J Gynecol Oncol. 2018 May;29(3):e27
https://doi.org/10.3802/jgo.2018.29.e27
pISSN 2005-0380·eISSN 2005-0399

JOURNAL OF GYNECOLOGIC ONCOLOGY

Original Article

Programmed death-1 (PD-1) expression in cervical intraepithelial neoplasia and its relationship with recurrence after conization

Hyeyoon Chang[1], **Jin Hwa Hong**[2], **Jae-Kwan Lee**[2], **Hyun Woong Cho**[2], **Yung Taek Ouh**[2], **Kyung Jin Min**[3], **Kyeong A So**[4]

[1]Department of Pathology, Korea University Guro Hospital, College of Medicine, Korea University, Seoul, Korea
[2]Department of Obstetrics and Gynecology, Korea University Guro Hospital, College of Medicine, Korea University, Seoul, Korea
[3]Department of Obstetrics and Gynecology, Korea University Ansan Hospital, College of Medicine, Korea University, Ansan, Korea
[4]Department of Obstetrics and Gynecology, Cheil General Hospital and Women's Healthcare Center, Dankook University College of Medicine, Seoul, Korea

Received: Aug 8, 2017
Revised: Dec 12, 2017
Accepted: Jan 14, 2018

Correspondence to
Jin Hwa Hong
Department of Obstetrics and Gynecology, Korea University Guro Hospital, College of Medicine, Korea University, 148 Gurodong-ro, Guro-gu, Seoul 08308, Korea.
E-mail: jhhong93@korea.ac.kr

ORCID iDs
Hyeyoon Chang
https://orcid.org/0000-0002-4274-4652
Jin Hwa Hong
https://orcid.org/0000-0002-6905-5363
Jae-Kwan Lee
https://orcid.org/0000-0003-3101-6403
Hyun Woong Cho
https://orcid.org/0000-0002-7114-6301
Yung Taek Ouh
https://orcid.org/0000-0001-5887-4497
Kyung Jin Min
https://orcid.org/0000-0002-5783-4968
Kyeong A So
https://orcid.org/0000-0002-3566-8436

ABSTRACT

Objective: Impaired local cellular immunity contributes to persistent human papillomavirus (HPV) infection and development of cervical intraepithelial neoplasia (CIN). Programmed death-1 (PD-1) and its ligands PD-ligand-1 (L1) and PD-L2 are negative regulators of T cell activity in various cancers, but few studies exist. The aim of this study was to determine the clinicopathologic and immunologic parameters (PD-1, PD-L1, and PD-L2) related to the persistence/recurrence of CIN after conization.
Methods: Medical records of 652 patients diagnosed with CIN and underwent conization were reviewed. The associations between clinicopathologic parameters (e.g., age, parity, initial HPV load, etc.) and persistence/recurrence of CIN were analyzed. Expression of PD-1, PD-L1, and PD-L2 was assessed on 100 conization specimens by immunohistochemistry (IHC) in women matched for propensity-score (50 with persistence/recurrence and 50 without).
Results: Initial HPV load (>1,000 relative light unit) and positive margin were shown to be significantly associated with CIN persistence/recurrence (p=0.012 and p<0.001, respectively). Multivariate analysis showed that margin status was an independent predictor of persistence/recurrence (hazard ratio=8.86; 95% confidence interval=1.67–16.81; p<0.001). On IHC analysis, none of the patients expressed PD-L1. PD-1+ T cells were observed in 25 of 100 patients. Also, PD-1+ T cells were significantly correlated with increasing grade of CIN (p=0.031). In addition, patients with persistence/recurrence had increased expression of PD-1 compared with those without (36% vs. 14%, respectively; p=0.020). Although PD-L2 expression did not differ between 2 groups, it was significantly higher in patients with high-grade CIN compared to low-grade (34.7% vs. 12%, respectively; p=0.041).
Conclusion: Positive surgical margin and expression of PD-1+ T cells were associated with CIN persistence/recurrence after conization.

Keywords: Cervical Intraepithelial Neoplasia; Programmed Cell Death-1; Papillomaviridae

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
Conceptualization: H.J.H.; Methodology: C.H.Y.; Formal Analysis: C.H.Y., S.K.A.; Funding Acquisition: H.J.H.; Investigation: M.K.J., L.J.K.; Resources: H.J.H., L.J.K.; Software: C.H.Y., O.Y.T.; Supervision: H.J.H.; Validation: C.H.Y., O.Y.T.; M.K.J.; Visualization: S.K.A.; Writing - original draft: C.H.Y.; Writing - review & editing: H.J.H.

