

Original Article

p16INK4a and Ki-67 measurement predict progression of cervical low-grade squamous intraepithelial lesion

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Abstract: Objective: To describe the natural history of low-grade squamous intraepithelial lesion/cervical intraepithelial neoplasia grade I (LSIL/CIN1), and to analyze the predictive values of p16INK4a and Ki-67 for LSIL/CIN1 progression. Methods: From January 2013 to January 2016, 264 patients were diagnosed with CIN1 by colposcopy-assisted biopsy and were followed up at 1-year intervals at Peking University People's Hospital. We measured expression levels of biomarkers p16INK4a and Ki67 to predict progression, persistence, or regression of the disease. We used chi-square tests and logistic regression analysis to explore the relationships among LSIL/CIN1 progression, p16INK4a/Ki-67 expression, and patient age. Results: Among 264 patients with LSIL/CIN1, p16INK4a, Ki-67 expression and patient age > 30 years old were significantly associated with progression. Univariate analysis showed that age was not a risk factor for progression ($P > 0.05$) but that p16INK4a and Ki-67 expression were significantly associated with the progression ($P < 0.05$). Multivariate analysis showed that p16INK4a-positivity and high expression of Ki-67 protein were associated with LSIL/CIN1 progression, with odds ratios (OR) and 95% confidence intervals (CI) of 10.95 (3.04-39.53), and 9.7 (2.77-34.03), respectively. Conclusion: p16INK4a-positivity and high expression of Ki-67 correlated with LSIL/CIN1 progression. These markers may be independent predictors of LSIL/CIN1 progression.

Keywords: LSIL/CIN1, p16INK4a, Ki-67, pathological outcome, follow-up

Introduction

Cervical cancer is the second most common malignancy among women in the world. In 2012, the American Society of Pathologists (ASP) and the American Society of Colposcopy and Cervical Pathology (ASCCP) generated a report from the squamous epithelial lesion group (LAST Project) suggesting that cervical lesions should be divided into LSIL and HSIL (high-grade squamous intraepithelial lesion), with CIN1 included in LSIL [1]. In the fourth version of the Women's Reproductive System Tumor Classification, this nomenclature was adopted for precancerous lesions of cervical squamous cell carcinoma [2]. The natural history of LSIL was characterized by either disease progression, persistence, or regression. Approximately 80% of LSIL/CIN1 were transient lesions that spontaneously regressed in 1-2 years [3].

According to the National Comprehensive Cancer Network (NCCN) cervical cancer screening

guide, the primary approach to CIN1 should be expectant management with follow-up. However, according to the literature, 10%-20% of patients progress to HSIL [4, 5]. Unfortunately, currently available methods cannot identify which LSIL/CIN1 lesions will progress to HSIL/CIN2-3. If all LSIL/CIN1 patients were managed uniformly, there would be a substantial risk of progression to HSIL/CIN2-3 or infiltrating carcinoma.

p16INK4a is a cyclin-dependent kinase inhibitor participating in the regulation of mammalian cell cycle [6]. Overexpression of p16INK4a has been observed in CIN associated with high-risk HPV (hrHPV) infection [7]. Currently, p16INK4a immunostaining is recommended to distinguish HSIL from unrelated neoplastic diseases. Studies have shown that immunohistochemical staining for p16INK4a may provide a useful biomarker for prediction of progression of CIN [8]. Ki-67 is a nuclear antigen associated with cell proliferation. High expression of Ki-67 correlates with progression to cancer. In cervical

