RESEARCH ARTICLE

Editorial Process: Submission:06/30/2024 Acceptance:03/23/2025

Role of Cyclooxygenase-2 (*COX-2*) Expression as a Prediction of Persistent Cervical Low Grade Squamous Intraepithelial Lesion (LSIL)

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Abstract

Background: Cervical cancer rates have been decreasing due to improved screening programs targeting HPV infections. Cervical Intraepithelial Neoplasia (CIN), including CIN 1, can regress, persist, or progress, leading to patient anxiety. The expression of Cyclooxygenase-2 (*COX-2*) may serve as an indicator of poor cancer outcomes and could potentially predict the persistence of CIN 1. **Objectives:** To assess the relationship between *COX-2* expression and the persistence of low-grade squamous intraepithelial lesions (LSIL) or CIN 1. Additionally, to compare baseline characteristics between patients with persistent and regressive LSIL/CIN 1. **Methods:** This case-control study included patients diagnosed with CIN 1 at least 12 months prior to the study started and followed up between May 2019 and April 2020. Pelvic examination and liquid-based cytology collection were performed. Participants were divided into two groups: regressive and persistent, based on current examination results. Previous cervical biopsy slides were reviewed by two gynecologic pathologists to confirm the CIN 1 diagnosis. Paraffin blocks from selected samples underwent immunohistochemistry staining to evaluate *COX-2* expression, which was assessed using the Allred score. Clinical risk factors, cervical cytology, HPV genotype, and Allred scores were analyzed. **Results:** Of the 161 patients recruited, 132 were in the regressive group and 29 in the persistent group, yielding a regression rate of 81.99%. *COX-2* expression was observed in 83.8% of the patients. In the regressive group, 110 out of 132 patients tested positive for *COX-2*, while 25 out of 29 patients in the persistent group were *COX-2* positive. Median Allred scores were similar between the groups, with no significant correlation between *COX-2* expression and persistent LSIL/CIN 1 ($p = 0.663$). Furthermore, there was no significant correlation between Allred scores, high-risk HPV infection, and high-risk HPV status ($p = 0.66$ and $p = 0.80$). Persistent detection of high-risk HPV was found to be a significant risk factor for persistent LSIL in univariate analysis ($p = 0.001$), but not in multivariate analysis. **Conclusion:** *COX-2* expression and HPV status do not appear to predict persistent LSIL/CIN 1. Further research is needed to identify reliable predictors for the persistence of LSIL/CIN 1.

Keywords: Cervical intraepithelial neoplasia- cyclooxygenase-2 expression- persistent- regression- LSIL

Asian Pac J Cancer Prev, **26 (3)**, 791-797

Introduction

Cervical cancer is one of the most common malignancies in women worldwide. However, its incidence has been decreasing due to improvements in healthcare screening programs. [1] Almost all cases of cervical cancer and preinvasive lesions are caused by persistent infection with human papillomavirus (HPV). Cervical Intraepithelial Neoplasia (CIN), the premalignant stage of cervical cancer, is classified based on the degree of dysplastic cells in the cervical squamous epithelium. CIN 1 is classified as a low-grade squamous intraepithelial lesion (LSIL), while CIN 2 and CIN 3 are considered high-grade lesions. Approximately 80% of CIN 1 cases regress spontaneously within two years [2, 3]. However, 9-16% of CIN 1 patients either continue to have CIN 1 or progress to more aggressive lesions [4, 5].

LSIL/CIN 1 is typically managed through regular follow-up with cervical cytology every 6-12 months or co-testing at 12 months. If CIN 1 persists or progresses after two years, treatment may be considered [6, 7]. During the follow-up period, many patients experience

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anxiety and stress, worrying about the potential outcomes. Therefore, a predictive test to determine whether the lesions will regress or persist could help alleviate these concerns and guide management decisions.