INTRODUCTION

Despite the dramatic reduction in incidence, cervical cancer is still one of the most common cancers in women worldwide. Persistent infection with high-risk human papillomavirus (HPV) is a known etiological factor for the development of cervical intraepithelial neoplasia (CIN) and cervical cancer [1]. High-grade CIN (CIN 2–3) is a precancerous lesion and can progress to cervical cancer if untreated. Conization has been widely accepted as both a diagnostic and therapeutic modality for the management of CIN 2–3 [2]. Cold knife conization (CKC) and the loop electrosurgical excision procedure (LEEP) are the 2 most common procedures. However, the prevalence of persistent or recurrent high-grade lesions after conization varies between 5% and 25%, leading to frequent follow-up and retreatment [3-6]. Previous studies have suggested that loss of follow-up might be related to increased risk of cervical cancer [7]. Therefore, knowledge of risk factors related to disease persistence or recurrence after conization is crucial for early detection.

Age, parity, pretreatment HPV load, lesion grade, glandular involvement, and margin status have been suggested as risk factors for persistent or recurrent cervical disease after conization for CIN [8-14]. However, their roles as risk factors have not been demonstrated in every previous report, and it is still difficult to predict the outcome for patients who undergo conization. Therefore, studies regarding molecular biomarkers might shed light on some of these questions.

HPV infection is a well-established cause of cervical cancer, and individuals with immunosuppression seem to be at an increased risk of cervical cancer with a higher rate of infection [15]. Moreover, several studies have demonstrated that local immunity is suppressed during HPV infection, especially in persistent infection with high-risk HPV [16-18]. Unfortunately, underlying molecular mechanisms remain largely unknown. In recent years, immunotherapies have been actively sought and tested as treatments for various cancers. Among them, the programmed death-1 (PD-1)/programmed death-ligand-1 (PD-L1) pathway is the most promising target for immunotherapy. In a study of cervical cancer, PD-1 was expressed by a vast number of infiltrating $CD8^+$ T cells, whereas relatively less expression of PD-L1 and PD-L2 was shown by 3-color fluorescent immunohistochemistry (IHC) [19]. A few studies have described the association between PD-1/PD-L1 and recurrence or resolution of CIN. PD-1 and PD-L1 expression in cervical T cells and dendritic cells (DCs), respectively, was associated with high-risk HPV positivity and increased in parallel with increasing CIN grade [20]. Another study showed that the proportions of cervical $CD4^+$ T cells that were regulatory T cells and PD-1+ cells were significantly lower in patients with regressed CIN compared with those with progressed CIN [21]. However, studies have thus far been few in number, have had a small sample size, and have not collected data on PD-L2; these limitations have prevented the elucidation of their exact role in the natural history of CIN.

In this study, we analyzed PD-1, PD-L1, and PD-L2 expression using IHC methods in conization specimens and assessed the association between the expression of these immune checkpoint molecules and persistence/recurrence of CIN after conization. In addition, we evaluated whether any clinicopathological factors could help identify subgroups of patients with a high-risk of persistence/recurrence.

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MATERIALS AND METHODS

1. Study population

We investigated all women who were diagnosed with CIN and underwent conization at Korea University Guro Hospital between January 2007 and December 2013. The inclusion criteria of this study consisted of 1) histologically confirmed CIN by colposcopy-directed biopsy, and 2) women who underwent conization. Some patients were excluded from this study according to the following exclusion criteria: 1) incomplete follow-up after conization (regular follow-up for at least one year after conization), 2) missing data on pretreatment HPV loads, 3) specimens collected from a procedure other than conization (e.g., hysterectomy), and 4) cervical cancer confirmed by conization. Medical records were obtained retrospectively from the hospital's database, including data regarding patient age, parity, HPV load (relative light units, RLUs), colposcopy-directed biopsy, type of conization, conization pathology, cone depth, glandular involvement, and margin status. The protocol of this study was approved by the Institutional Review Board of Korea University Guro Hospital (KUGH16004-001) before data collection, and all participating patients submitted written formal informed consent for research use of their specimens.

Formalin-fixed, paraffin-embedded (FFPE) tissue specimens were retrospectively collected from patients who underwent conization. The conization procedures used were CKC or LEEP. The type of conization was chosen by the surgeon. The specimens were examined for cone depth, lesion grade, and surgical margin status. Margins were reported as positive if any grade of CIN existed at or near (≤1 mm) the resection surface.

