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Usefulness of p16^{INK4a} staining for managing histological high-grade squamous intraepithelial cervical lesions

Ester Miralpeix[1], Jordi Genovés[1], Josep Maria Solé-Sedeño[1], Gemma Mancebo[1], Belen Lloveras[2], Beatriz Bellosillo[2], Francesc Alameda[2,3] and Ramon Carreras[1,3]

[1]Department of Obstetrics and Gynecology, Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona, Spain and [2]Department of Pathology, Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona, Spain

p16^{INK4a} (p16) tumor-suppressor protein is a biomarker of human papillomavirus (HPV) oncogenic activity that has revealed a high rate of positivity in histological high-gade squamous intraepithelial lesion/cervical intraepithelial neoplasia grade 2 (HSIL/CIN2) lesions. However, there is a paucity of data regarding p16 status as a surrogate marker of HSIL/CIN2 evolution. The aim of this study was to evaluate the outcome of HSIL/CIN2 patients followed up without treatment for 12 months according to p16 immunohistochemical staining. Patients diagnosed with HSIL/CIN2 colposcopy-directed biopsy, were recruited prospectively between December 2011 and October 2013. p16 staining was performed in all HSIL/CIN2 diagnostic biopsies. Follow-up was conducted every 4 months by cytology, colposcopy and biopsy if suspicion of progression and once the 12 months of follow-up completed. Complete regression, partial regression, persistence, and progression rates of HSIL/CIN2 were defined as a final outcome. A total of 96 patients were included in the analysis. The rate of spontaneous regression was 64%, while 28% had persistent disease, and 8% progressed at 12 months of follow-up. p16 was positive in 81 (84%) initial HSIL/CIN2 biopsies. Regression was observed in all 15 p16 negative cases and in 46 of 81 (57%) p16 positive cases ($P=0.001$). In conclusion, patients with p16 negative HSIL/CIN2 biopsy had a high rate of regression during first 12 months of follow-up. Status of p16 staining could be considered for HSIL/CIN2 management.

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Cervical cancer and its precursor lesions, induced by persistent infection with human papillomavirus (HPV), represent a significant public health problem. Low-grade squamous intraepithelial lesion/cervical intraepithelial neoplasia grade 1 (LSIL/CIN1) are usually followed up without treatment because around 80% regress spontaneously and only 10% progress to high-grade squamous intraepithelial lesion (HSIL) or cervical cancer.[1] In contrast, CIN grades 2 and 3 are collectively classified as HSIL and excisional treatment is the standard way to manage these lesions. Although HSIL/CIN3 has been shown to carry a higher risk of subsequent cancer, HSIL/CIN2 is not so obviously precancerous as the spontaneous regression and progression rates are around 40 and 20%, respectively.[2–4] Unfortunately HSIL/CIN2 commonly occurs in women who desire preservation of fertility and their overtreatment may increase the risk of subsequent obstetric complications.[5] Accordingly, the last consensus of the American Society for Colposcopy and Cervical Pathology (ASCCP) recommends conservative management with 24 months of follow-up in adolescent and young women with HSIL/CIN2 lesions.[6]

An accurate diagnosis of cervical grade is important guiding the clinical management of women diagnosed with HSIL/CIN2-3. However, it may be difficult as there is often inter-observer variability.[7,8] Adding p16^{INK4a} (p16) immunohistochemistry staining to the hematoxylin and eosin (H&E) morphological interpretation has been proposed to increase the accuracy of cervical histopathological diagnosis.[9–11] p16 is a tumor suppressor protein that

Correspondence: Professor G Mancebo, MD, PhD, Department of Obstetrics and Gynecology, Hospital del Mar, Universitat Autònoma de Barcelona, Passeig Marítim 25-29, Barcelona E-08003, Spain.
E-mail: 94490@parcdesalutmar.cat
[3]These authors equally contributed to this work.
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inhibits the cyclin-dependent kinases (CPK) which regulates progression through the cell cycle by phosphorylating the retinoblastoma protein (pRb). The overexpression of p16 has been shown to indicate advanced interference of the viral oncoproteins with cellular proteins involved in cell-cycle regulation.[12,13] At present, p16 staining is recommended to help clarify the diagnosis between low-grade and precancerous disease in cases in which there is a disagreement in morphological interpretation.[14]

Furthermore, some studies have evaluated the role of p16 as a predictive marker in low-grade lesions. LSIL/CIN1 cases with diffuse p16 staining had a significantly higher tendency to progress to a high-grade lesion than p16 negative cases.[15–18] However, the natural history of HSIL/CIN2 lesions according to p16 staining is not well known and could represent a contribution to the clinical management of those patients.