Cyclooxygenase (COX) is an enzyme that converts arachidonic acid into prostaglandins and thromboxanes. There are two isoforms of the COX enzyme: *COX-1* and *COX-2*. *COX-1* is constitutively expressed and regulates several housekeeping functions, such as cytoprotection of the gastric mucosa and platelet aggregation. In contrast, *COX-2* is inducible and is stimulated by various mediators, including growth factors, cytokines, and some oncogenes. *COX-2*-derived prostaglandins can stimulate cell proliferation, promote angiogenesis, enhance cell invasiveness, inhibit apoptosis, and reduce immune surveillance [8]. Numerous studies have shown that increased *COX-2* expression is associated with unfavorable outcomes and poor prognosis in several cancers, including esophageal, breast, lung, gastric, colon, and cervical cancers [9-14].

Previous studies have demonstrated *COX-2* expression in cervical intraepithelial lesions and cervical cancer, with its expression correlating to the degree of lesion severity. However, there have been no reports examining the correlation between *COX-2* expression and persistent LSIL/CIN 1 [15-17]. Therefore, the aim of this study was to evaluate the association between *COX-2* expression and persistent CIN 1. A secondary objective was to examine the baseline characteristics of patients with persistent versus regressive LSIL/CIN 1 and to determine the regression rate of CIN 1.

Materials and Methods

This case-control study was conducted at the Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, from May 16, 2019, to April 30, 2020, with approval from the Institutional Review Board (IRB 126/62). The sample size was calculated using the formula for two independent proportions. Approximately 80% of CIN 1 cases regress within two years [2, 3]. The proportion of *COX-2* expression, assessed by immunohistochemistry (IHC) in CIN 1 specimens in a previous study, was 50% [16]. We assumed the proportion of *COX-2* expression in normal cervical specimens to be 25%. Using $\alpha = 0.05$ and $\beta = 0.2$ for the sample size calculation, a total of 175 participants was required, with a ratio of persistent to regressive cases of 1:4.

Women aged 21 to 65 years with a history of CIN 1 diagnosed at least 12 months prior to the current visit and who returned for follow-up during the study period were eligible for inclusion. Exclusion criteria included: previous treatment with cryotherapy, laser ablation, conization, or loop electrosurgical excision procedure (LEEP); prior radiotherapy or hysterectomy; a known history of endometrial or cervical cancers; unavailability of histological slides for review; pregnancy; or refusal to undergo cervical cytology smears.

All participants were provided with detailed protocol information and signed informed consent voluntarily. Papanicolaou (Pap) smears (liquid-based cytology) were performed for each participant. If cytology results showed atypical squamous cells of undetermined significance (ASC-US) or worse, colposcopy and cervical biopsy were performed. Histopathological diagnoses were made by a gynecologic pathologist and reported according to the 2014 WHO classification system. If abnormal results were detected, they were managed according to the ASSCP 2012 guidelines.

Participants were divided into two groups based on the results of the current examination: persistent or regressive LSIL/CIN 1, with a follow-up period of at least 12 months since the prior biopsy. Persistent LSIL/CIN 1 was defined as abnormal cytology with histologically confirmed CIN at the current visit. Regression was defined as normal cervical cytology and/or negative histology at the current visit.

For HPV infection, high-risk HPV status was classified as persistent if high-risk HPV was detected in both the initial diagnosis and follow-up visit, or as transient if high-risk HPV was detected only once, either at the initial diagnosis or follow-up visit. Demographic data, including age, parity, number of sexual partners, age at first intercourse, contraceptive methods, underlying diseases, history of sexually transmitted diseases (STDs), smoking, and alcohol use, were recorded prospectively in a database and obtained from electronic medical records.