During the postoperative period, recurrence was assessed using Pap smear (ThinPrep; Cytyc Corp., Marlborough, MA, USA) and HPV test (Hybrid Capture 2; Digene, Gaithersburg, MD, USA), which detected 13 high-risk HPV types (16/18/31/33/35/39/45/51/52/56/58/59/68). Colposcopy-directed biopsy was performed during the follow-up period if abnormal cytology and/or persistent HPV infection were noted during the one year following CKC or LEEP. Follow-up visits occurred after 3, 6, and 12 months and every 12 months thereafter. The pathologic diagnosis was re-assessed by review of pathology slides by an experienced pathologist (CHY). The level of squamous intraepithelial lesion was assessed according to the 2014 World Health Organization classification [22]. Any grade of CIN on histological confirmation during the follow-up visit was defined as persistence/recurrence.

Finally, 652 patients were enrolled, from which 50 patients were found harboring CIN persistence/recurrence, and another 50 patients without CIN persistence/recurrence were identified to match them by propensity-score matching. Therefore, a total of 100 conization specimens were used to evaluate immunologic markers.

2. Antibodies and immunohistochemical staining

IHC staining for PD-1, PD-L1, and PD-L2 was performed on CIN tissue as follows. Paraffin tissue blocks were cut into 4-μm sections. A standard streptavidin-biotin-peroxidase complex method was used. After deparaffinization with xylene, rehydration was performed via a graded alcohol series and treatment with 3% hydrogen peroxide for 20 minutes for endogenous peroxidase blocking. Subsequently, antigen retrieval was accomplished using 10 mM citrate buffer (pH 6.0) for 20 minutes. Bond-maX autostainer (Leica, Wetzlar, Germany) was used for IHC studies. The antibodies used were as follows: anti-PD-1 antibody (1:100, clone NAT105, mouse monoclonal; Abcam, Cambridge, MA, USA), anti-PD-L1 antibody

https://doi.org/10.3802/jgo.2018.29.e27

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(1:200, clone 28-8, Rabbit monoclonal; Abcam), and anti-PD-L2 antibody (1:200, clone 176611, mouse monoclonal; R&D systems, Minneapolis, MN, USA). Human tonsillar tissue was used as a positive control for antibodies. PD-1 expression was assessed on intratumoral tumor infiltrating lymphocytes (TILs) and was considered positive if membranous staining could be seen.

PD-1-positive cells were counted in the 5 highest numbered high-power fields (HPFs). Normal or reactive hyperplastic human tonsil tissue was used as a positive control for PD-1.

For PD-L1 and PD-L2, IHC staining was assessed based on the intensity and proportion of membranous and/or cytoplasmic staining in dysplastic squamous epithelium and scored as follows: 0, negative; 1, weak or moderate in <10% of tumor cells; 2, moderate in ≥10% of tumor cells; 3, strong in ≥10% of tumor cells. An overall score of 2 or more was considered positive for PD-L1 or PD-L2 expression [23-25].

3. Statistical analysis

The clinicopathological parameters of age, parity, initial HPV load, colposcopy-directed biopsy, type of conization, conization pathology, conization depth, glandular involvement, and margin status were compared between patients with persistence/recurrence and those without persistence/recurrence using χ^2 and Fisher's exact tests. Odds ratio and 95% confidence interval (CI) were calculated using logistic regression analysis. The associations between PD-1, PD-L1, and PD-L2 expression and clinicopathological parameters were assessed using χ^2 test.

For propensity-score matching, following clinicopathological factors were applied: age, parity, initial HPV load, colposcopy-directed biopsy, type of conization, conization pathology, conization depth, glandular involvement, margin status. In addition, we used caliper method and the caliper width was 0.1.

For PD-1, differences in the number of PD-1+ TILs between the recurrence group and the non-recurrence group were compared using the Mann-Whitney U test. For PD-Ls, we used χ^2 and Fisher's exact tests to compare the levels of expression between the groups.

All p-values resulting from the tests of significance were 2-sided and were considered significant at $p<0.05$. Data analysis was performed using SPSS 20.0 (SPSS, Chicago, IL, USA).

RESULTS

A total of 652 patients were included in this study. According to our predefined criteria, 50 (7.7%) patients were classified as persistence/recurrence, and the remaining 602 (92.3%) had no persistence/recurrence after a median follow-up of 34 months (range: 2–73). The median age of the patients was 38 years (range: 15–78), and the baseline clinicopathological characteristics are listed in **Table 1**. An initial HPV test showed that high-risk HPV was positive in all patients, and the median HPV viral load was 88.7 (range: 1.1–66,647.7). The cut-off value of HPV load was designated as 1,000 RLU based on previous report [26]. In addition, the median conization depth in this study was 22 mm (range, 4–50), so patients were arbitrarily divided into 2 groups based on a conization depth of either ≤20 or >20 mm. Age, parity, colposcopy-directed biopsy, type of conization, conization pathology, conization

