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Impact of antiretroviral drugs on PD-L1 expression and copy number gains with clinical outcomes in HIV-positive and -negative locally advanced cervical cancers

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Abstract. Cervical cancer has become a leading cause of death in both HIV-infected and uninfected women. Previous studies have revealed that antiretroviral therapy (ART) possesses anti-human papillomavirus (HPV) and antitumour properties, potentially serving as an anticancer agent and improving functional immunity in HIV-positive individuals. However, to the best of our knowledge, no studies have examined the association between ART and the clinical outcome of patients with pre-existing invasive cervical cancer. The current study analysed 48 HIV-positive and 123 HIV-negative patients with locally advanced stage IB2-IVA cervical cancer between December 2008 and December 2016. Tumours were categorized based on programmed cell death-ligand 1 (PD-L1) immunoreactivity and copy number alterations in the PD-L1 gene, as determined by fluorescence *in situ* hybridization. The results revealed that ART-treated patients exhibited a lower prevalence of PD-L1 immunopositivity, PD-L1 amplification and polysomy compared with patients that did not receive ART and those that were HIV-negative. Furthermore, ART-treated patients with PD-L1 immunonegativity exhibited an improved recurrence-free survival (RFS) compared with patients that did not receive ART and HIV-negative individuals with PD-L1 immunopositivity (P=0.041 vs. P=0.030). Additionally, ART-exposed patients with PD-L1 disomy demonstrated improved locoregional recurrence-free survival (LRR) when compared with HIV-negative patients with PD-L1 amplification and polysomy (P=0.039 vs. P=0.007), RFS (P<0.001 vs. P=0.006) and cancer-specific survival (CSS) (P=0.021 vs. P=0.025). ART-exposed patients with PD-L1 disomy also exhibited improved RFS (P<0.001) and CSS (P<0.001) compared with HIV-negative patients with PD-L1 amplification. Improved LRRs were demonstrated in ART-exposed patients with PD-L1 disomy (P=0.028) compared with non-HIV patients with polysomy. Following multivariate analysis, International Federation of Gynaecology and Obstetrics stage and PD-L1 amplification were determined to be predictors of poor a RFS [hazard ratio (HR), 2.43; 95% confidence interval (CI), 1.37-4.30; P=0.002 vs. HR, 7.03; 95% CI, 2.79-17.74; P<0.001) and CSS (HR, 11.47; 95% CI, 4.70-27.99; P<0.001 vs. HR, 4.05; 95% CI, 1.64-9.98; P=0.002). However, only PD-L1 polysomy was determined to be a predictor of poor LRR (HR, 2.50; 95% CI, 1.11-5.63; P=0.027). HIV status was not associated with poor outcomes, as determined using Cox models. The results of the current study indicated that ART may be used for the treatment of cervical cancer in both HIV-infected and uninfected patients. However, additional research is required to further elucidate these results.

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Introduction

Cervical cancer is one of the most common cancer types in women living with human immunodeficiency virus (HIV) (1). Most patients present with locally advanced disease (2), defined as stages IB2-IVA by the International Federation of Gynaecology and Obstetrics (FIGO), and concurrent chemo-radiation remains the standard of treatment for these patients. However, the majority of recurrences occur within two years after treatment (3,4). A defective immune surveillance might contribute to poor outcomes. Theoretically, tumours can evade immune surveillance by upregulating programmed cell death-ligand 1 (PD-L1) expression. PD-L1 is known to play a key role in the inhibition of T cell-mediated immune responses, leading to the progression of tumours. PD-L1 on malignant cells is often upregulated within the cancer

microenvironment (5). Several mechanisms contributing to the upregulation of PD-L1 on malignant cells, including epigenetic factors, oncogenic signalling and acquired immune responses, have been identified. Constitutive oncogenic signalling has been discovered to induce PD-L1 expression on malignant cells either through the phosphatidylinositol-3-kinase-protein kinase B (PI3K-AKT) pathway or signal transducer and activator of transcription (STAT) 3 signalling (6,7). In addition, the acquired immune response is considered to manifest through PD-L1 upregulation on malignant cells by endogenous antitumour immunity-related factors in the cancer microenvironment, such as interferon-γ (IFN-γ) produced by tumour-infiltrating lymphocytes (8).

PD-L1 overexpression has been identified in many solid cancer types (9), such as malignant melanoma (10), pulmonary cancer (11) and colorectal cancer (12). Wu *et al* (9) demonstrated that PD-L1 overexpression is related to worse overall survival in gastric carcinoma, hepatocellular carcinoma, oesophageal carcinoma, and transitional cell carcinoma, whereas this relationship is not present in pulmonary cancer and malignant melanoma.

Interestingly, amplification of chromosome 9p24.1 has recently been demonstrated as an essential mechanism for increased PD-L1 protein expression in nodular sclerosing classical Hodgkin lymphoma and primary mediastinal large B-cell lymphoma (13). Consequently, 9p24.1 gene locus amplification has been discovered in subsets of colorectal carcinoma, triple-negative breast cancer, glioblastoma and gastric adenocarcinoma (14,15).

More recently, the genetic basis of increased PD-L1 expression was identified in cervical and vulvar squamous cell carcinoma. The genes encoding PD-L1 and PD-L2, *CD274* and *PDCD1LG2*, respectively, were coamplified or overexpressed due to chromosomal gains in 67% of cervical and 43% of vulvar squamous cell carcinoma cases assessed by fluorescence in situ hybridization (FISH) (16). The data show that 9p24.1 gene copy number alterations are an important mechanism of increased PD-L1 expression in cervical squamous cell carcinoma. However, this study did not investigate the correlation of genetic changes with clinical outcomes.

In the highly active antiretroviral therapy era, several studies have demonstrated that the incidence of AIDS-defining cancer among HIV-positive patients has significantly decreased over the past few decades (17-19). Regarding cervical cancer, a recent meta-analysis showed a reduction in the incidence and progression of cervical intraepithelial neoplasia and the incidence of invasive cervical cancer after antiretroviral therapy (ART) (20). However, the interactions between ART and high-risk human papillomavirus (HPV) and invasive cervical cancer in HIV-positive patients are poorly understood. Several previous studies have shown that ART possesses anti-HPV and anticancer properties in addition to improving functional immunity (21).