Immunohistochemical Staining and Interpretation

Formalin-fixed, paraffin-embedded biopsy tissue from the initial CIN 1 diagnosis was obtained from the Division of Pathology and Cytology, Department of Obstetrics and Gynecology, King Chulalongkorn Memorial Hospital. All hematoxylin and eosin (H&E)-stained slides of the previous biopsy tissues were reviewed, and the most representative slide from each case was selected by gynecologic pathologists. *COX-2* immunohistochemical staining was performed using the Ventana XL BenchMark automated slide stainer. Tissue samples were sectioned into 2-micron thick slices and mounted onto SuperFrost Plus adhesive-coated slides. Ezprep Roche concentrate solution was used for paraffin removal and rehydration.

Antigen retrieval was performed using Ventana Cell Conditioning 1 (CC1) solution for 30 minutes, followed by incubation with 3% hydrogen peroxide (H_2O_2) to block endogenous peroxidase activity. The primary monoclonal anti-human *COX-2* antibody was applied at a 1:100 dilution for 30 minutes at 37°C, followed by the application of a secondary anti-mouse antibody. For visualization, the slides were exposed to diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate and counterstained with hematoxylin for 10 minutes. Subsequently, all slides were dehydrated with 95% alcohol, cleared with xylene, and mounted using permanent mounting media. Normal lung tissue or colonic carcinoma tissue were used as positive controls, and a section without primary antibody staining was used as a negative control.

COX-2 immunohistochemical expression was evaluated by two gynecologic pathologists who were blinded to the participants' clinical history and outcomes.

In the case of discordant results, the pathologists discussed the findings and reached a final consensus. For squamous epithelium, only cytoplasmic staining was considered indicative of positive *COX-2* expression. Semi-quantitative analysis was performed based on the Allred score, which consists of two components: the Proportion Score (PI) and the Intensity Score (IS).

The Proportion Score (PI) was calculated by determining the percentage of stained squamous epithelial cells in the lesion. The PI scale ranged from 0 to 5:

- 0 = no staining
- 1 = <1% stained cells
- 2 = 1-10% stained cells
- 3 = 10-33% stained cells
- 4 = 33-66% stained cells
- 5 = >66% stained cells

The Intensity Score (IS) measured the average intensity of the staining in the cells, using a scale from 0 to 3:

- 0 = no staining
- 1 = weak staining
- 2 = moderate staining
- 3 = strong staining (see Figure 1).

The Allred score, widely used to evaluate hormonal receptor expression in breast cancer, [18] is the sum of the PI and IS scores, yielding a final score ranging from 0 to 8.

Statistical Analysis

Data were analyzed using IBM SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics are reported as mean ± SD, number, and percentage. Clinical factors were presented as odds ratios (OR) with 95% confidence intervals (95% CI). Categorical variables were compared using the Chi-square test or Fisher's exact test. Continuous variables were analyzed using the Student's t-test. *COX-2* expression comparisons were performed using the nonparametric Mann-Whitney U test. Multivariate logistic regression was used to determine factors significantly associated with persistent LSIL. The presence of high-risk HPV infection at initial diagnosis and the persistence of high-risk HPV at follow-up were evaluated. A p-value of less than 0.05 was considered statistically significant.

Results

A total of 164 participants were recruited for the study. One patient was excluded due to pregnancy, and two patients were excluded because their previous biopsy results were normal upon review. As a result, 161 participants were included in the final analysis. Baseline characteristics are summarized in Table 1. The mean age of women in the regressive group was 40.8 ± 9.7 years, compared to 43.2 ± 9.2 years in the persistent group.

The majority of participants were premenopausal women with a single sexual partner and no history of sexually transmitted infections. Baseline cervical cytology results were similar between the two groups. HPV DNA testing at the time of initial diagnosis was performed in 71 cases (44.0%). Non-16/18 high-risk HPV genotypes were the most common, followed by HPV 16, multiple HPV types, and HPV 18 (Table 1).