Thus, the objective of this study was to evaluate the usefulness of p16 immunohistochemical staining to predict the evolution of HSIL/CIN2 in patients followed without treatment for 12 months.

Materials and methods

Study Design and Patient Selection

This prospective observational study included consecutive women of first-time HSIL/CIN2 colposcopy-directed biopsy diagnosis from December 2011 to October 2013 who attending the Cervical Pathology Unit of the Hospital del Mar, Barcelona. All HSIL/CIN2 patients were evaluated by gynecologic colposcopists who proposed conservative management in the framework of this study *versus* immediate excisional treatment according to local guidelines.

Women older than 18 years old were considered candidates for the study if they fulfilled the following inclusion criteria: (1) preferred expectant management rather than immediate treatment; (2) had an exocervical histological diagnosis of HSIL/CIN2; (3) the entire squamocolumnar junction of the cervix was visible; (4) could be followed-up every 4 months during 1 year; and (5) signed the consent form. Patients with unsatisfactory colposcopy, endocervical histological diagnosis of HSIL/CIN2, previous cervical treatment, or immunosuppressive treatment/immunodeficiency disease were excluded.

The gynecologic colposcopists followed patients every 4 months with cervical cytology and colposcopy. A biopsy was performed when colposcopy showed a worsening of suspicious lesion in relation to previous examination.[19] When the biopsy revealed HSIL/CIN2 or less, patients continued on surveillance; conversely when the biopsy revealed HSIL/CIN3, patients underwent immediate treatment using loop electrosurgical excision procedure. At 12 months of follow-up, all women who still showed abnormal liquid-based cytology or colposcopy abnormalities, a colposcopy-directed biopsy was taken from the most severe and suspicious areas.

The final outcome of HSIL/CIN2 was classified as complete regression when two consecutive cervical cytology, colposcopy and biopsy results showed no lesion at 12 months of follow-up; partial regression if final biopsy showed LSIL/CIN1; persistence when biopsy showed HSIL/CIN2; or progression if a HSIL/CIN3 biopsy was detected at any time during the follow-up. Patient who desired immediate treatment during follow-up or discontinued their participation, a loop electrosurgical excision procedure was performed.

The study has been evaluated and approved by the institutional ethics committee and all patients signed an informed consent. Institutional Review Board Project No. 2011/4293/I was approved on 8 September 2011.

Histologic Data and Immunohistochemical Detection of $p16^{INK4a}$

Histologically diagnosis of CIN was established according to the criteria of the World Health Organization based on morphologic criteria using H&E staining.[20] Proliferating cells restricted to the lower third of the epithelium characterized LSIL/CIN1, one-third to two-thirds dysplastic cells described HSIL/CIN2, and HSIL/CIN3 referred to full-thickness dysplasia. All histologic diagnosis of HSIL/CIN2 was reviewed and confirmed by two experienced gynecological pathologists (F.A. and B.L.).

p16 immunohistochemical staining was performed in all HSIL/CIN2 diagnostic biopsies. Formalin-fixed and paraffin-embedded specimens were analyzed using a CINtec p16 Histology Kit (Ventana Roche Diagnostics, Tucson, Arizona) according to the manufacturer's guidelines. In the study, nuclear and cytoplasmic reactivity were considered to define p16 staining. Diffuse, continuous and strong block-positive p16 staining reaction involving at least the lower third of the epithelium (basal and parabasal layers) was considered a positive result, following the LAST recommendations.[14] Focal, weak, and isolate or non-block-positive staining expression was considered p16 negative. Afterwards p16 positive cases were quantitatively classified according to their p16 expression in three epithelial layers: lower third of the epithelium, two thirds, and more than two thirds up to full epithelial thickness. The pathologist and gynecologist were blinded to follow-up outcomes.