Several antitumour mechanisms have been discovered, including inhibition of angiogenesis, invasion of cancer cells and induction of apoptosis (21). Consequently, ART might hold promise for treating cancer. Furthermore, it is possible that ART might participate in a variety of anticancer mechanisms and associate with PD-L1 expression via the downregulation of common signalling pathways or cytokines. Hence, we aimed to explore this relationship using PD-L1, a prognostic and predictive biomarker in various solid tumours. The associations of ART, PD-L1 protein expression, and PD-L1 gene copy number status with clinical outcomes were studied by comparing ART-exposed subjects with ART-unexposed controls.

Materials and methods

Patients. The retrospective cohort consisted of 48 HIV-infected patients and 123 uninfected controls with International Federation of Gynaecology and Obstetrics stage (FIGO) stage IB2-IVA cervical cancer who underwent tissue biopsies of squamous cell carcinoma and adenocarcinoma of the cervix between December 2008 and December 2016 at the Faculty of Medicine, Navamindradhiraj University, the National Cancer Institute, and Rajavithi Hospital. The present study was approved by the Institutional Review Boards of Navamindradhiraj University, the National Cancer Institute, and Rajavithi Hospital. All patients provided informed consent. H&E-stained sections were reviewed by two pathologists (KL and NP). Complete clinicopathologic data were available for all patients. The inclusion and exclusion criteria are described below.

Inclusion criteria: Subjects were eligible if they i) had stage IB2-IVA cervical cancer; ii) were HIV-positive and had previously been exposed to ART more than one year before the diagnosis of cervical cancer (classified as the ART-exposed group); and iii) were HIV-positive and had never been exposed to ART before cervical cancer diagnosis (classified as the ART-untreated group).

Exclusion criteria: Subjects were excluded from the study for the following reasons: i) Previous exposure to chemoradiation therapy before cervical cancer diagnosis; ii) known history of the following underlying illnesses (autoimmune diseases, diabetes mellitus, and hepatitis B or C virus coinfection); iii) taking immunosuppressive or antituberculous drugs within one year before the diagnosis of cervical cancer; and iv) presence of synchronous or metachronous malignancy.

Immunohistochemistry. Immunohistochemistry (IHC) was performed in all cases with a monoclonal antibody recognizing PD-L1. Whole tissue sections (4 μm) were cut and stained for PD-L1 (clone SP263; Ventana Medical Systems, Inc.) on an automated staining platform (Benchmark ULTRA; Ventana Medical Systems, Inc.). An OptiView DAB IHC Detection Kit (Ventana Medical Systems, Inc.) was used according to the manufacturer's instructions for the visualization of the primary anti-PD-L1 antibody. Human placental tissue was used as a positive control in all immunohistochemical reactions. Immunohistochemical expression of PD-L1 in malignant cells was evaluated by counting the proportion of positive malignant cells and quantifying IHC staining intensity in a 4-tiered scoring system according to Hofmann's criteria (22) as follows: Score 0 indicated no appreciable staining or staining in less than 10% of malignant cells; score 1+ indicated weak appreciable partial membranous staining in >10% of malignant cells; score 2+ indicated moderate complete membrane staining in >10% of malignant cells; and score 3+ indicated intense complete membrane staining in >10% of malignant

cells. Based on a previous study involving other malignancies (23), tumours with 5% or more cells showing positive PD-L1 staining, regardless of the intensity, were considered 'positive'.

For p16 IHC, 4-μm tissue microarray (TMA) sections were cut from formalin-fixed paraffin-embedded tissue, and IHC was performed on a Leica Bondmax platform (Leica Micro-systems) according to the manufacturer's instructions. Mouse monoclonal anti-p16 (clone JC8, 1:600 dilution; Santa Cruz Biotechnology) was used as a primary antibody. p16 IHC was scored as positive if there was strong and diffuse nuclear and cytoplasmic staining present in greater than 70% of malignant cells.

PD-L1 fluorescence in situ hybridization. Tissue microarrays (TMAs) with 3 mm core diameter were obtained from representative cervical cancer tissues. Dual-colour FISH analysis was performed on 4 μm FFPE TMA sections. The SPEC CD274, PDCD1LG2/CEN9 Dual Color Probe (Zytovision) was used according to the manufacturer's guidelines.

At least 50 malignant cells were detected based on DAPI-stained nuclei. PD-L1 amplification was defined as a PD-L1/CEP9 ratio ≥2.0. Polysomy was defined as a mean copy number of PD-L1 ≥3.0, with a PD-L1/CEP9 ratio <2.0. All other instances were considered disomy as previously reported (24).

Statistical analysis. Statistical analysis was performed using Stata Statistical Software (College Station, TX: StataCorp LP; http://www.stata.com). The distribution of qualitative data was compared between groups using χ^2 tests or Fisher's exact tests, depending on the cell counts of corresponding contingency tables. For survival analysis, the Kaplan-Meier method was used to compute recurrence-free survival (RFS), cancer-specific survival (CSS), and locoregional recurrence-free survival (LRR). Univariate and multivariate analyses were performed using Cox proportional hazards models, and the differences between groups were analysed using the log-rank test. For all statistical analyses, P<0.05 was considered statistically significant.

Results

Patient Characteristics. The clinicopathological characteristics of the cervical cancer patients in the HIV-positive or HIV-negative cohorts are shown in Table I. The median follow-up time was 40 (range: 1-120) months for the HIV-positive cohort and 28 (range: 2-82) months for the HIV-negative cohort. The median CD4 count was 312 (interquartile range (IQR): 158.5-439.0). Among the HIV-positive patients (n=48), there was no significant difference in the mean age of ART-exposed patients (n=23) and ART-untreated patients (n=25) [43.70 (9.07) vs. 40.68 (9.83) years; P=0.276]. The median time on ART was 21 (range: 12.5-91) months. Compared to ART-untreated patients, ART-exposed patients (n=23) usually had FIGO stage IB2-IIB disease (82.6 vs. 48.0%; P=0.012), increased CD4 counts (74.0 vs. 40.0%; P=0.022), undetectable viral loads (82.6 vs. 40.0%; P=0.003), reduced tumour sizes (34.8 vs. 4.0%; P=0.009) and reduced likelihood of having parametrial invasion (52.2 vs. 92.0%; P=0.002). There were no differences in the histologic subtypes of squamous cell carcinoma (73.9 vs. 92.0%; P=0.130), presence of metastatic lymph nodes (30.4 vs. 36.0%; P=0.683) or use of radio (chemo) therapy (100.0 vs. 96.0%; P=1.000) between the two groups. No significant correlation was observed between the NRTI+NNRTI group and the NRTI+PI group with regard to patient age [40.69 (10.14) vs. 41.43 (5.97) years; P=0.441], higher CD4 counts (75.0 vs. 71.4%; P=1.000), undetectable viral loads (81.2 vs. 85.7%; P=1.000), FIGO stage 1B2-IIB disease (81.3 vs. 85.7%; P=1.000), tumour size ≥4 cm (75.0 vs. 42.9%; P=0.182), the presence of parametrial invasion (75.0 vs. 71.4%; P=1.000), histologic subtype of squamous cell carcinoma (68.8 vs. 85.7%; P=0.621), or the presence of metastatic nodes (37.3%; P=0.366). Additionally, ART-exposed patients had younger age [median age, 43.70 (9.07) vs. 55.15 (12.67) years; P<0.001], more likelihood of FIGO stage IB2-IIB disease (82.6 vs. 62.6%; P=0.063), and lower prevalence of parametrial invasion (52.2 vs. 79.7%; P=0.005) than HIV-negative patients (n=123). No other correlations were observed between the two groups.