The regressive group included 132 patients, while the persistent group had 29 patients. The regression rate in this study was 81.99% (132/161). Twenty-nine patients (18.01%) still had lesions at the follow-up visit. Of the 29 patients in the persistent group, five were diagnosed with CIN 2 or CIN 3 at follow-up. The cytology results for these five cases included two with atypical squamous

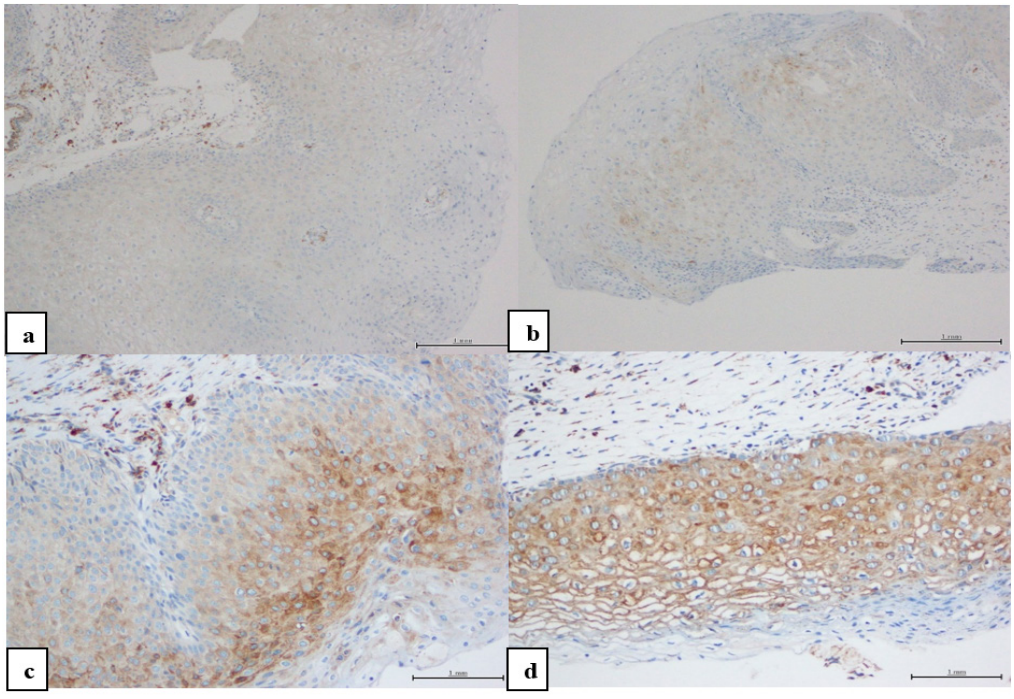

Figure 1. (a) and (b): weak cytoplasmic intensity. (c) and (d): moderate and diffuse cytoplasmic intensity and negative staining in stroma.

Table 1. Baseline Characteristics of Participants in Regressive and Persistent Group

Participants Characteristics	Regression (n=132)	Persistentce (n=29)
Age (mean±SD) (yr)	40.8±9.7	43.2±9.2
Parity, n (%)		
Nulliparous	44 (33.3%)	12 (41.4%)
Multiparous	88 (66.7%)	17 (58.6%)
BMI (kg/m^2), n (%)		
< 18.5	13 (9.8%)	4 (13.8%)
18.6 – 24.9	85 (64.4%)	19 (65.5%)
25.0 – 29.9	25 (18.9%)	3 (10.3%)
≥ 30.0	9 (6.8%)	3 (10.3%)
Number of partners, n median(range)	1 (1-9)	2 (1-10)
Age of 1st Sexual intercourse, year median (range)	20 (14-46)	20 (14-35)
Menopause status, n (%)		
Premenopausal	108 (81.8%)	24 (82.8%)
Postmenopausal	24 (18.2%)	5 (17.2%)
Sexual transmitted disease, n (%)		
No	117 (88.6%)	26 (89.7%)
Yes	15 (11.4%)	3 (10.3%)
Medical disease, n (%)		
None	96 (72.7%)	21 (72.4%)
Diabetes mellitus	3 (2.3%)	0 (0.0%)
Hypertension	8 (6.1%)	2 (6.9%)
Dyslipidemia	4 (3.0%)	0 (0.0%)
Autoimmune disease	4 (3.0%)	1 (3.4%)
HIV infection	8 (6.1%)	2 (6.9%)
Other	9 (6.8%)	3 (10.3%)
Initial cytology results , n (%)		
NILM	7 (5.3)	3 (10.3)
ASCUS	51 (38.6)	9 (15.0)
LSIL	74 (56.1)	17 (58.6)
Initial HPV genotype, n (%)		
Unknown	76 (57.7)	14 (48.4)
Negative	4 (3.0)	1 (3.4)
HPV 16	11 (8.4)	4 (13.8)
HPV 18	1 (0.6)	0 (0.0)
Other Hr-HPV	33 (25.0)	7 (24.1)
≥ 2 type HPV	7 (5.3)	3 (10.3)