Statistical Analysis

Statistical analysis was performed using SPSS 18.0 (Chicago, IL, USA) with two-sided tests assuming a significance level of 5% ($P < 0.05$). Socio-

demographic characteristics of HSIL/CIN2 patients and their final outcome were described and stratified by baseline p16 staining. Mann–Whitney *U*-test for continuous variables and Pearson's Chi-square tests for categorical variables, when appropriate, was used. Considering persistence and progression risk as non-regression outcome (HSIL/CIN2+), values of sensitivity, specificity, and positive and negative predictive value for p16 positivity were also assessed.

Estimated sample size considering a HSIL/CIN2 regression rate of 40% was 79 patients.

Results

From 158 patients with a histologic diagnosis of HSIL/CIN2, 105 fulfilled the inclusion criteria. Three patients did not complete 12 months of follow-up and six were excluded for insufficient material for p16 immunohistochemical staining. Therefore, a total of 96 patients were finally considered. After a 12-month follow-up period, there was complete regression in 25 patients (26%), partial regression in 36 patients (38%), persistent HSIL/CIN2 in 27 patients (28%), and 8 patients (8%) progressed to HSIL/CIN3 (Figure 1). No case of progression to invasive carcinoma was observed.

The median age of HSIL/CIN2 patients included in the study was 30 years (range, 18–56). Table 1 summarizes baseline demographic features of HSIL/CIN2 patients according to p16 staining. No significant differences were observed between the groups. The results of p16 staining in the initial HSIL/CIN2 biopsy and the final outcome after 12 months of follow-up are presented in Table 2. Among the 96 patients included, 81 (84%) were p16 positive and 15 (16%) were p16 negative. All 15 patients with p16 negative status regressed, of which 8 presented complete regression and 7 partial regression ($P=0.008$).

All 35 patients who had persistence or progression of HSIL/CIN2 lesion (HSIL/CIN2+) were p16 positive in the initial biopsy. Nevertheless, 46 of 81 (57%) patients positive for p16 regressed. To attempt to predict final outcome of these 81 p16 positive cases, we graded p16 staining based on the thickness of the epithelium involved. Biopsies were categorized as lower third involvement, lower two-thirds involvement, or full thickness. Statistical analyses showed no significant association between p16 positive epithelial distribution and HSIL/CIN2 final outcome (Table 3; Figure 2).

Table 4 shows regression rate according to different variables, we observed that only p16 staining was able to distinguish those HSIL/CIN2 lesions that regress ($P=0.001$). There was no significant difference between other demographic features like age, smoking status, sexual behavior or parity. The sensitivity, specificity, positive predictive value, and negative predictive value for HSIL/CIN2+ of p16 positive staining were 100% (95% confidence interval (CI) 87.7; 100), 25% (14.8; 37.6), 43% (32.4; 54.7), and 100% (74.7; 100), respectively.

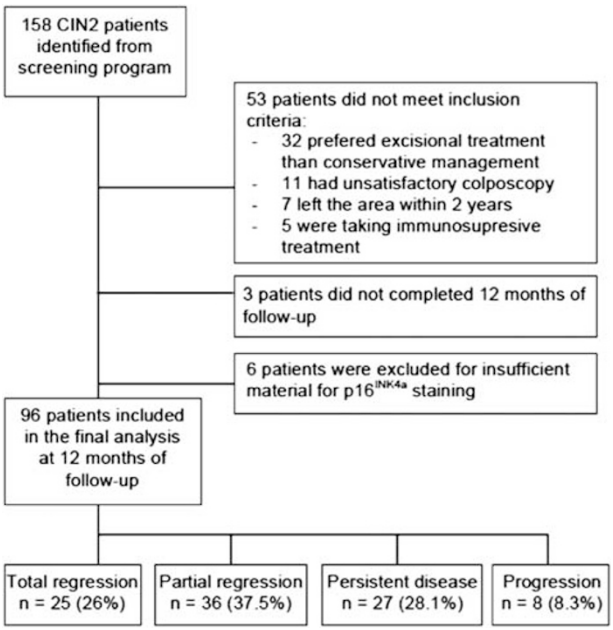

Figure 1 Flow diagram and final outcomes of HSIL/CIN2 patients. At 12 months of follow-up, 25 of 96 HSIL/CIN2 patients (26.0%) who were managed conservatively presented total regression, 36 patients (37.5%) showed partial regression to LSIL/CIN1, 27 (28.1%) had persistent HSIL/CIN2, and 8 (8.3%) progressed to HSIL/CIN3 during follow-up. No case of progression to invasive carcinoma was observed.