Status of PD-L1 expression. For the entire cohort, PD-L1 expression in at least 5% of tumour cells was identified in 130/171 (76%) of cervical carcinoma cases. The mean percentage of positive tumour cells (any intensity of staining) was 60% (range: 15-90%). Strong membranous staining (3+) was identified in 24/171 (14%) cases, moderate staining (2+) in 46/171 (27%) cases, and weak staining (+1) in 39/171 (23%) cases (Fig. 1). Fig. 3 shows the proportion of PD-L1 immunoreactivity in each patient group. There was a significant difference in PD-L1 overexpression between the HIV-positive cohort and HIV-negative cohort (56.3 vs. 83.7%; P<0.001). Among the HIV-positive patients, compared to ART-untreated patients, ART-exposed patients showed a significant decrease in PD-L1 protein expression (26.1 vs. 84%; P<0.001). Additionally, ART-exposed patients had a lower prevalence of PD-L1 immunopositivity than HIV-negative patients (26.1 vs. 83.7%; P<0.001).

Status of p16 expression. P16 positivity was not altered by the HIV status, ART use, or antiretroviral drug regimen. All cases in the HIV cohort displayed p16 immunopositivity, whereas nearly all cases in the HIV-negative cohort were p16-positive except for two cases of adenocarcinoma. No significant correlation was observed in any of the groups with regard to any of the relevant parameters mentioned above (data not shown).

Status of PD-L1 gene copy number alterations. Overall, 12/171 (7%) tumours were positive for amplification. Gene copy number gain was restricted to tumour cells and was not present in the inflammatory cell component. Polysomy was observed in 60/171 (35%) cases. A total of 99/171 (58%) cases were disomic for the PD-L1 gene locus at 9p24.1 (Fig. 2). Fig. 3 shows the proportion of PD-L1 copy number alterations in each patient group. There was no significant difference in PD-L1 amplification, polysomy, or disomy (64.6 vs. 55.3%, 27.1 vs. 38.2% and 8.3 vs. 6.5%, respectively; P=0.387) between the HIV-positive cohort and the HIV-negative cohort. Among the HIV-positive cohort, ART-exposed patients had a lower prevalence of amplification (0 vs. 28.6%;

Table I. Clinicopathological characteristics of patients with locally advanced cervical cancer (n=171).

	HIV					ART regimen		
Variable	ART-treated (n=23)	ART-untreated (n=25)	Non-HIV (n=123)	P-value[a]	P-value[b]	NRTI+NNRTI (n=16)	NRTI+PI (n=7)	P-value[c]
Age (years)								
<60	22 (95.7)	23 (92.0)	81 (65.9)	>0.05	0.004	15 (93.8)	7 (100.0)	>0.05
≥60	1 (4.3)	2 (8.0)	42 (34.1)			1 (6.3)	0 (0.0)	
Histology								
SCC	17 (73.9)	23 (92.0)	110 (89.4)	0.130	0.083	11 (68.8)	6 (85.7)	0.621
Adeno	6 (26.1)	2 (8.0)	13 (10.6)			5 (31.3)	1 (14.3)	
Tumor size (cm)								
<4	8 (34.8)	1 (4.0)	30 (24.4)	0.009	0.297	4 (25.0)	4 (57.1)	0.182
≥4	15 (65.2)	24 (96.0)	93 (75.6)			12 (75.0)	3 (42.9)	
FIGO stage								
Stage IB2-IIB	19 (82.6)	12 (48.0)	77 (62.6)	0.012	0.063	13 (81.3)	6 (85.7)	>0.05
Stage IIIA-IVA	4 (17.4)	13 (52.0)	46 (37.4)			3 (18.8)	1 (14.3)	
CD4 count (cells/μl)								
≤350	6 (26.0)	15 (60.0)		0.022		4 (25.0)	2 (28.6)	>0.05
>350	17 (74.0)	10 (40.0)				12 (75.0)	5 (71.4)	
HIV viral load (copies/μl)								
Undetectable	19 (82.6)	10 (40.0)		0.003		13 (81.2)	6 (85.7)	>0.05
≥50	4 (17.4)	15 (60.0)				3 (18.8)	1 (14.3)	
Parametrial invasion								
Absent	11 (47.8)	2 (8.0)	25 (20.3)	0.002	0.005	8 (50.0)	3 (42.9)	>0.05
Present	12 (52.2)	23 (92.0)	98 (79.7)			8 (50.0)	4 (57.1)	
Metastatic lymph node								
Absent	16 (69.6)	16 (64.0)	93 (75.6)	0.683	0.541	10 (62.5)	6 (85.7)	0.366
Present	7 (30.4)	9 (36.0)	30 (24.4)			6 (37.5)	1 (14.3)	
Treatment								
CTRT	23 (100.0)	24 (96.0)	119 (96.7)	>0.05	>0.05	16 (100.0)	7 (100.0)	
Radical RT	0 (0.0)	1 (4.0)	4 (3.3)			0 (0.0)	0 (0.0)	

Values are presented as n (%). Data are presented as the mean ± standard deviation or the median (interquartile range). [a]ART-treated group vs. the ART-untreated group; [b]ART-treated group vs. the non-HIV group; [c]NRTI+NNRTI group vs. the NRTI+PI group. SCC, squamous cell carcinoma; Adeno, adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; CTRT, concurrent chemoradiotherapy; RT, radiation therapy; ART, antiretroviral therapy; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