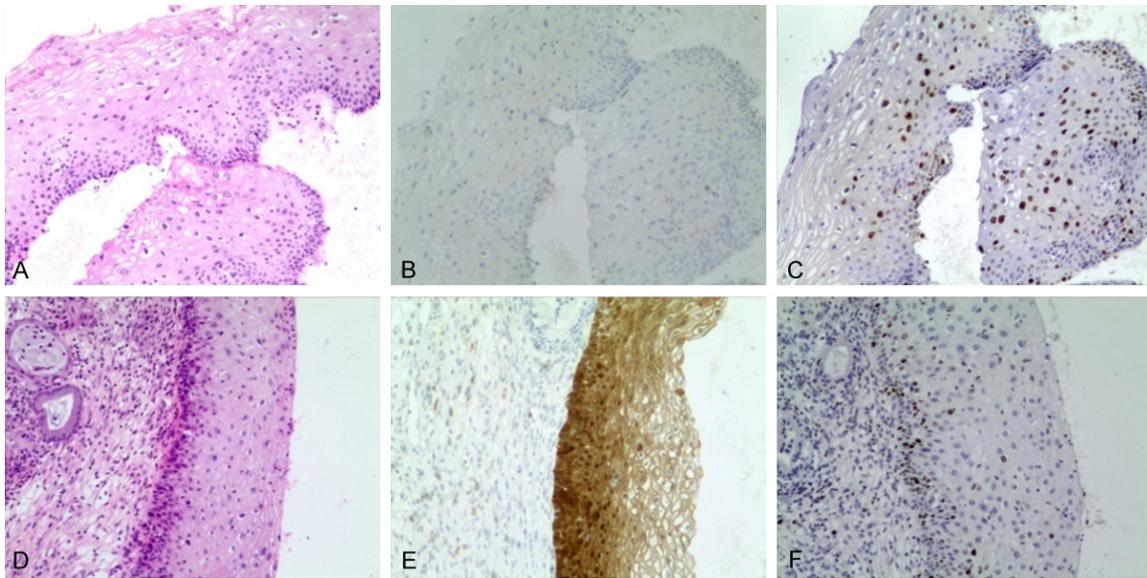


Figure 1. A: HE stain (×100); B: Negative p16INK4a (×100); C: Ki-67 expression 10-30% (×100); D: HE stain (×100); E: Positive p16INK4a stain (×100); F: Ki-67 expression < 10% (×100).

lesions, Ki-67 expression intensity correlates with lesion level. Positive expression in two-thirds of cervical epithelium can be used to identify LSIL/HSIL [9].

This study retrospectively analyzed expression of p16INK4a and Ki67 in patients with CIN1 presenting to our hospital from January 2013 to January 2016, with follow-up. The aim was to evaluate the usefulness of p16INK4a and Ki67 in predicting the progression of LSIL/CIN1 lesions, specifically whether these markers served as independent prognostic factors for progression. If so, measurement of these markers may prove useful in clinical practice.