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Table 1. Baseline clinicopathological characteristics

Characteristics	No persistence/recurrence (n=602)	Persistence/recurrence (n=50)	p-value
Age (yr)			0.067
≤50	513 (85.2)	40 (80)	
>50	89 (14.8)	10 (20)	
Parity			0.442
0	170 (28.2)	10 (20)	
1	143 (23.7)	14 (28)	
≥2	289 (48.1)	26 (52)	
Initial HPV load (RLU)			0.012
1–1,000	475 (78.9)	28 (56)	
>1,000	127 (21.1)	22 (44)	
Colposcopy-directed biopsy			0.071
CIN1	106 (17.6)	9 (18)	
CIN2	155 (25.7)	20 (40)	
CIN3	341 (56.7)	21 (42)	
Type of conization			0.649
LEEP	317 (52.7)	28 (56)	
CKC	285 (47.3)	22 (44)	
Conization pathology			0.180
No residual dysplasia	62 (10.3)	1 (2)	
CIN1	90 (15)	8 (16)	
CIN2	89 (14.8)	11 (22)	
CIN3	361 (59.9)	30 (60)	
Conization depth (mm)			0.857
≤20	309 (51.3)	25 (50)	
>20	293 (48.7)	25 (50)	
Glandular involvement			0.190
Negative	116 (19.3)	15 (30)	
Positive	269 (44.6)	19 (38)	
N/A	217 (36.1)	16 (32)	
Margin status			<0.001
Negative	506 (84.1)	14 (28)	
Positive	75 (12.5)	35 (70)	
N/A	21 (3.4)	1 (2)	

Values are presented as number (%).
CIN, cervical intraepithelial neoplasia; CKC, cold knife conization; HPV, human papilloma virus; LEEP, loop electrosurgical excision procedure; N/A, not applicable; RLU, relative light unit.

depth, and glandular involvement were not significantly different between the 2 groups. In contrast, the proportion of patients with initial HPV load >1,000 RLU was significantly higher in the persistence/recurrence group than the no persistence/recurrence group (44% vs. 21%, p=0.012). Moreover, the proportion of patients with a positive surgical margin was also significantly higher in the persistence/recurrence group than the no persistence/recurrence group (70% vs. 12.4%, p<0.001).

Using univariate analysis, we found that age, parity, colposcopy-directed biopsy, type of conization, conization pathology, conization depth, and glandular involvement were not associated with persistence or recurrence of CIN after conization (**Table 2**). On the other hand, initial HPV load and margin status were significantly associated with CIN persistence or recurrence (p=0.039 and p<0.001, respectively). Using multivariate analysis, only margin status showed a statistically significant association with CIN persistence or recurrence (hazard ratio=8.86; 95% CI=1.67–16.81; p<0.001).

To evaluate the role of immune checkpoint molecules in disease persistence/recurrence, we found 50 patients with CIN persistence/recurrence and then matched them with 50 patients without CIN persistence/recurrence by propensity-score matching (**Table 3**). Univariate

Table 2. Univariate and multivariate analyses of clinicopathological parameters for persistence/recurrence after conization

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Age (>50 yr)	1.74 (0.94–3.22)	0.153		
Parity (≥2)	1.66 (0.72–3.86)	0.547		
Initial HPV load (>1,000 RLU)	4.93 (1.67–32.37)	0.039	1.14 (0.64–2.04)	0.118
Colposcopy-directed biopsy (CIN3)	1.52 (0.67–3.47)	0.618		
Type of conization (LEEP)	1.14 (0.64–2.05)	0.841		
Conization pathology (CIN3)	2.09 (0.83–5.23)	0.072		
Conization depth (≤20 mm)	1.06 (0.59–1.87)	0.549		
Glandular involvement (positive)	0.54 (0.27–1.11)	0.358		
Margin status (positive)	16.87 (8.67–32.82)	<0.001	8.86 (1.67–16.81)	<0.001

CI, confidence interval; CIN, cervical intraepithelial neoplasia; HPV, human papilloma virus; HR, hazards ratio; LEEP, loop electrosurgical excision procedure; RLU, relative light unit.