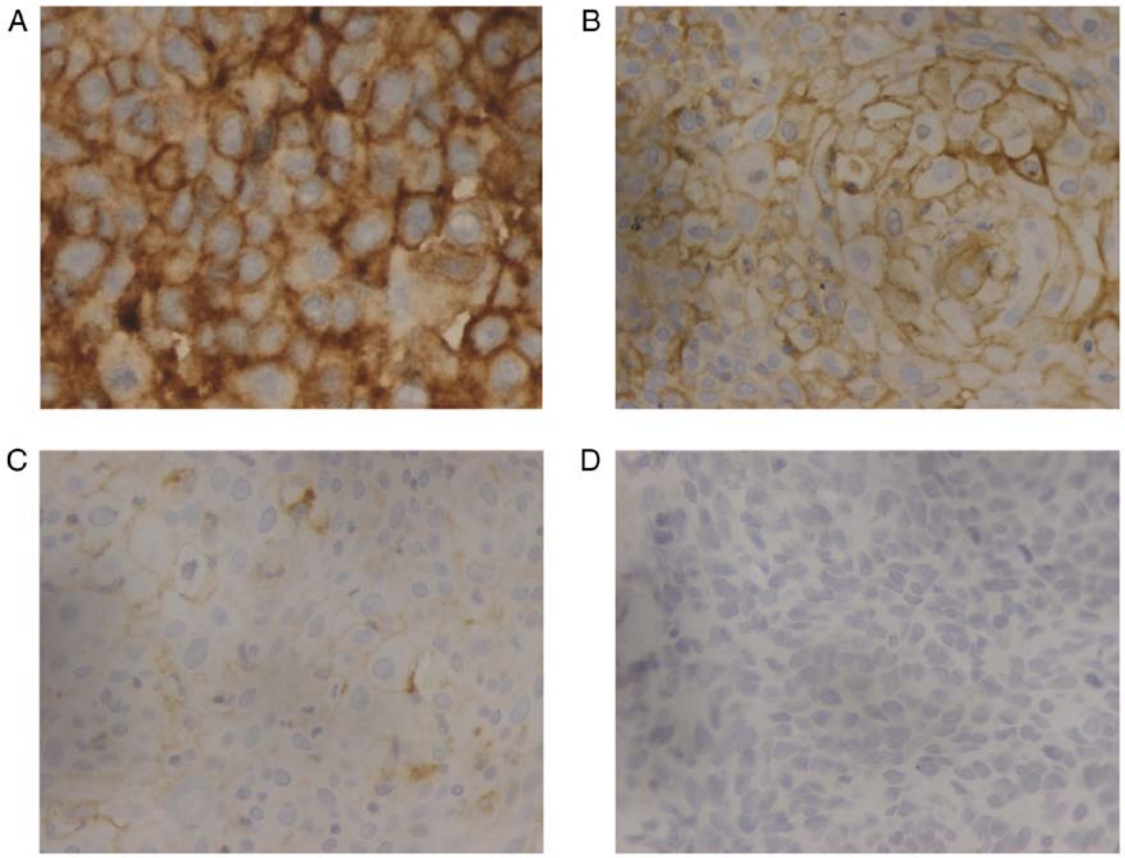

Figure 1. Intensities of PD-L1 immunohistochemical reactions (original magnification, x60). (A) Representative image of 3+ intensity staining, revealing a strong circumferential cell membrane reaction. (B) Representative image of intensity 2+, exhibiting moderate complete circumferential cell membrane staining. (C) Intensity 1+, presenting weak appreciable partial cell membrane staining and (D) intensity 0, presenting no cell membrane reaction. PD-L1, programmed cell death-ligand 1.

P=0.019) and polysomy (8.7 vs. 52.4%; P=0.002) and a higher prevalence of disomy (91.3 vs. 40%; P<0.001) than their ART-untreated counterparts. Additionally, ART-exposed patients had a lower prevalence of polysomy (8.7 vs. 40.9%; P=0.003) and a higher prevalence of disomy (91.3 vs. 55.3%; P=0.001) than HIV-negative patients. There were no differences in amplification between the two groups (0 vs. 10.5%; P=0.195).

Correlation between PD-L1 copy number gain and PD-L1 protein expression. Results for both PD-L1 immunohistochemistry and PD-L1 FISH are shown in Table II. Tumours with PD-L1 gene amplification and polysomy displayed membranous PD-L1 immunostaining (scores 1+ to 3+) by immunohistochemistry in 11/12 (92%) and 46/60 (76%) cases, respectively. A significantly higher frequency of cases with PD-L1 amplification were PD-L1 immunopositive than cases without amplification (92 vs. 61.6%; P=0.03). Likewise, the proportion of PD-L1 immunopositive tumours with PD-L1 polysomy were significantly higher than those of tumours with disomy (76.7 vs. 52.5%; P=0.002). Furthermore, 7/12 carcinoma cases with strong membranous PD-L1 immunostaining (score 3+) showed PD-L1 amplification, 11/60 showed a polysomy, and 6/99 cases displayed a disomy.

Survival outcomes. Fig. 4 shows the Kaplan-Meier survival curves for exposed (ART-exposed) vs. unexposed ART (ART-untreated and HIV-negative) patients, according to the IHC-based and FISH-based expression status of PD-L1 in tumours. Overall, ART-exposed patients had longer survival with regard to LRR, RFS, and CSS than ART-unexposed patients. The results of univariate and multivariate analyses evaluating the impact of various known prognostic factors on LRR, RFS and CSS are summarized in Table III (Tables SI-III).