Materials and methods

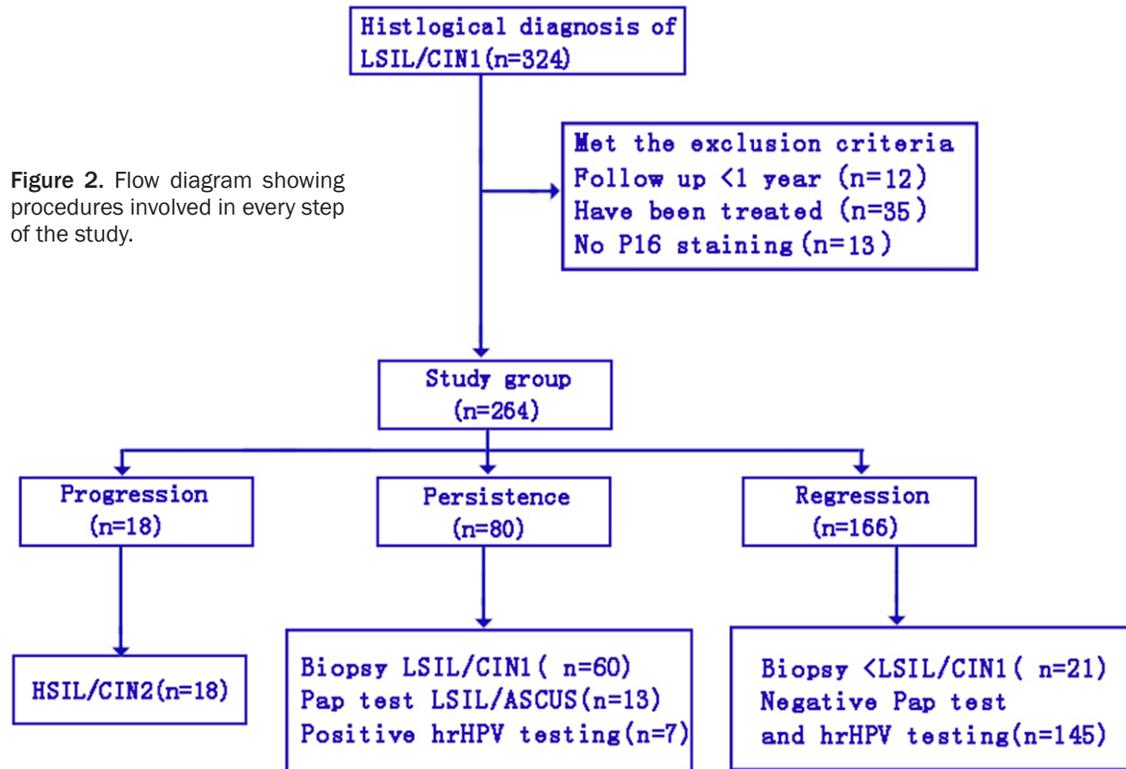
Study subjects

324 patients with LSIL/CIN1 were collected from January 2013 to January 2016 in the Department of Obstetrics and Gynecology of Peking University People's Hospital. All slides were reviewed by two gynecological pathologists. Exclusion criteria were as follows: (1) pregnancy; (2) immunocompromised status, or use of immunosuppressive drugs; (3) treatment with cervical conization, LEEP (Loop Electrosurgical Excision Procedure), or hysterectomy; and (4) other serious disease, including invasive squamous cell carcinoma and adenocarcinoma. This study was approved by the ethics committee of our hospital.

Inspection method

1. TCT (Thin Prep Cytologic test) performed using the Thin-Prep T2000 slide processor (Hologic), with staining by the Papanicolaou method. Cytology slides were evaluated by a cytotechnologist and senior pathologist. Cytological diagnoses were made according to the Bethesda System, 2015 [10]. 2. hrHPV testing was performed on cervical specimens collected in PreservCyt solution using the commercially available Hybrid Capture 2 (HC2) system (Qiagen, Beijing). 3. Histological evaluation and immunohistochemical detection of p16INK4a were prepared as follows: all histological samples were fixed in 10% neutral buffered formalin and embedded in paraffin following routine procedures. The diagnosis of LSIL/CIN1 was made based on inspection of slides stained with hematoxylin and eosin (HE). 1) p16INK4a detection: Paraffin sections of cervical biopsy tissue were obtained and cut into 3-5 μ m continuous slices. p16INK4a was detected using the CINtec Histology Kit (clone E6H4, Roche Diagnostics Products (Shanghai Limited Company) following manufacturer's instructions. Immunohistochemistry was performed with the Ventana Benchmark XT. An automatic staining machine was used for immunohistochemical staining. 2) Ki-67 protein detection: Ki-67 rat anti-human monoclonal antibody was obtained from Beijing Zhongshan Golden Bridge Biotechnology Co. LTD. Clone number: 7B11.

Figure 2. Flow diagram showing procedures involved in every step of the study.



Result interpretation

1. We employed diagnostic criteria according to the WHO 2014 Female reproductive system tumor pathology and genetic classification criteria [2] (See **Figure 1A, 1D**). For p16INK4a and Ki-67 staining, each slide was examined alongside a positive control (cervical squamous cell carcinoma) and a negative control (normal cervical squamous epithelium). 2. According to the LAST-Project Histology interpretation guide, “positivity” was defined as nuclear and/or cytoplasm staining. A diffuse positive reaction was defined as continuous staining of cells in the basal and para-basal layers, with or without staining of superficial squamous cell layers. A “negative” slide was a slide with no staining, staining of only isolated cells or small cell clusters, or discontinuous staining (i.e., focal staining pattern) [1, 11] (**Figure 1B, 1E**). 3. Ki-67 was considered positive in the presence of substantial numbers of brown yellow particles in the nucleus. Positive cells at the basal layer were regarded as negative. In addition to the basal layer, the positive range of other sites was divided into three groups: 1%-10% (+), 10%-30% (++), and > 30% (+++) (**Figure 1C, 1F**).