*BMI, body mass index; HR-HPV, High risk human papilloma virus; NILM, Negative for intraepithelial lesion/malignancy; ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial lesion; LSIL, Low grade squamous cell intraepithelial lesion; HSIL, High grade squamous cell intraepithelial lesion; ASC-H, Atypical squamous cells cannot exclude HSIL; AGC, Atypical glandular cells.

cells of undetermined significance (ASC-US), two with low-grade squamous intraepithelial lesion (LSIL), and one with atypical glandular cells (AGC). Final excision (LEEP) results revealed CIN 2 in two women (6.9%) and CIN 3 in three women (10.3%).

HPV infection rates were significantly higher in the persistent group, with 81.3% of persistent cases testing positive for high-risk HPV, compared to 32.3% in the regressive group (p = 0.001). The rate of persistent high-risk HPV infection was also significantly higher in the persistent group (42.1% vs. 6.9%, p = 0.001) compared to the regressive group (Table 2). However, no significant association was found in the multivariate analysis (Table 3).

COX-2 Expression

COX-2 expression was observed as brown cytoplasmic staining in squamous epithelial cells (Figure 1). No staining or weak intensity was seen in some cervical stromal cells. *COX-2* expression was present in 135 out of 162 cases (83.33%) of the total participants. In the persistent LSIL group, 110 out of 132 cases (84.09%) were positive for *COX-2*, and in the regressive group, 25 out of 29 cases (86.21%) showed *COX-2* expression.

The Allred scores are presented in Tables 3 and 4. The median Allred score was similar between both groups. The majority of cases (31.7%) had an Allred score of 3. Two cases in the persistent group (6.9%) had strongly positive *COX-2* staining with an Allred score ≥ 6, and 9 out of 132 cases (6.8%) in the regressive group had a similar strong *COX-2* expression. However, the difference in *COX-2* expression between the persistent and regressive groups was not statistically significant (p = 0.663).

Among the five patients with CIN 2 or CIN 3 at follow-up, four had positive *COX-2* expression with Allred scores ranging from 2 to 4, which was not significantly different from the other participants. Furthermore, the correlation between *COX-2* expression, current HPV infection, and HPV status (classified as intermittent or persistent infection) is shown in Table 4. No significant correlation was found between the Allred score and current HPV infection or HPV status (p = 0.66 and p = 0.80, respectively).

Discussion

Persistent high-risk HPV infection remains a major concern in the management of HPV-related cervical lesions, including preinvasive lesions such as CIN1. Once HPV integrates into cervical epithelial cells, the viral oncoproteins E6, E7, and E5 trigger an inflammatory response through the cyclooxygenase (COX)/prostaglandin (PG) pathway. While persistent HPV infection is a key factor in cervical carcinogenesis, it alone may not be sufficient for neoplastic transformation. Chronic inflammation is another potential factor that alters immunological mechanisms, leading to changes in cellular and immune function, which in turn may promote tumor angiogenesis, enhance tumor growth, reduce apoptosis, and impair immune surveillance.