For the entire cohort, FIGO stage (HR, 2.87; 95% CI, 1.76-4.69; P<0.001 vs. HR, 14.73; 95% CI, 6.18-35.09; P<0.001), tumour size (HR, 2.30; 95% CI, 1.17-4.52; P=0.016 vs. HR, 6.79; 95% CI, 1.64-28.08; P=0.008), nodal status (HR, 1.98; 95% CI, 1.20-3.27; P=0.007 vs. HR, 2.19; 95% CI, 1.19-4.03; P=0.012), PD-L1 amplification (HR, 8.37; 95% CI, 3.67-19.12; P<0.001 vs. HR, 7.46; 95% CI, 3.15-17.69; P<0.001) and polysomy (HR, 2.39; 95% CI, 1.44-3.99; P=0.001 vs. HR, 2.08; 95% CI, 1.07-4.05; P=0.031) were univariately associated with RFS and CSS. Nevertheless, on multivariate analysis, FIGO stage and PD-L1 amplification continued to show a significant impact on RFS (HR, 2.43; 95% CI, 1.37-4.30; P=0.002 vs. HR, 7.03; 95% CI, 2.79-17.74; P<0.001) and CSS (HR, 11.47; 95% CI, 4.70-27.99; P<0.001 vs. HR, 4.05; 95% CI, 1.64-9.98; P=0.002). FIGO stage and PD-L1 polysomy showed a significant impact on LRR in univariate analysis (HR, 2.55; 95% CI, 1.23-5.28; P=0.012 vs. HR, 3.46; 95% CI, 1.61-7.45; P=0.002); however, only PD-L1 polysomy remained an independent predictor of LRR in the multivariate analysis (HR, 2.50; 95% CI, 1.11-5.63; P=0.027). In subgroup analyses,

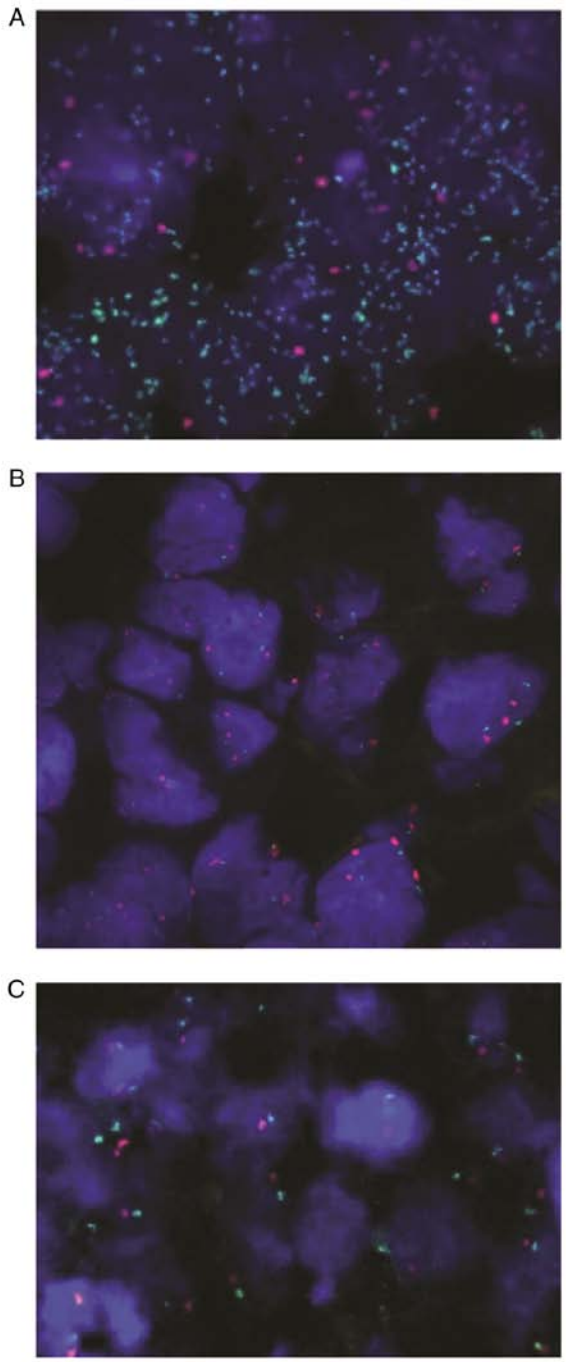

Figure 2. FISH analysis of the PD-L1 gene locus. (A) Amplification of the PD-L1 gene locus, (B) PD-L1 polysomy and (C) PD-L1 disomy. Green indicates the PD-L1 gene and red indicates centromere 9. FISH, fluorescence in situ hybridization; PD-L1, programmed cell death ligand 1.

ART exposure was univariately correlated with CSS (HR, 0.21; 95% CI, 0.04-0.96; P=0.044) in the HIV-positive cohort; however, no significant difference was observed in the multivariate analysis (HR, 0.55; 95% CI, 0.12-2.63; P=0.455).

There was no significant difference in LRR, RFS, or CSS between the ART-exposed group and the HIV-negative group (HR, 0.26; 95% CI, 0.03-1.94; P=0.187, HR, 0.60; 95% CI, 0.24-1.47; P=0.265, and HR, 0.50; 95% CI, 0.12-2.20; P=0.362, respectively) or between the ART-untreated group and the HIV-negative group (HR, 0.95; 95% CI, 0.36-2.5; P=0.913, HR, 1.07; 95% CI, 0.55-2.09; P=0.833, and HR, 1.09; 95% CI, 0.49-2.42; P=0.826, respectively) on multivariate analysis. The data demonstrated that HIV status was not associated with worse outcomes in Cox models.

Discussion

In the present study, our results showed an association between ART and PD-L1 expression. We found that ART-exposed patients had a significantly lower prevalence of PD-L1 protein expression, PD-L1 amplification and polysomy, and better clinical outcomes than ART-untreated HIV-positive and HIV-negative patients. As reported in earlier studies (25), HIV-positive women tended to have aggressive cervical cancer with a poor prognosis. Nevertheless, a recent analysis of surveillance data pertaining to the post-antiretroviral era showed a comparable prognosis for HIV-positive and HIV-negative populations (18). Although several factors that contribute to poor prognosis of cervical cancer in HIV-positive patients have been proposed, the definite aetiology in this respect has not been clearly identified. From the standpoint of anticancer immunity, suppression of the T cell-mediated anticancer immune response is likely to underlie the association between HIV infection and poor prognosis for cervical cancer.

In addition to improving functional immunity, ART could exert a combined effect on oncogenic HPV infection and cervical cancer. Different antiretroviral drug combinations may show a wide spectrum of activity and improved potency (i.e., synergistic or additive effects) against HPV infection or cancer cells. In a recent meta-analysis, Kelly *et al* demonstrated that ART may reduce the risk for cervical cancer and its precursor lesions in women living with HIV (20). Interestingly, these effects remained after adjusting for immune restoration indicators, such as CD4 cell count and duration of ART use.

In vitro studies have shown that lopinavir in some ART regimens may have activity against oncogenic HPV through the inhibition of the viral oncogene E6 (26). Several studies have shown that protease inhibitors (PIs) and other anti-HIV drugs possess several pleiotropic anticancer properties, including inhibition of cancer cell invasion, angiogenesis, inflammatory cytokine production, and proliferation and induction of apoptosis (21,27). Several intracellular signalling pathways have been identified, and some of these pathways might be linked to PD-L1 expression.