Follow-up

A follow-up visit occurred at least 1 year after first diagnosis. At each follow-up, cervical samples were processed for Pap tests and hrHPV testing. Some patients underwent colposcopy and cervical biopsy. Disease progression was defined as histologic diagnosis of CIN2 and/or a more severe lesion. Regression was defined as negative hrHPV testing with negative Pap test and cervical biopsy pathology results (grade < CIN1, if available). Persistence was defined as: (1) LSIL/CIN1 diagnosed by a colposcopy-directed biopsy or endocervical curettage, independently of Pap test or hrHPV result; (2) Pap test showing ASCUS (a typical squamous cell of undetermined significance) or LSIL, independent of the hrHPV test result; (3) persistence of a positive hrHPV with a normal Pap test and/or normal biopsy results; or (4) regardless of TCT result, cervical biopsy remaining CIN1 [3, 12].

Statistical analysis

SPSS-19.0 software was used for data processing. Chi-square or Fisher exact tests were used for comparisons between categorical vari-

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Table 1. The relationship of patient age, expression of p16INK4a and Ki67 in CIN lesion outcome

Category	Total numbers	Progression	Persistence	Regression	P-Value
p16 (+)	56 (21.2%)	14 (25%)	18 (32.14%)	24 (42.86%)	
p16 (-)	208 (78.8%)	4 (1.92%)	62 (29.81%)	142 (68.27%)	< 0.001
≤ 30	44 (16.7%)	4 (9.09%)	13 (29.55%)	27 (61.36%)	
> 30	220 (83.3%)	14 (6.37%)	67 (30.45%)	139 (63.18%)	< 0.001
Ki67 ≤ 10%	216 (81.8%)	7 (3.24%)	53 (24.54%)	156 (72.22%)	
Ki67 in 10-30%	48 (18.2%)	11 (22.92%)	27 (56.25%)	10 (20.83%)	< 0.001

ables. The ANOVA test was used to compare quantitative variables between the different categories. Multivariate logistic regression models were used to adjust for possible confounders and to evaluate interaction effects with regard to risk of progression. *P*-value < 0.05 was considered statistically significant.

Results

Pathological characteristics

We considered 324 patients with LSIL/CIN1. Age range was 18-69 years (mean 40.5 years). Among these, 60 patients were excluded for the following reasons: in 12, the follow-up time was < 1 year; p16INK4a staining was not performed in 13; in 35, LEEP, cervical conization, or hysterectomy was performed following the first biopsy. Among the 264 remaining patients, 18 were diagnosed with CIN2, and 60 with CIN1; no patients with CIN3 or invasive carcinoma were seen; 13 had ASCUS/LSIL; 145 were normal and hrHPV negative; 21 were high-risk HPV positive and/or TCT results were abnormal. However, colposcopy biopsy results were normal. We considered two age groups separately: ≤ 30 years old and > 30 years old. Average follow-up time was 14.3 months (range 12-30 months, **Figure 2**).

p16INK4a and Ki-67 expression in LSIL/CIN1

Of 264 patients, p16INK4a was positive in 56 (21.2%). There were no negative patients and no patients with Ki67 staining > 30% (**Figure 1C, 1F**). In 216 patients, Ki67 staining was ≤ 10%, and 48 patients showed Ki67 staining between 10% and 30%.

The relationship of patient age, p16INK4a protein, Ki67 intensity, patient age and CIN outcome

At follow-up for 56 patients with p16INK4a-positivity, 14 showed progression, 18 showed per-

sistence, and 24 showed regression. In 208 patients where p16INK4a was negative, four showed progression, 52 showed persistence, and 142 showed regression. The differences between p16INK4a-positivity and negativity were statistically significant among all three groups. The progression rate in p16INK4a-positive patients was 25% (14/56), while the progression rate in p16INK4a-negative cases was 1.9% (4/208) (relative risk 13).

Of 216 with Ki67 staining ≤ 10%, seven patients showed progression, 43 showed persistence, and 156 showed regression. Of 48 with Ki67 staining between 10% and 30%, 11 showed progression, 27 showed persistence, and 10 showed regression. The difference in expression of Ki67 staining was statistically significant in different pathological outcome groups. The progression rate of the Ki67 ≤ 10% was 3.24% (7/216). The progression rate for Ki67 staining 10%-30% was 22.92% (11/48) (relative risk is 7).

For patients < 30 years old, four showed progression, 13 showed persistence, and 27 showed regression. For patients > 30 years old, 14 cases showed progression, 67 showed persistence, and 139 showed regression. There was a statistically significant difference among various age groups (**Table 1**).

