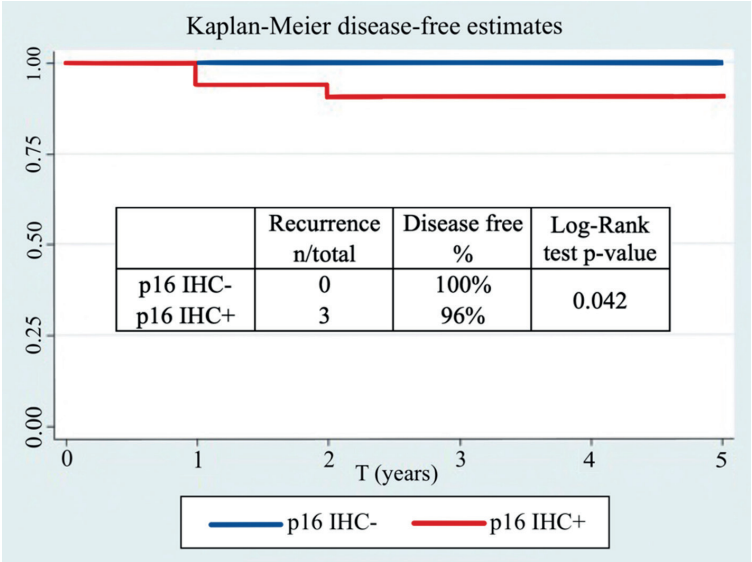

Figure 2. CIN2 progression to CIN3, 5-year progression estimates and Log-Rank test according to p16 IHC.

Table VI. Accuracy of p16 IHC in predicting the presence of intraepithelial disease after excision, both in the entire follow-up period and after 24-60 months of follow-up (T> 2).

	Follow-up T<2 + T>2			Follow-up T>2		
	SIL	HSIL	CIN3	SIL	HSIL	CIN3
Sensitivity	52.63%	66.67%	100.00%	85.75%	100.00%	100.00%
Specificity	51.16%	51.79%	52.54%	64.52%	58.33%	58.33%
PPV	31.26%	12.90%	9.18%	35.29%	11.76%	11.76%
NPV	**70.97%**	**93.55%**	**100.00%**	**95.24%**	**100.00%**	**100.00%**

Unlike other studies that have included patients of all ages and diagnosed with HSIL enclosed CIN3, we have evaluated the prognostic role of p16 IHC selectively in young women aged 35 years or less and diagnosed with HSIL (only CIN2). This difference is crucial if we consider that the natural history and the recurrence of disease after excisional treatment, are strongly influenced by factors such as the age of the patients, the degree of intraepithelial neoplasia, the state of the resection margins, and the HR HPV persistence. Indeed, CIN2 has a higher regression rate than CIN3 (43% *vs.* 32%)[26], especially in the category of young women where it reaches regression in 60% of cases[32,33]. Also, to minimize the influence of one of the most significant prognostic factors of persistence and recurrence of disease after treatment of HSIL, i.e., "the state of the margins of excision"[60,61], we excluded from the study all patients with positive endocervical and/or exocervical or both excision margins, equal to 17.3% of all group. This percentage is within the range established by the European Federation for Colposcopy as an indicator of good clinical practice in excisional treatments, defined as the positivity of the excision margins less than 20%[62].

According to the literature data, the overall percentages of persistence and/or recurrence of disease in the first 24 months decreased overtime during the follow-up[9,10,60,61,63,64].

Specifically, our data suggest that p16 IHC is significantly associated with the presence of SIL after two years of follow-up (p-value = 0.031). This data is in accordance with Fonseca et al[56], who in 2016 assessed the role of p16 IHC in predicting disease recurrence in the 18 months following conization in patients of different ages (16 to 86 years) diagnosed with HSIL (both CIN2 and CIN3). Despite differences in the inclusion parameter, this study could be compared with our data for the same reference period. In detail, at time T<2, the results obtained in both studies do not show a significant correlation between the expression of the p16 protein and the presence of disease. Also, in accordance with our findings, Fonseca et al[56] also observe a similar rate of SIL recurrence (36.14%) and prognostic accuracy values of the protein: NPV (72%), sensitivity (66%), PPV (36%), and specificity (49%), reaching percentages that do not differ much from ours (SIL recurrence 30.6%, NPV 70.97%, sensitivity 52.63%, PPV 31.6%, specificity 51.16%).