An *in vitro* study in cultured squamous cell carcinoma of the head and neck (SCCHN) cell lines demonstrated that PD-L1 expression is significantly upregulated in response to interferon-γ (IFN-γ), a key cytokine triggering *de novo* PD-L1 induction in tumour cells and normal tissues (28).

PD-L1 expression can be stimulated by autocrine/paracrine mediators within the cancer microenvironment, especially IFN-γ. Interactions between extrinsic stimuli and the IFN-γ receptor could lead to the expression and activation of various downstream signalling pathways, including nuclear factor-κ light chain enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR) and Janus kinase/signal transducer and activator of transcription (JAK/STAT), which promote cell cycle progression and the activation of transcription factors. Such signalling pathways

Table II. PD-L1 FISH and PD-L1 IHC.

PD-L1 FISH	Cases (n=171)	PD-L1 IHC Score 3+	PD-L1 IHC Score 2+	PD-L1 IHC Score 1+	PD-L1 IHC Score 0
Amplification	12 (7%)	7/12	2/12	2/12	1/12
Polysomy	60 (35%)	11/60	18/60	17/60	14/60
Disomy	99 (58%)	6/99	26/99	20/99	47/99

PD-L1, programmed cell death ligand 1; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.

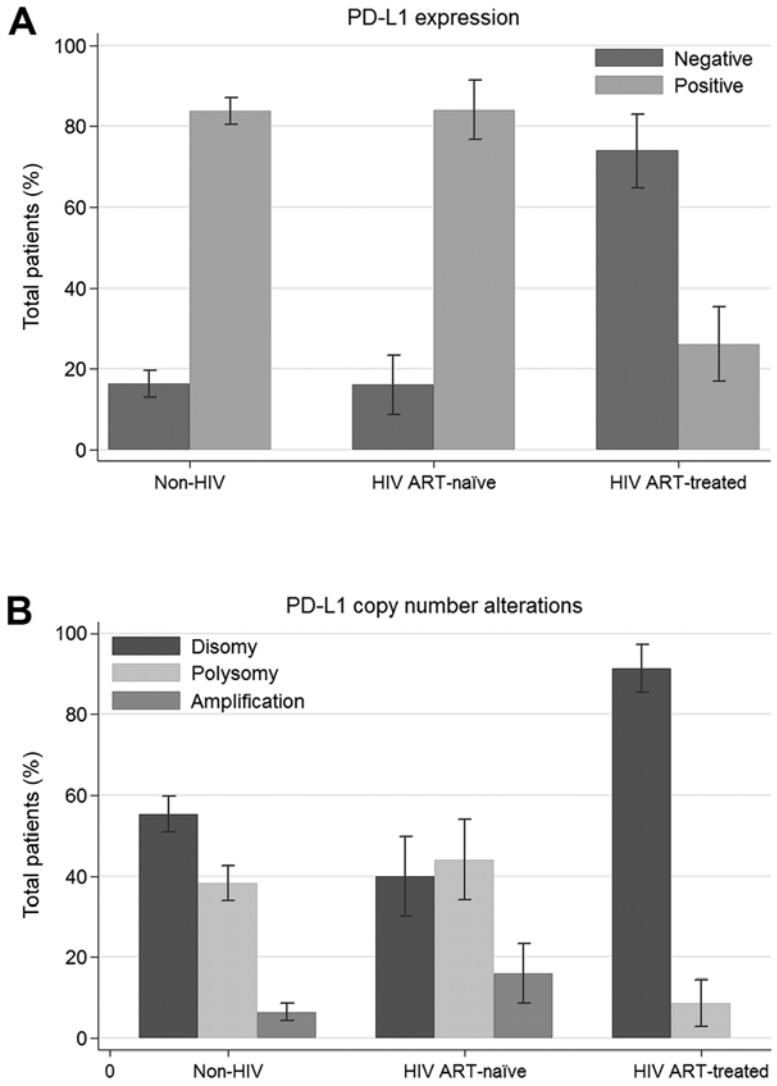

Figure 3. PD-L1 immunoreactivity and PD-L1 copy number alterations in non-HIV patients, ART-naïve patients and ART-treated patients with cervical carcinomas. (A) Percentage of PD-L1 immunoreactivity across patient groups. (B) Percentage of PD-L1 copy number alterations (disomy, polysomy and amplification) across patient groups. Data are presented as the mean ± standard error of the mean. PD-L1, programmed cell death ligand 1; ART, antiretroviral therapy.

further regulate the nuclear translocation of transcription factors to the PD-L1 promoter (29).

In the setting of HIV infection, several cytokines are produced by infected cells and cells of the immune system. Both innate and adaptive immune responses are activated during the disease course. $CD4^+$ T helper cells play a crucial role in the immune system by secreting cytokines that regulate the immune response. Th1 $CD4^+$ subsets produce IL-2 and IFN-γ. IFN-γ acts by stimulating macrophages and is important for eliminating intracellular pathogens (30). Previous studies conducted by De Luca *et al* documented that the production of various inflammatory cytokines [macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), regulated on activation, normal T cell expressed and secreted (RANTES), and IFN-γ] can be significantly inhibited at 8 weeks and partially recovered at 24 weeks after the commencement of