Risk factors for progression of CIN1

Age, p16INK4a and Ki67 expression were analyzed in 264 patients. ANOVA single factor analysis showed that there was no significant correlation between patient age and progression of LSIL/CIN1 (*P* > 0.05). However, expression of p16INK4a protein and Ki67 positivity significantly correlated with the progression of LSIL/CIN1 (*P* < 0.05). Multivariate logistic regression analysis showed that when p16INK4a and Ki67 expression variables were selected into a multi-factor analysis model, p16INK4a-positivity and

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Table 2. ANOVA single factor analysis of patient age, p16INK4a, and Ki67 expression intensity in lesion progression

Category	Total numbers	Progression		F-value	P-value
		Numbers	Percentage (%)		
Ages (years)				0.213	0.809
≤ 30	44	4	9.09%		
> 30	220	14	6.36%		
P16				22.669	0.000
Positive	56	14	25%		
Negative	208	4	1.92%		
Ki67				31.877	0.000
≤ 10% +	216	7	3.24%		
10-30%	48	11	22.9%		

Ki67 expression emerged as risk factors for the progression of lesions (**Tables 2 and 3**).

Discussion

p16INK4a is a widely-studied biomarker. It has been studied for cervical cancer as well as for precancerous lesions. The marker represents a cell-cycle protein-dependent kinase inhibitor (CDK4/6). The expression of p16INK4a is usually low in normal cell cycles, however, when epithelial cells are infected with HPV (especially during persistent infection), E6/E7 protein is produced. This activates transcription factor E2F, making the cell cycle proceed continuously until stopped by the CDK4/6 molecular switch. Persistent HPV infection leads to more production of p16. However, E2F no longer modifies CDK4/6 action, and the accumulation of p16INK4a expression over time does not play a role in the regulation of cell cycle. Therefore, overexpression of p16INK4a can be observed in both CIN and cervical cancer. The intensity and range of expression increased with the increase of lesion grade [13]. Overexpression of p16INK4a may be a marker of disease progression [14]. p16INK4a was diffusely positive in HSIL, and the rate of positivity was high, but the expression in LSIL/CIN1 was often variable, and in some cases, could be expressed in only small amounts.

In our study, p16INK4a-positivity was 21.2% (56/264) in LSIL/CIN1. The results were similar to those of previous studies [15]. The three groups (progression, persistence, and regression) were significant with respect to p16INK4a-positivity. By single-factor ANOVA, p16INK4a-positive and negative expression were signi-

ficantly different in the progression group ($P < 0.05$). Therefore, we included it in multivariate logistic regression. The OR of progression was 10.95 (95% CI 3.04-39.53). p16INK4a-positive lesions progressed to HSIL significantly more frequently than did those of p16INK4a negative patients. Marker positivity may be an independent predictor of the progression of LSIL/CIN1. This result is similar to that of previous reports [16, 17]. There are data suggesting that overexpression of p16INK4a may precede the develop-

ment of HSIL by several years. Overexpression of p16INK4a might be a prelude to a chain of events that leads to HSIL. There was significantly different expression in the progression and regression groups ($P < 0.05$). However, the OR (95% CI) for persistence was 1.02 (0.46-2.26), suggesting no significant difference between the persistence and regression groups ($P > 0.05$).

In a clinical study comprising 739 patients, 12.3% of p16INK4a-positive protein showed lesions progression at 1-year follow-up, while 2.2% of p16INK4a-negative lesions progressed (relative risk 5.6) [3]. Negri et al. [18] demonstrated that LSIL/CIN1 patients with p16INK4a overexpression showed a significantly higher tendency towards progression to CIN3 than did those without p16INK4a overexpression. However, clinical information was limited, because they only selected patients whose lesions regressed or progressed to CIN3. CIN2 was not included. Moreover, follow-up information was limited, and there was no persistence group. Therefore, they could not completely characterize the developmental process of the lesion.

According to LAST, p16INK4a-positivity in LSIL/CIN1 lesions did not significantly correlate with progression to HSIL/CIN2-3. They suggested that the role of p16INK4a in LSIL/CIN1 lesions should be limited to equivocal cases in which HSIL/CIN2 was included in the differential diagnosis [19].