Table 3. Clinicopathological characteristics of patients after propensity-score matching

Characteristics	No persistence/recurrence (n=50)	Persistence/recurrence (n=50)	p-value
Age (yr)			0.195
≤50	41 (82)	40 (80)	
>50	9 (18)	10 (20)	
Parity			0.861
0	9 (18)	10 (20)	
1	14 (28)	14 (28)	
≥2	27 (54)	26 (52)	
Initial HPV load (RLU)			0.875
1–1,000	30 (60)	29 (58)	
>1,000	20 (40)	21 (42)	
Colposcopy-directed biopsy			0.715
CIN1	12 (24)	10 (20)	
CIN2	17 (34)	20 (40)	
CIN3	21 (42)	20 (40)	
Type of conization			0.686
LEEP	25 (50)	27 (54)	
CKC	25 (50)	23 (46)	
Conization pathology			0.904
No residual dysplasia	3 (6)	2 (4)	
CIN1	6 (12)	8 (16)	
CIN2	10 (20)	11 (22)	
CIN3	31 (62)	29 (58)	
Conization depth (mm)			0.840
≤20	24 (48)	25 (50)	
>20	26 (52)	25 (50)	
Glandular involvement			0.369
Negative	10 (20)	16 (32)	
Positive	22 (44)	19 (38)	
N/A	18 (36)	15 (30)	
Margin status			0.828
Negative	13 (26)	14 (28)	
Positive	34 (68)	34 (68)	
N/A	3 (6)	2 (4)	
PD-1			0.587
Negative	43 (86)	32 (64)	
Positive	7 (14)	18 (36)	
PD-L2			0.724
Negative	38 (76)	33 (66)	
Positive	12 (24)	17 (34)	

Values are presented as number (%).
CIN, cervical intraepithelial neoplasia; CKC, cold knife conization; HPV, human papilloma virus; LEEP, loop electrosurgical excision procedure; N/A, not applicable; PD-1, programmed death-1; PD-L2, programmed death-ligand-2; RLU, relative light unit.

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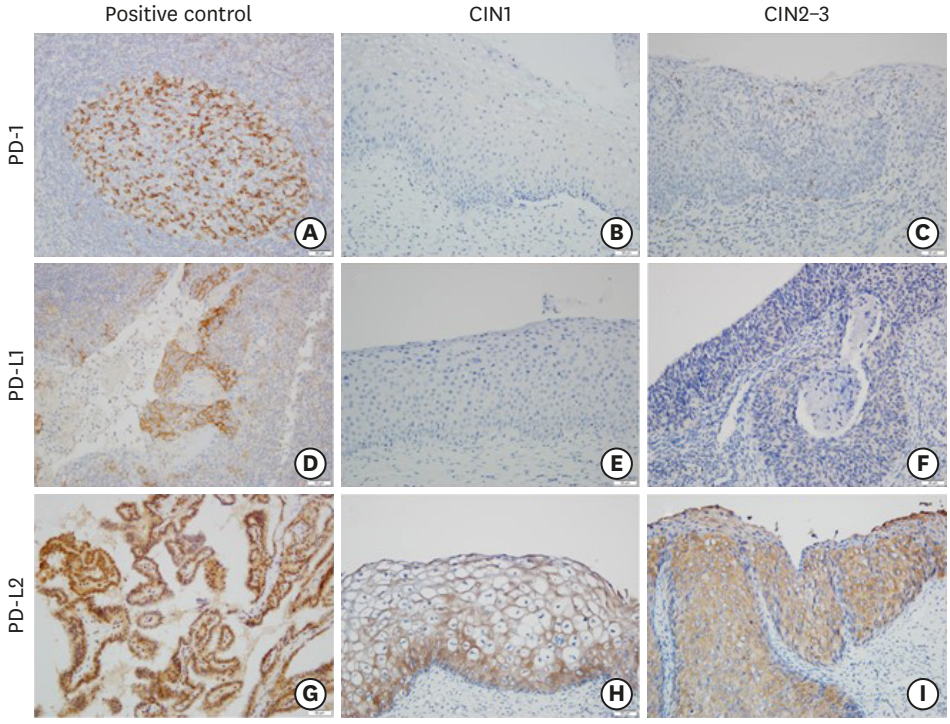

Fig. 1. Representative images of immunohistochemical staining for cervical intraepithelial neoplasms. Positive control: Human tonsil (A, D) and papillary thyroid carcinoma (G), negative for PD-1+ TIL (B), positive for PD-1+ TILs (C), no expression of PD-L1 (E, F), negative for PD-L2, score 1 (H), and positive for PD-L2, score 2 (I) (scale bars, 50 μm; original magnification, ×200).
CIN, cervical intraepithelial neoplasia; PD-1, programmed death-1; PD-L1, programmed death-ligand-1; PD-L2, programmed death-ligand-2; TILs, tumor infiltrating lymphocytes.