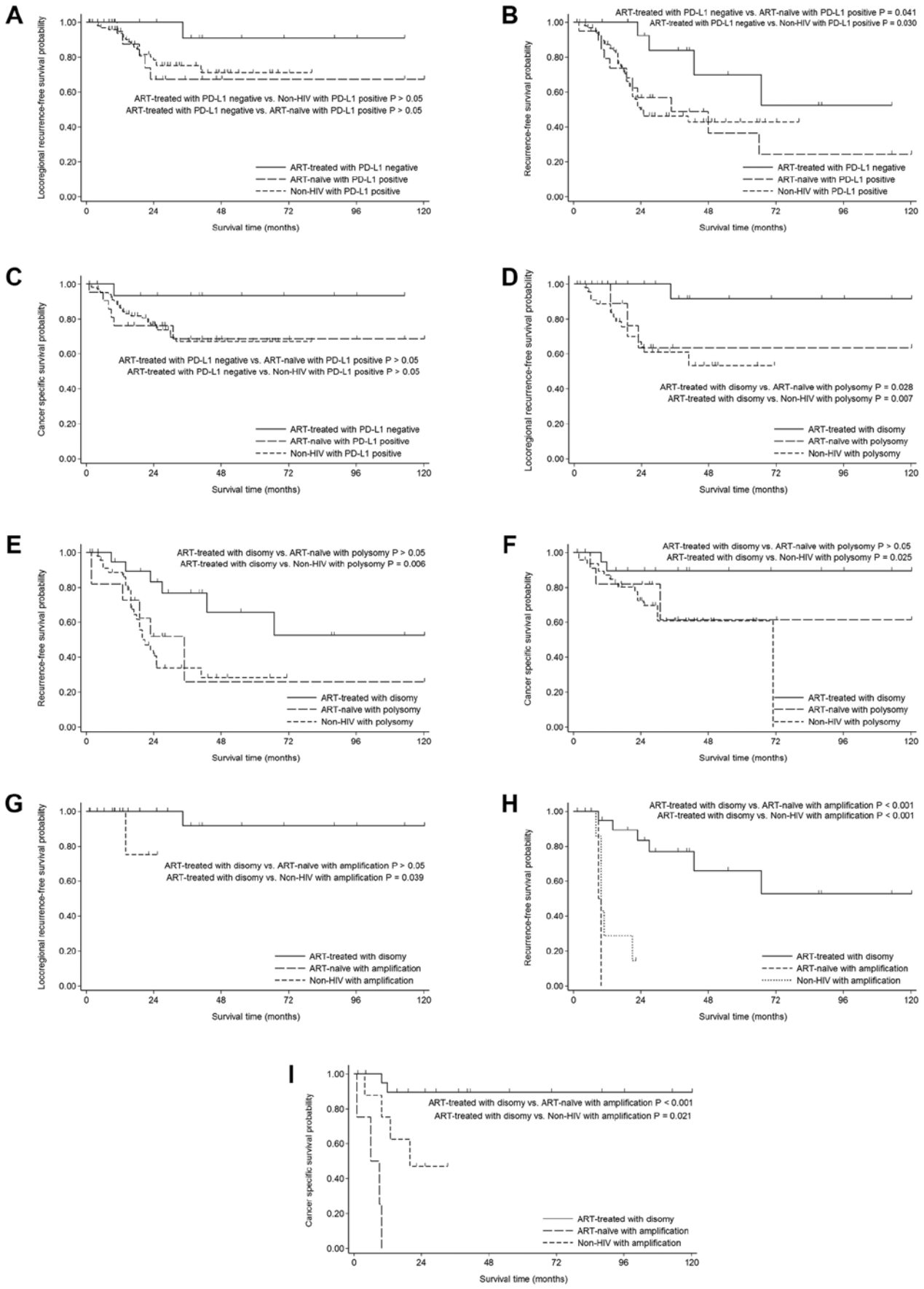

Figure 4. Kaplan-Meier survival curves for exposed vs. unexposed ART patients in relation to PD-L1 immunoreactivity and genetic category. (A) LRR (B) RFS, and (C) CSS based on exposed ART patients that are PD-L1 negative vs. unexposed ART patients that are PD-L1 positive. (D) LRR, (E) RFS and (F) CSS based on exposed ART patients with disomy vs. unexposed ART patients with polysomy. (G) LRR, (H) RFS and (I) CSS based on exposed ART patients with disomy vs. unexposed ART patients with amplification. ART, antiretroviral therapy; PD-L1 programmed cell death ligand 1; LRR, locoregional recurrence-free survival; RFS, recurrence-free survival; CSS, cancer-specific survival.

Table III. Univariate and multivariate survival analysis (n=171).

Variables	Number (n)	Univariate analysis (HR, 95% CI, P-value)			Multivariate analysis (HR_{adj}, 95%CI, P-value)		
		LRR	RFS	CSS	LRR	RFS	CSS
Patient's subgroups							
Non-HIV vs. ART-naïve	123 vs. 25	1.11, 0.42-2.90, >0.05	1.11, 0.59-2.08, >0.05	1.39, 0.66-2.93, >0.05	1.06, 0.40-2.78, >0.05	0.93, 0.48-1.81, >0.05	0.91, 0.41-2.03, >0.05
ART-treated vs. ART-naïve	23 vs. 25	0.15, 0.02-1.32, >0.05	0.44, 0.17-1.11, >0.05	0.21, 0.04-0.96, 0.044[a]	0.24, 0.03-2.13, >0.05	0.64, 0.22-1.85, >0.05	0.55, 0.12-2.63, >0.05
ART-treated vs. non-HIV	23 vs. 123	0.17, 0.02-1.27, >0.05	0.48, 0.22-1.07, >0.05	0.29, 0.07-1.20, >0.05	0.26, 0.03-1.94, >0.05	0.60, 0.24-1.47, >0.05	0.50, 0.12-2.20, >0.05
Age (years)							
<60 vs. ≥60	126 vs. 45	0.81, 0.33-1.97, >0.05	0.95, 0.54-1.67, >0.05	1.45, 0.77-2.76, >0.05			
Histology							
SCC vs. adeno	150 vs. 21	1.75, 0.71-4.29, >0.05	0.92, 0.44-1.92, >0.05	0.49, 0.15-1.59, >0.05			
Tumor size (cm)							
<4 vs. ≥4	39 vs. 132	0.90, 0.4-02.02, >0.05	2.3, 1.17-4.52, 0.016[a]	6.79, 1.64-28.08, 0.008[a]	>0.05	1.42, 0.69-2.95, >0.05	2.39, 0.55-10.42, >0.05
FIGO stage							
Stage IB2-IIB vs. Stage IIIA-IVA	108 vs. 63	2.55, 1.23-5.28, 0.012[a]	2.87, 1.76-4.69, <0.001[a]	14.73, 6.18-35.09, <0.001[a]	1.74, 0.80-3.78, >0.05	2.43, 1.37-4.30, 0.002[a]	11.47, 4.70-27.99, <0.001[a]
Parametrial invasion							
Absent vs. present	38 vs. 133	1.21, 0.52-2.84, >0.05	1.97, 1.05-3.71, 0.035[a]			0.81, 0.37-1.77, >0.05	
Metastatic lymph node							
Absent vs. present	125 vs. 46	1.63, 0.77-3.45, >0.05	1.98, 1.20-3.27, 0.007[a]	2.19, 1.19-4.03, 0.012[a]		1.35, 0.79-2.32, >0.05	1.46, 0.77-2.77, >0.05
Treatment							
CTRT vs. radical RT	166 vs. 5						
PDL1 expression							
Negative vs. positive	41 vs. 130	1.66, 0.63-4.34, >0.05	1.78, 0.95-3.34, >0.05	1.25, 0.60-2.61, >0.05		0.85, 0.40-1.83, >0.05	
PDL1 copy number alterations							
Polysomy vs. disomy	60 vs. 99	3.46, 1.61-7.45, 0.002[a]	2.39, 1.44-3.99, 0.001[a]	2.08, 1.07-4.05, 0.031[a]	2.5, 1.11-5.63, 0.027[a]	1.73, 0.96-3.12, >0.05	0.92, 0.45-1.87, >0.05

Table III. Continued.