Discussion

To the best of our knowledge, this is the largest prospective study that evaluates p16 status as a predictor of HSIL/CIN2 behavior. The key findings from this study were that the regression rates are highest in the setting of p16 ^{INK4a} negative HSIL/CIN2 biopsies, and that it supports the safety of conservative management of HSIL/CIN2 in selected patients.

The management of HSIL/CIN2 lesions is still a dilemma for a gynecologist. Several reports demonstrating HSIL/CIN2 spontaneous regression in young women have been published during the past decades, but predictors of this are poorly understood.[2–4] The results of the present study are in agreement with most of the published studies, showing 64% of HSIL/CIN2 spontaneous regression rate during the first 12 months of follow-up in patients with satisfactory colposcopy and not immunosuppressed, independent of their age. Therefore, a conservative approach could be considered in these cases to reduce unnecessary cone excision.

In order to identify additional prognostic factors involved in HSIL/CIN2 evolution, we evaluated the predictive value of p16 and other demographic features. In our study, only p16 immunohistochemistry

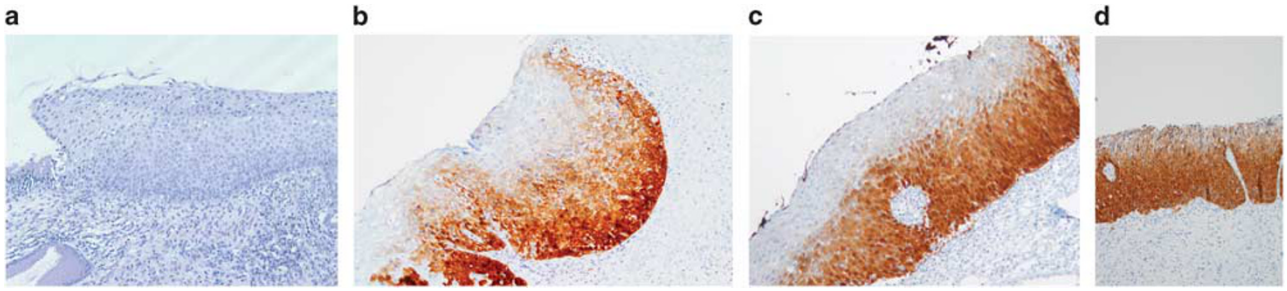

Figure 2 Representative staining of p16. Representative figures of immunohistochemical grading for $p16^{INK4a}$ in HSIL/CIN2 tissues (**a**) with focal $p16^{INK4a}$ staining was classified as negative, (**b**) with positive $p16^{INK4a}$ staining in the lower third of the epithelium, (**c**) with positive $p16^{INK4a}$ staining in two lower thirds of the epithelium, (**d**) with positive $p16^{INK4a}$ staining more than two thirds up to full epithelial thickness.

Table 1 Baseline demographic features of HSIL/CIN2 patients according to $p16^{INK4a}$ staining

	Subjects in analysis	*$p16^{INK4a}$ negative* (n = *15*)	*$p16^{INK4a}$ positive* (n = *81*)	P-*values*
Age (years); mean (range)	30 (18–56)	33 (24–56)	30 (18–47)	0.724
Smokers; n (%)				
Yes	43 (45)	5 (33)	38 (47)	0.331
No	53 (55)	10 (67)	43 (53)	
Contraception method; n (%)				
Condom	43 (45)	5 (33)	38 (47)	0.165
Hormonal	37 (39)	5 (33)	32 (40)	
None or IUD	16 (17)	5 (33)	11 (14)	
Age at first sexual intercourse (years); mean (range)	17 (13–29)	17 (13–22)	17 (14–29)	0.803
Number of sexual partners; mean (range)	9 (1–40)	8 (2–20)	9 (1–40)	0.550
Parity; n (%)				
Nulliparous	71 (74)	11 (73)	60 (74)	0.952
Parous	25 (26)	4 (27)	21 (26)	

Abbreviation: IUD, intrauterine devices.