analysis was performed to find out which parameter was associated with CIN persistence/recurrence among 100 patients, but any parameter did not show a statistical significant association. As a result, age, parity, initial HPV load, colposcopy-directed biopsy, type of conization, conization pathology, conization depth, glandular involvement, and margin status were all well-balanced between the 2 groups. We analyzed PD-L1 and PD-L2 expression in the dysplastic squamous epithelium and PD-1 expression in the TILs by IHC in FFPE tumor specimens of the 100 patients (50 patients with persistence/recurrence, 50 patients without persistence/recurrence). The patient demographics and clinicopathological characteristics and their correlations with protein expression are summarized in **Table 4**. Representative IHC images are shown in **Fig. 1**. Of the 100 cases, 39 had glandular involvement, 20 cases from recurred patients, and 19 cases non-recurred. The mean depth of glandular involvement was 1.12 mm. The volume of CIN was estimated by the number of cervical quadrants. In 18 cases, the involved number of quadrants could not be determined due to fragmentation of tissue. One quadrant was involved by dysplasia in 25 cases, 2 quadrants in 24, 3 quadrants in 21, and 4 quadrants in 12 cases. None of the cases expressed PD-L1. PD-1 was expressed on TILs with a membranous pattern. PD-1+ TILs were observed in 25 of 100 patients (25%; 95% CI=16%–34%). PD-1+ TILs were significantly correlated with a higher grade of CIN (p=0.031). In addition, patients with persistence/recurrence had increased expression of PD-1+ TILs compared with those without persistence/recurrence (36% vs. 14%, respectively; p=0.020).

PD-L2 expression was noted in the dysplastic squamous epithelium with both membranous and cytoplasmic patterns. High PD-L2 expression was shown in 29 of 100 patients (29%;

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Table 4. Expression levels of PD-1 and PD-L2 and their associations with the clinicopathologic characteristics

Characteristics	Total papulation	PD-1 (TILs)			PD-L2		
		Negative	Positive	p-value	Negative	Positive	p-value
No. of patients		75	25		71	29	
Age (yr)				0.775			0.054
≤50	81	60 (80)	21 (84)		54 (76.1)	27 (93.1)	
>50	19	15 (20)	4 (16)		17 (23.9)	2 (6.9)	
Parity				0.775			0.260
≤2	81	60 (80)	21 (84)		55 (77.5)	26 (89.7)	
>2	19	15 (20)	4 (16)		16 (22.5)	3 (10.3)	
Initial HPV load (RLU)				0.440			0.211
1–1,000	35	27 (36)	8 (32)		30 (42.3)	5 (17.2)	
>1,000	65	48 (64)	17 (68)		41 (57.7)	24 (82.8)	
Pap smear				0.696			0.442
Negative	2	2 (2.7)	0		2 (2.9)	0	
ASCUS, LSIL	37	28 (37.3)	9 (36)		28 (39.4)	9 (31.1)	
ASC-H, HSIL	61	45 (60)	16 (64)		41 (57.7)	20 (68.9)	
Colposcopy-directed biopsy				0.774			0.415
Negative	0	0	0		0	0	
LSIL	20	16 (21.3)	4 (16)		16 (22.5)	4 (13.8)	
HSIL	80	59 (78.7)	21 (84)		55 (77.5)	25 (86.2)	
Conization pathology				0.031			0.041
No residual dysplasia	0	0	0		0	0	
LSIL	25	23 (30.7)	2 (8)		22 (30.9)	3 (10.3)	
HSIL	75	52 (69.3)	23 (92)		49 (69.1)	26 (89.7)	
Persistence/recurrence				0.020			0.378
Negative	50	43 (57.3)	7 (28)		38 (53.5)	12 (41.4)	
Positive	50	32 (42.7)	18 (72)		33 (46.5)	17 (58.6)	

Values are presented as number (%).
ASC-H, atypical squamous cells cannot exclude HSIL; ASCUS, atypical squamous cells of undetermined significance; HPV, human papilloma virus, HSIL; high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; PD-1, programmed death-1; PD-L2, programmed death-ligand-2; RLU, relative light unit; TILs, tumor infiltrating lymphocytes.

95% CI=20%–38%). There were no differences in PD-L2 expression by age, parity, initial HPV load, Pap smear results, and colposcopy-directed biopsy. PD-L2 expression in dysplastic squamous epithelium was not different among patients with persistence/recurrence and those without persistence/recurrence (34% vs. 24%, respectively; p=0.378). On the other hand, pathologic diagnosis of high-grade squamous intraepithelial lesion (HSIL) was significantly correlated with high PD-L2 expression (34.7% vs. 12%, respectively; p=0.041).