On the other hand, it can be inferred that in our study, the protein is unable to predict the recurrence of HSIL alone after excision either before or after two years, probably related to the reduced sample size. This is in accordance with data published by Charoonwatana et al[58] in 2019. The authors observed the risk of recurrence of HSIL for 48 months in patients of all ages with previous HSIL diagnosis (CIN2 and CIN3) correlating it with p16 IHC. However, they found no statistically significant association between the two variables. Still, they stated the recurrence rates of HSIL twice as high in the p16 IHC positive (17.9%) compared to the negative (8.3%) cases with percentages similar to those observed by us (12.9% *vs.* 6.5%). A further agreement between the two studies was that new HSIL in p16 IHC negative cases occurred only during the first 24 months of follow-up and not appeared any more after two years. Our findings (9.5%) and that of Charoonwatana et al[58] (13.1%) are in accordance with the rate of recurrence and/or persistence of CIN2 after treatment reported in the literature (4-18%)[65]. Moreover, in agreement with literature data, most of the relapses occurred in the first two years post-treatment of our patients[66,67].

Neither Charoonwatana et al[58] nor other authors delved into the accuracy values of the protein in predicting the presence of high-grade lesions after excision, which instead are relevant from our analysis, showing that the p16 IHC has a high

NPV (93.55%) which reaches 100% in predicting the long-term HSIL disease-free follow-up.

Another aspect revealed by our study was related to the progression to CIN3 after excisional treatment in young women diagnosed with HSIL (CIN2). Patients with negative p16 IHC observed for five years in our study population never, in no case and no follow-up period, progressed to CIN3+, demonstrating a statistically significant difference compared to the positive immunostaining ones (p-value = 0.042). This data was further confirmed by the statistically significant correlation between the p16 IHC and the progression to CIN3 in the subgroup of patients with complete adherence to 5-year follow-up (p-value = 0.029) and by predictive accuracy of the protein that reaches 100% values both in NPV and in sensitivity at all times of follow-up.

Different studies that considered the role of p16 IHC in the natural history of CIN2 have reported that the patients' p16 IHC positive CIN2 progress to CIN3 about ten times more than p16 IHC negative. In detail, according to Miralpeiz et al[52], the p16 IHC positive women progress in 10% of cases compared to 0% of the p16 IHC negative while Maniar et al[46] reported 25.8% *vs.* 2.4% of progression, respectively.

Recently Umphress et al[59] described the ability of p16 IHC to predict the progression to CIN3 in 117 patients diagnosed with HSIL (CIN2). By comparing the rate of progression to CIN3 observed in the p16 IHC positive subgroup *vs.* the negative one, those with p16 positivity seemed to progress with about ten times higher rates than p16 negative, even in our (9.7% *vs.* 0%) and in Umphress et al[59] study (26.9% *vs.* 2.9%). However, by comparing the rates of progression of our research (4.8% in the whole series) with Umphress et al[59] (19.5% in total), their higher rate of progression could be explained by the various inclusion criteria considered. Umphress et al[59] included women of any age (20 to 78 years), of which a small number, although not specified, has not been subjected to excisional treatment. Moreover, the state of the excision margins of the treated patients was not specified. Therefore, both the older age, the failure to excise the lesion in some patients, and the state of the margins not considered would make the different progression rate consistent since they represent critical prognostic factors for recurrence and/or persistence of disease after excisional treatment[60,61,63,66].