Table 2 Results of $p16^{INK4a}$ staining in the initial HSIL/CIN2 biopsy and final outcome after follow-up

Evolution at follow-up	n	*$p16^{INK4a}$ negative* n *(%)*	*$p16^{INK4a}$ positive* n *(%)*	P-*values*
Total regression	25	8 (53)	17 (21)	0.008
Partial regression	36	7 (47)	29 (36)	
Persistence	27	0 (0)	27 (33)	
Progression	8	0 (0)	8 (10)	
Overall	96	15 (16)	81 (84)	

$p16^{INK4a}$ positive was defined as continuous and strong staining of the basal and suprabasal cells in an area, independent of whether superficial cells of the squamous epithelium was stained or not. $p16^{INK4a}$ *negative* was defined as either discontinuous, focal and weak staining of isolated basal cells or any type of staining in superficial and/or suprabasal layers.

showed high accuracy in predicting HSIL/CIN2 evolution. All patients with HSIL/CIN2 biopsy negative for p16 regressed, and conversely, HSIL/CIN2 lesions positive for p16 were a higher risk of evolving into HSIL/CIN2 or HSIL/CIN3 lesions at 12 months of follow-up. Thus, there was a significant relationship between initial p16 staining and HSIL/CIN2 evolution, indicating that p16 is an important marker to distinguish HSIL/CIN2 lesions with different biological behavior. It should be pointed out that, although HSIL/CIN2 lesions positive for p16 showed a significantly higher tendency to persist or progress than p16 negative, 57% of them regressed at first 12 months of follow-up. Given that, if we considered all HSIL/CIN2 lesions positive for p16 as high-grade lesions, we will overtreat 57% of HSIL/CIN2 that could regress spontaneously. In addition, we subsequently stratified all 81 HSIL/CIN2 p16 positive lesions in three epithelial layers according to their p16 staining through the epithelium and no association was found to predict the evolution of HSIL/CIN2 p16 positive lesions. This suggests that further biomarkers and other factors may be required to distinguish those HSIL/CIN2 p16 positive lesions that are more likely to progress from those will regress.

Besides, the findings of our study, where almost 84% of HSIL/CIN2 biopsies were p16 positive, confirm previous data suggesting that lesions with strong and diffuse block-positive p16 results support a categorization of precancer; in contrast, negative or non-block-positive staining favors an interpretation

Table 3 Results of $p16^{INK4a}$ positive epithelial distribution in the initial HSIL/CIN2 biopsy and final outcome after follow-up

	n	*$p16^{INK4a}$ positive epithelial distribution*			
		1/3 positive n *(%)*	*2/3 positive* n *(%)*	*3/3 positive* n *(%)*	P-*values*
Total regression	17	0 (0)	8 (28)	9 (17)	0.702
Partial regression	29	0 (0)	10 (35)	19 (37)	
Persistence	27	0 (0)	8 (28)	19 (37)	
Progression	8	0 (0)	3 (10)	5 (10)	
Overall	81	0 (0)	29	52	

$p16^{INK4a}$ immunostaining epithelial distribution: 1/3, lower one third of the epithelium; 2/3, two thirds of the epithelium; 3/3, more than two thirds up to full thickness of the epithelium.

Table 4 Regression rate of HSIL/CIN2 patients at 12 months of follow-up according to different variables

Variables	*Subjects in analysis* n	*Regression < CIN2* n *(%)*	*Non-regression CIN2+* n *(%)*	P-*values*
Overall	96	61 (64)	35 (37)	
$p16^{INK4a}$				
Negative	15	15 (100)	0 (0)	0.001
Positive	81	46 (57)	35 (43)	
Age				
≤25	25	17 (68)	8 (32)	0.590
>25	71	44 (62)	27 (38)	
Smokers				
No	43	27 (63)	16 (37)	0.890
Yes	53	34 (64)	19 (36)	
Contraception method				
Condom	43	30 (70)	13 (30)	0.310
Hormonal	37	20 (54)	17 (46)	
None or IUD	16	11 (69)	5 (31)	
Age at first intercourse				
≤18	76	46 (61)	30 (39)	0.231
>18	20	15 (75)	5 (25)	
Lifetime sexual partners				
≤10	74	48 (65)	26 (35)	0.621
>10	22	13 (59)	9 (41)	
Parity				
Nulliparous	71	48 (68)	23 (32)	0.163
Parous	25	13 (52)	12 (48)	

Abbreviation: IUD, intrauterine devices.