Among 50 patients who suffered persistence/recurrence of CIN during post-conization follow-up, 24 were diagnosed as CIN1, 9 CIN2, and 17 CIN3. Of them, 13 underwent re-conization, and 6 underwent hysterectomy. Of 9 patients with CIN2, 5 underwent re-conization and 2 hysterectomy. Of 17 patients with CIN3, 8 underwent re-conization and 4 hysterectomy. Based on the role of adverse predictors, long-term risk of persistence/recurrence of CIN was evaluated using Kaplan-Meier curve analysis (**Fig. 2**). The estimated rate of recurrence-free at 60 months was 40%.

DISCUSSION

Cervical conization achieves cure rates for high-grade CIN in excess of 95% [27]. Despite this high cure rate, 5.5%–31.6% of patients develop persistent/recurrent disease [28-31]. Consistent with those studies, the persistence/recurrence rates of CIN after conization in this study was 7.7% (50/652). Regular follow-up and effective treatment of persistence/recurrence

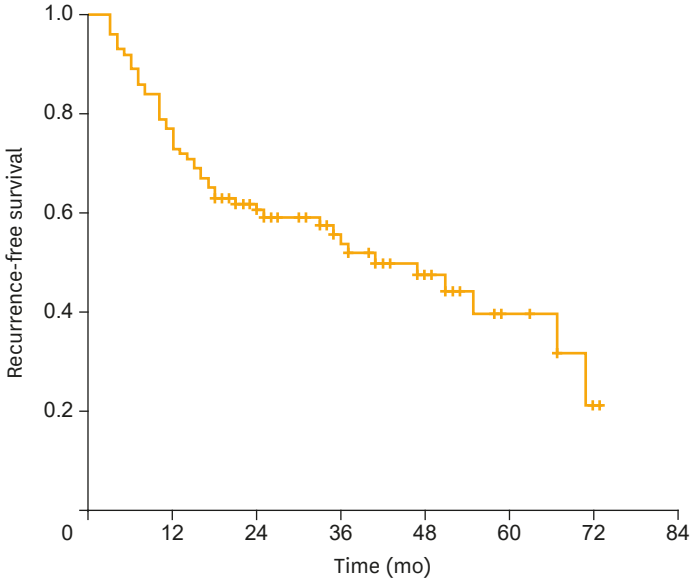

Fig. 2. Kaplan-Meier curve analysis of long-term risk of persistence/recurrence.

are crucial because of the risk of progression to invasive carcinoma in the absence of timely intervention. It is known that a substantial proportion of cervical cancer developed after prior treatment of CIN was attributed to loss of follow-up [32]. Therefore, identification of clinicopathological predictors of CIN persistence/recurrence will help identify the high-risk group so that clinicians can emphasize the importance of follow-up.

Although previously reported risk factors for persistence/recurrence following conization include age, parity, CIN grade, glandular involvement, and margin status, not all of these factors show consistent findings in the literature [12,13,33]. In this study, age, parity, CIN grade confirmed by either colposcopy-directed biopsy or conization, conization depth, and glandular involvement were not associated with an increased risk of persistence/recurrence of CIN after conization. In contrast, pre-conization HPV load and positive cone margin were associated with increased risk of disease persistence/recurrence. Alonso et al. reported that increased high-risk HPV load (>1,000 RLU) and positive cone margin were significantly associated with higher risk of recurrence [26]. Park et al. [34] also revealed that patients with pre-conization high-risk HPV load >100 RLU had a higher rate of persistent/recurrent disease after conization for CIN. Recently, 2 meta-analyses revealed that positive cone margin was a strong predictive factor of recurrent disease [35,36]. In our cohort, CIN persistence/recurrence developed in 45% of patients with positive cone margins, which is consistent with previous studies [11,12].

Binding of PD-1, which is expressed by activated T cells, with its ligands PD-L1 and PD-L2, which are expressed on antigen-presenting cells including tumor cells, down-modulates T cell function by inhibiting T cell activation via various pathways, resulting in a blockade of anti-tumor immune response of the host [37]. In this study, we found that high PD-1+ TILs and high PD-L2 expression were significantly correlated with higher grade CIN on conization specimens. In addition, high PD-1+ TIL expression was also significantly correlated with CIN persistence/recurrence. According to our results, we suggest that high PD-1 expression is related to a host immune response to HPV and plays an important role in recurrence associated with viral immunity. Similar to our report, Yang et al. [20] reported that high

https://doi.org/10.3802/jgo.2018.29.e27

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expression of PD-1 and PD-L1 expression on cervical T cells and DCs, respectively, was associated with high-risk HPV positivity and higher CIN grade.