Cyclooxygenase enzymes, particularly *COX-2*, have been proposed as potential biomarkers for cervical intraepithelial neoplasia (CIN), given their role in inflammation and cancer progression. According to the American Society for Colposcopy and Cervical

Table 2. Univariable and Multivariable Analysis of HPV and Cytology Results at Initial and Follow up Visit in Participants with Regressive or Persistent LSIL/CIN1

Factors	Participants, No. (%)		Univariable analysis		Multivariable analysis	
	Regression (n=132)	Persistence (n=29)	OR (95% CI)	P-valve	OR (95% CI)	P-valve
HR-HPV status at initial diagnosis	(N= 56)	(N= 15)				
Negative	4 (7.1%)	1 (6.7%)	1	0.999[b]	N/A	N/A
Positive	52 (92.9%)	14 (93.3%)	1.07 (0.11- 10.41)			
Cytology at initial diagnosis						
NILM	7(5.3%)	3 (10.3%)	1	0.388[b]	N/A	N/A
Low grade group	125 (94.7%)	26 (89.7%)	0.48 (0.11- 2.00)			
HR-HPV status at follow up	(N=31)	(N=16)				
Negative	21 (67.7%)	3 (18.8%)	1	0.001[a]		
Positive	10 (32.3%)	13 (81.3%)	9.10 (2.10- 39.33)		3.75 (0.54-26.04)	0.181
HR-HPV status over time	(N=58)	(N=19)				
Intermittent	54 (93.1%)	11 (57.9%)	1	0.001[b]		
Persistent	4 (6.9%)	8 (42.1%)	9.81 (2.51-38.41)		2.40 (0.44- 12.98)	0.309

NILM, Negative for intraepithelial lesion; ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial lesion; LSIL, Low grade squamous cell intraepithelial lesion; HSIL, High grade squamous cell intraepithelial lesion; ASC-H, Atypical squamous cells cannot exclude HSIL; AGC, Atypical glandular cells; Low grade group: ASC-US, LSIL; High grade group, HSIL; ASC-H, AGC and cervical cancer; [a], Chi-square test; [b], Fisher exact test; N/A, non applicable

Table 3. The association between Cyclooxygenase-2 Expression and Persistent LSIL

	Regressive LSIL/CIN1 (n=132)	Persistent LSIL/CIN1 (n=29)	Total N, (%)	P-valve
Proportion score (PS), %				0.221[a]
0	21 (15.9%)	6 (20.7%)	27 (16.8%)	
1	12 (9.1%)	2 (6.9%)	14 (8.7%)	
2	52 (39.4%)	15 (51.7%)	67(41.6%)	
3	38 (28.8%)	5 (17.2%)	43 (26.7%)	
4	9 (6.8%)	1 (3.4%)	10 (6.2%)	
Intensity score (IS), %				0.839[a]
0	22 (16.7%)	4 (13.8%)	26 (16.1%)	
1	91 (68.9%)	21 (72.4%)	112 (69.6%)	
2	19 (14.4%)	4 (13.8%)	23 (14.3%)	
Allred score, %				0.663[a]
0	22 (16.7%)	4 (13.8%)	26 (16.1%)	
2	13 (9.8%)	6 (20.7%)	19 (11.8%)	
3	43 (32.6%)	8 (27.6%)	51 (31.7%)	
4	33 (25.0%)	7 (24.1%)	40 (24.8%)	
5	12 (9.1%)	2 (6.9%)	14 (8.7%)	
6	9 (6.8%)	2 (6.9%)	11 (6.8%)	

[a], Mann-Whitney test.