of low-grade disease, to help inter-observer agreement in the pathological diagnosis of cervical specimens.[21–23] In particular, the Lower Anogenital Squamous Terminology (LAST) considered p16 immunohistochemical staining in their revised nomenclature for lower genital tract lesion to replace histopathological diagnosis of CIN nomenclature with a two-tiered classification system: low-grade squamous intraepithelial lesion (LSIL), and high-grade squamous intraepithelial lesion (HSIL), not distinguishing HSIL/CIN2 from HSIL/CIN3 lesions. Moreover, the LAST group supported the use of p16 complementing morphology only in equivocal HSIL/CIN2 cases to guide the decision between LSIL (p16 negative) and HSIL (p16 positive).[14] Although these data are consistent with the findings in our study, we suggest performing p16 staining in all HSIL/CIN2 cases to avoid overtreating morphologically HSIL, but p16 negative, lesions that will probably regress.

To our knowledge, there is only one other study that evaluated the predictive value of p16 as a marker of HSIL/CIN2 outcome.[24] Among 45 HSIL/CIN2 patients who were followed up for at least 12 months, 42% had spontaneous regression, 20% had partial regression to LSIL/CIN1, 11% showed persistence and 22% progressed to HSIL/CIN3. Interestingly p16 positive and negative cases showed practically identical final outcomes of their HSIL/CIN2 lesions. They reported a HSIL/CIN2 failed regression rate of 24% in p16 positive and 16% in p16 negative cases without significant differences. To a certain extent, different outcomes rates could be explained by a different definition of p16 staining. Although the present recommendation only considers strong and diffuse staining to be a positive result, Guedes *et al*[24] considered weak, moderate and strong intensity p16 staining to be a positive result.

Nevertheless, our study agrees with some authors that evaluated the prognostic value of p16 in low-grade lesions. In these studies, p16 positive was associated with significantly higher rates of progression and, in contrast, those low-grade lesions negative for p16 had a higher tendency to regress.[15–18,25,26] Thus, at present, although p16 is a promising biomarker for predicting the outcome of low-grade lesions, there is insufficient evidence to recommend clinical management of low-grade lesions based on p16 staining.

In our study, we recognize that there may be possible limitations that should be considered. The follow-up period of 12 months may not be sufficient to determine the true rate of patients who experience regression or progression of disease. Another concern based on histologic diagnosis of HSIL/CIN2 is the non-reproducibility of the diagnosis and the inter- and intra-observer variation described in the

literature.[7,8] However, in our study the histologic diagnosis of HSIL/CIN2 was reviewed and confirmed by two experienced gynecological pathologists. Furthermore, it is appropriate to take into account that though HSIL/CIN2 initial diagnosis and their final outcome were defined as changes confirmed by colposcopy-directed biopsy on the worsening suspicious zone of colposcopy, the biopsy contains only a portion of the lesion and the biopsy might have altered the evolution of the lesions. Nevertheless, studies using both cytology and histology to follow the natural history have shown no effect of punch biopsy on the disease in the short term, and similar probabilities of persistence and progression have been reported.[27]

There are several strengths of our study. One is the fact that this is the largest prospective study evaluating p16 as a prognostic marker of HSIL/CIN2 lesions. In our study only three patients were lost to follow-up. In addition, the present study used a well-defined inclusion criteria and follow-up routine protocol. The final outcome assessment using cytology, colposcopy, and histology was blinded to initial p16 staining which was evaluated in the same initial diagnosis HSIL/CIN2 biopsy. Furthermore, it is important to highlight that the p16 negative and p16 positive groups were comparable for baseline characteristics.

In conclusion, our results showed that all patients with HSIL/CIN2 negative for p16 regressed in the first 12 months of follow-up. Conversely, HSIL/CIN2 lesions positive for p16 have higher tendency to persist or progress to HSIL/CIN3, and might require closer follow-up. Thus, p16 immunohistochemical staining may have an important role as an adjunctive stain to improve the reliability of HSIL/CIN2 histopathological diagnosis and could be useful for planning the clinical management of patients with HSIL/CIN2. This study supports the safety of conservative management of HSIL/CIN2 in select patients; however, further larger follow-up studies are encouraged to identify other biomarkers to predict the evolution of HSIL/CIN2 patients, in particular looking for predictors of HSIL/CIN2 patients with p16 positive staining.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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