PD-L2 expression has been reported in tumor cells, DCs, and a subset of B and T cells [38]. PD-L2 is known to play an important role in the Th2 response and is not a dominant mediator of T cell inhibition [38,39]. Unlike PD-L1, the mechanism by which PD-L2 affects tumor immune evasion remains unclear. Previously, expression of PD-L2 was reported in the esophagus [40], pancreas [41], ovary [42], and hepatocellular carcinoma [43], and an association with poor prognosis was reported in esophageal cancer. In this study, PD-L2 expression was significantly correlated with higher grade CIN on conization specimens and, to the best of our knowledge, this is the first study reporting the relationship between PD-L2 expression and persistence/recurrence of CIN after conization.

The PD-L1 antibody showed no immunoreactivity with the dysplastic squamous epithelium of the uterine cervix. As a positive control, we performed IHC staining of PD-L1 on human tonsils. Because immunoreactivity of anti-PD-L1 was reported in several invasive lesions of squamous epithelium including the esophagus [40] and uterine cervix [19], 5 cases of invasive squamous cell carcinoma samples were submitted for validation of IHC staining. Throughout those sections, we observed that at least 3 of the 5 cases exhibited more extensive immunoreactivity against the PD-L1 antibody at the invasive front of the carcinoma (**Supplementary Fig. 1**). According to a previous study analyzing 156 squamous cell carcinomas and 49 adenocarcinomas of the uterine cervix, PD-L1 positivity was observed in 54% of the squamous cell carcinomas and in 14% of all adenocarcinomas (cut-off: >5% of the tumor cells, $p<0.001$) [44]. Consistent with our results, PD-L1 expression was not demonstrated in high-grade or low-grade intraepithelial neoplasms of the esophagus, where 1.7% of esophageal adenocarcinoma tumor cells expressed PD-L1 [45]. Interestingly, Yang et al. [20] demonstrated expression of PD-L1 on DCs in CIN and reported a correlation between expression of PD-1 on T cells and PD-L1 on DCs and increasing CIN grades in patients with high-risk HPV infection, suggesting that the PD-1/PD-L1 pathway is associated with altered production of pro-inflammatory and anti-inflammatory cytokines. On the other hand, Mezache et al. [46] reported enhanced expression of PD-L1 in cervical intraepithelial neoplasms and invasive cervical cancers and demonstrated PD-L1 expression on HPV-infected cells using IHC and in situ hybridization analyses. They reported PD-L1 positivity in up to 95% of CIN1 or CIN2 lesions (when a “positive case” was defined as observed immunoreactivity in more than 10% of the tumor cells). Furthermore, Zhang et al. [47] noted that PD-L1 positivity by IHC staining was not significantly different between patients with CIN recurrence and those without (60% vs. 46%, respectively; $p=0.161$). However, it was significantly correlated with higher grade CIN. Based on the aforementioned contradictory studies, there is a need for further research on PD-L1 expression in intraepithelial lesions of the uterine cervix and its clinical significance on CIN persistence/recurrence.

Among 50 patients who suffered persistence/recurrence of CIN during post-conization follow-up, 24 were diagnosed as CIN1, 9 CIN2, and 17 CIN3. Of them, 13 underwent reconization, and 6 underwent hysterectomy. We performed IHC studies in recurrent lesions to compare the protein expression levels between the primary tumor and the recurrent tumor. Only 3 cases were available for IHC studies and they showed no significant differences (**Supplementary Fig. 2**). In a previous study analyzing PD-1, PD-L1, and PD-L2 expressions using salivary gland tumors, there also showed no statistically significant difference despite small numbers of cases [48]. More large-scale studies with larger numbers of cases will provide more validated results.

https://doi.org/10.3802/jgo.2018.29.e27

Our study sought to find out the clinicopathologic risk factors related to CIN persistence/recurrence with relatively large number of patients. As a result, positive margin of conization specimen, which was a well-known predictor of disease recurrence, was re-confirmed to be an independent prognostic factor for CIN persistence/recurrence. Furthermore, our study also shows the clinical utility of PD-1 and PD-L2 in predicting higher grade lesion as well as disease persistence/recurrence. Those findings might help identify subgroup of patients with a high-risk of persistence/recurrence and continue close surveillance.

In conclusion, positive surgical margin and expression of PD-1+ T cells are associated with persistence/recurrence of CIN after conization.

SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

Programmed death-ligand-1 expression at invasive front of squamous cell carcinoma samples.

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Supplementary Fig. 2

PD-1 and PD-L2 expressions in primary and recurrent tumors. Uterine cervical cone biopsy with high-grade squamous intraepithelial lesion with PD-1 (A, B) and PD-L2 (C, D) expression in primary (left) and recurrent tumor (right). No significant differences were observed.

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