In a large retrospective study (507 CIN1), Amaia studied p16INK4a expression in biopsies from women with LSIL/CIN1. They suggested that

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Table 3. Multiple logistic regression adjustment for different variables of the lesion outcome

Variables	Progression			Persistence		
	Numbers	OR (95% CI)	P-Value	Numbers	OR (95% CI)	P-Value
p16INK4a						
Positive	14	10.95 (3.04-39.53)	0.000	18	1.02 (0.46-2.26)	0.960
Negative	4	1		62	1	
Ki67						
≤ 10% +	7	9.72 (2.77-34.03)	0.000	53	7.17 (3.12-16.46)	0.000
10-30% +	11	1		27	1	

Regression group was used as the reference category.

p16INK4a was a poor predictor of risk of progression to HSIL/CIN2-3 and had very low value as marker of progression of LSIL/CIN1 in clinical practice [12]. Taken together, the literature suggested that p16INK4a alone was insufficient to predict progression.

Ki-67 is a nucleic antigen associated with proliferating cells and may reflect the activity of tumor cells. Ki67 expression is closely related to the pathogenesis of precancerous cervical lesions. Ki67 staining may indicate the classification and progression of cervical lesions [20]. In our study, there were no patients with negative or Ki67 > 30% positivity. We found only Ki67 ≤ 10% and 10%-30%. The difference in expression of Ki67 was statistically significant among the various pathologic outcome groups. ANOVA single factor analysis showed that expression of Ki67 was correlated significantly with progression of CIN1 ($P < 0.05$). In multivariate logistic regression analysis, the ORs (95% CI) of progression and persistence were 9.72 (2.77-34.03) and 7.17 (3.12-16.46), respectively. Expression of Ki67 was significantly related to both progression and regression of the lesion. Therefore, Ki67 can be used as an independent factor to predict the progress of the disease. Similar to our result, Baak et al. [21] found that expression of Ki67 showed high predictive value in LSIL/CIN1. Kruse et al. [22] studied the relationship between Ki67 and progression of CIN1/2, with an 11% rate of progression at follow-up; multifactor analysis showed that high expression of Ki67 may be a risk factor for the progression and persistence of CIN1 lesion after six months.

When stratified by age (cut-off 30 years), we found significantly different risks of progression. However, ANOVA single factor analysis showed no significant correlation between age

and the outcome of LSIL/CIN1 ($P > 0.05$). A study by del Pino suggested transient infection in young women carried a low risk of lesion progression. In some patients, HPV can be eliminated by the immune system for a certain period of time. The likelihood of persistent infection is greater in older women [4]. In a recent prospective study, Matsumoto et al. examined risk factors for progression from CIN1 and CIN2 to CIN3. They found that lesions in women aged 18-29 years old more often regressed than did those of women aged 30-54 years old. The latter had a higher rate of progression. This effect may be related to sexual activity in women in this age group, and the self-clearing ability of HPV infection was stronger [23]. However, it has also been suggested that age as the boundary indicator predicted only the degree of disease and that the application value was low. Large sample sizes are needed to further validate these findings.

This study has some limitations. First, prior to the first biopsy, the number of HPV-positive patients was not counted. HC2 was used only in the follow-up and was not used for HPV types. It was not possible to assess whether the same HPV serotype was detected before and after follow-up. However, we may have failed to identify all the cases with high-risk HPV infection. A recent study concluded that hrHPV genotyping was a poor predictor compared with p16INK4a immunohistochemistry for predicting the outcomes of CIN 1-2 patients [24]. Second, the biopsy procedure might alter the natural evolution of lesions, particularly of small lesions. Some small lesions may have been completely removed at biopsy. The progress of LSIL/CIN1 lesions is not a unilateral pathophysiological disease progression unlike other diseases; therefore, it will introduce some problems to the experimental design. Third, the

follow-up time is short. The patients we chose were not in the middle/long-term follow-up.

p16INK4a-positivity and high expression of Ki-67 may be risk factors for progression and may be independent predictors of progression of LSIL/CIN1 lesions. More precise, rigorous study methods with large sample sizes are needed to further evaluate the role of p16INK4a and Ki67 in LSIL/CIN1 lesions.

Disclosure of conflict of interest

None.

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