Even if our results appear relevant, the limitations of our research are mainly related to the reduced sample size and its retrospective nature; for this reason, we have not included in this longitudinal observation other variables notoriously crucial in the natural history of HPV disease like smoking, HPV vaccination, long term use of contraceptive pill and HR HPV DNA persistence as not available for all patients, for all follow-up periods and with the same diagnostic tool. Remarkably, the correlation of the p16 IHC expression with HR-HPV persistence, a known prognostic factor for recurrence and/or persistence of disease after excision[67,68], could have given greater credibility to the prognostic value of the oncoprotein. Another limit is related to the lack of dosage of Ki67, a proliferation marker increased in HPV-infected mature squamous epithelia, which could have confirmed already known or revealed new correlations with the other biological parameters. Finally, immunohistochemistry is the technique with some limits since a negativity p16 IHC on the cut of the biological material examined does not exclude that the patient may present a positivity for the protein in the cervical tissue adjacent or distant from the observed section. This aspect must always be considered when using the high negative predictive value of the p16 IHC in clinical management.

Recent recommendations emphasize that observational management for HSIL (CIN2) in very young women under 25 years only by colposcopy and cytology has limited ability to exclude persistent high-grade disease[69] and that biopsy should be performed at 12 months even in very young patients. In the perspective of fertility-sparing management of young women[70], the biopsy samples could be processed for prognostic markers such as p16 IHC.

To our knowledge, this is the first study that takes into account the prognostic role of p16 IHC in young patients treated for CIN2 high-grade squamous intraepithelial lesions of the cervix.

Conclusions

In this group of young women aged less than or equal to 35 years and diagnosed with HSIL (CIN2), we found a significant association between the overexpression of p16 IHC and the long-term recurrence of the intraepithelial disease after "cervix sparing" excisional treatment. Indeed, the expression of this protein seems to correlate specifically with SIL's presence after two years of follow-up and with the progression of HSIL (CIN2) to HSIL (CIN3). The p16 IHC accuracy evaluation further confirmed these results:

the high NPV relative to the disease-free is the greater, the higher the degree of the intraepithelial lesion, reaching 100% values when it comes to CIN3 progression.

Throughout the observation period of approximately five years, the percentage of intraepithelial lesions (LSIL or HSIL) in p16 IHC positive patients had always been greater than negative ones, highlighting a different trend that widened over time and became statistically significant after two years. This could speculate that young patients with negative p16 IHC have a better clinical course and outcome after HSIL (CIN2) treatment than those with positive p16 IHC showing:

- a lower tendency of recurrent or persistent intraepithelial lesions after excision, which is significantly accentuated after the two years of follow-up, so much that in the latter case there was a statistically significant difference compared to the positive subgroup;
- a lower tendency to recur in the form of HSIL, presenting this lesion only in the first two years and, unlike the p16 IHC positive subgroup, completely delete after this period;
- a lower tendency to progress to HSIL (CIN3) in the five years of follow-up. Contrary to what was observed in patients with p16 IHC positivity, the progression of the disease had never occurred for p16 IHC negative lesions, also showing, in this case, a statistically significantly different prognosis between the two subgroups.

Considering that other authors have recently published similar results, we may suggest that the clinical utility of p16 IHC is not only as a diagnostic test but also as a prognostic factor in the post-treatment clinical management of young women. This already standardized histological parameter could offer valuable information above all on the risk of long-term progression of the high-grade intraepithelial neoplasia useful to both the patient and the clinician to avoid or postpone further excisional treatments, thus reducing the risk of obstetric morbidity.

However, our findings are not sufficient to define p16 IHC as a prognostic factor of the recurrence or progression of HSIL (CIN2) disease after excision in young patients since they are based on a retrospective observational study limited by the drop-out of cases during follow-up and by the lack of sufficient data on HPV DNA test throughout the follow-up period.

Therefore, they would need confirmation from future investigations that mainly concern larger statistics and consider other risk factors such as HR HPV status, since currently considered the most accurate risk factor in predicting persistence or recurrence after excisional treatment of the cervix. The efficacy of the HPV vaccine's post-operative administration in the HSIL (CIN2) p16 positive young women is to be evaluated as part of the fertility-sparing treatment.

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