Table 4. Correlation between *COX-2* Expression, HPV Status and High-Risk HPV Infection at Initial Diagnosis

Allred score	HPV status			
	Intermittent (n=65), %	Persistent (n=12), %	OR (95% CI)	P-value[a]
0-3	35 (53.85%)	6 (50%)	Reference	0.8
3-6	30 (46.15%)	6 (50%)	1.1(0.34-4.00)	
Allred score	HR-HPV infection			
	Negative (n=5), %	Positive (n=66), %	OR (95% CI)	P-value[b]
0-3	2 (40%)	35 (53.03%)	Reference	0.66
3-6	3 (60%)	31 (46.97%)	0.6(0.09-3.76)	

[a], Chi-square test; [b], Fisher exact test.

Pathology (ASCCP) guidelines, patients with CIN1 are typically followed up with co-testing at 12-month intervals. However, the ability to predict whether CIN1 will persist or regress has not been well-established. This study aimed to evaluate *COX-2* expression as a potential predictor of persistent low-grade squamous intraepithelial lesions (LSIL), which could inform the need for repeat colposcopic examinations.

In our study, 3.1% (5/161) of patients progressed from CIN1 to CIN2+ based on colposcopic-directed biopsies, a finding that aligns with previous studies [19]. However, this rate is slightly lower than that reported in other studies, which have observed progression rates ranging from 5% to 7% [5-7]. Univariate analysis showed that persistent high-risk HPV infection was significantly associated with persistent LSIL/CIN1, but multivariate analysis did not confirm this association. This result contrasts with the study by Ho GY et al. [20], who found that persistent high-risk HPV infection, as well as undetected HPV infections, were crucial risk factors for the development of high-grade lesions.

In our study, *COX-2* expression, as measured by the Allred score, did not significantly differ between the persistent and regressive groups. Previous studies have not established a definitive cut-off value for *COX-2* positivity using the Allred scoring system. In our study, most cases (31.7%) had an Allred score of 3, which is consistent with the findings of Balan et al. [17]. However, there were differences in the methods: Balan et al. used specimens obtained from LEEP procedures, while our study used tissue from colposcopic-directed biopsies. Additionally, we focused exclusively on women with previous CIN1, which may express weaker *COX-2* compared to high-grade lesions like CIN2/3.

We also observed a lack of significant correlation between *COX-2* expression and HPV infection status (both recent and persistent infections) in our study ($p = 0.66$ and $p = 0.80$, respectively). This finding is in line with the study by Sarian LO et al. [21], which reported no correlation between HPV detection and the severity of histological cervical intraepithelial lesions (OR = 1.7, 95% CI 0.7-4.2). This study has several strengths. All participants had a histologic diagnosis of CIN1 preceded by low-grade cervical cytology, and *COX-2* expression was evaluated by two independent pathologists using the same criteria for interpretation. However, there are some limitations. First, we did not reach the targeted sample size (162 out of 175) due to the COVID-19 pandemic, which led to deferrals in follow-up visits for low-grade lesions, and some patients declined to continue with regular follow-up (approximately 25 patients). Despite this, the statistical power of the study was 0.71, which is still considered acceptable. Second, we did not obtain histological confirmation in the regressive group because colposcopic biopsy is not a standard procedure for women with normal cervical cytology. Moreover, the small sample size in the persistent group limited the analysis of some secondary objectives. Lastly, HPV testing was not performed in all participants, limiting our ability to definitively assess persistent HPV infection in this study. Given these limitations, further prospective studies with larger, more specific cohorts are needed to address the questions raised in this study.

In conclusion, neither *COX-2* expression nor HPV status serves as a reliable predictor for persistent CIN1. Further research is needed to identify better predictors of CIN1 persistence, which could improve patient management and reduce unnecessary interventions.

Author Contribution Statement

Pitchaya Homsup: literature review, study concept design, data acquisition, statistical analysis, and primary manuscript drafting. Chai Ariyasriwatana: data acquisition. Natkrita Pohthipornthawat: study concept design, primary manuscript editing and manuscript revision for intellectual content. Associate Professor Shina Oranratanaphan: study concept design and manuscript revision for intellectual content

Acknowledgements

None.

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