Variables	Number (n)	Univariate analysis (HR, 95%CI, P-value)			Multivariate analysis (HR_{adj}, 95%CI, P-value)		
		LRR	RFS	CSS	LRR	RFS	CSS
Amplification vs. disomy	12 vs. 99	1.73, 0.22-13.67, >0.05	8.37, 3.67-19.12, <0.001[a]	7.46, 3.15-17.69, < 0.001[a]	1.44, 0.18-11.41, >0.05	7.03, 2.79-17.74, <0.001[a]	4.05, 1.64-9.98, 0.002[a]

[a]P<0.05. LRR, locoregional recurrence; RFS, recurrence-free survival; CSS, cancer-specific survival; HR, hazard ratio; HR_{adj}, adjusted hazard ratio; CI, confidence interval; SCC, squamous cell carcinoma; Adeno, adenocarcinoma; CTRT, concurrent chemoradiotherapy; RT, radiation therapy.

protease inhibitor therapy in patients with advanced HIV infection (31).

In cross-sectional and longitudinal clinical studies, comparison of ART-untreated to ART-exposed subjects demonstrated high serum levels of many cytokines, which were significantly reduced when ART was initiated. A cross-sectional study of pre- and post-ART showed lower serum levels of IFN-γ with the initiation of ART (32). A longitudinal study displayed a statistically significant reduction in IFN-γ after ART for 60 days or longer. In addition, a study conducted by Piconi *et al* found that different NRTI combinations (AZT+ddI and AZT+3TC) could exert different effects on IFN-γ and interleukin-2 (IL-2) production (33).

Nevertheless, IFN-γ-induced PD-L1 expression can fluctuate at different time points during the disease course. In contrast, PD-L1 expression can be continuously activated via gene amplification events involving a gene locus on chromosome 9p24.1. The additional somatic copy number alterations resulting in an increase in the fraction of DNA regions could be associated with carcinogenesis and cancer progression. Several important genes are known to be amplified and have been identified as prognostic markers, factors associated with drug resistance, or treatment targets in some cancer types, such as non-small cell lung cancer (34).

The 9p24.1 chromosomal region contains the genes encoding PD-L1, PD-L2 and JAK2.

Amplification of the chromosomal region 9p24.1 has recently been demonstrated as an essential mechanism for increased PD-L1 protein expression in nodular sclerosing classical Hodgkin lymphoma and primary mediastinal large B-cell lymphoma (13) and has also been identified in colorectal carcinoma, triple-negative breast cancer, glioblastoma and gastric adenocarcinoma (14,15),and cervical and vulvar carcinoma (16).

In the present study, our results demonstrated that PD-L1 copy number gains (amplification and polysomy) can be observed in a subset of cervical cancer patients using FISH analysis (35.4% for HIV-positive patients and 44.7% for HIV-negative patients).

In contrast to the results of a previous study (16), our results showed that PD-L1 amplification can be identified in only a minority of cases (8.3% for HIV-positive patients and 6.5% for HIV-negative patients). These conflicting results can be explained by differences in sample size, disease stage, or underlying diseases in the studied population.

More importantly, we found that ART was correlated with PD-L1 copy number status in addition to protein expression. In the present study, we demonstrated a novel genetic association between PD-L1 copy number gain and ART in cervical carcinoma. However, the exact molecular mechanism for this phenomenon is still unknown.

Based on previous genetic studies, several signalling pathways might be downregulated after treatment with ART, which may lead to a decrease in PD-L1 expression (35,36). The results showed that a variety of genes are downregulated following ART and that these genes might share common pathways with PD-L1, such as the NF-κB, MAPK, and JAK/STAT pathways (35,36). Moreover, several ART-responsive genes have been identified, and the biological processes and functions of a large number of these genes are still unknown (35). The expression of some of these genes could be changed as a

direct consequence of exposure of human cells to ART, rather than as a consequence of ART-mediated viral suppression (35). Further studies are needed to clarify the relationship between ART and PD-L1 gene expression.

Considering survival outcomes, we found that both copy number gains in the PD-L1 gene and PD-L1 overexpression indicated poor prognosis in univariate analysis. However, PD-L1 copy number gains were superior to PD-L1 overexpression and could act as independent and strong predictors of survival outcomes in cervical carcinoma.

Our results may identify a new subgroup of cervical cancer with a disease-specific genetic alteration. Further studies are required to evaluate the impact of PD-L1 copy number gain on pathogenesis, disease progression and prognosis in this newly identified subgroup of cervical cancer patients. In addition, the identification of PD-L1 gene copy number gain as a powerful mechanism for PD-L1 expression in the present study may provide a rationale for the treatment of cervical cancer patients in this subgroup.

In addition to the abovementioned mechanisms, ART could affect the response to radiation, as some PIs potently sensitize cancer cells to radiation (37). *In vitro* results have demonstrated that the commonly used combination of tenofovir, emtricitabine, and efavirenz sensitizes tumours to external beam radiation therapy (EBRT) (<4 Gy per fraction) but protects tumours from brachytherapy (≥4 Gy per fraction) (38).

In conclusion, although the immunotherapeutic drug pembrolizumab has been approved for locally advanced cervical cancer, the cost of cancer treatment is relatively high, and the response to pembrolizumab alone remains low. In addition to immune-checkpoint inhibitors, various therapeutic options, such as HPV vaccines and adoptive T-cell therapy, are currently being developed for the treatment of cervical cancer (39). However, the development of new drug treatments is both time-consuming and expensive. Subsequently, the repositioning of previously approved drugs for alternative purposes, such as cancer treatment, is reasonable. Hopefully, our preliminary data will be useful and lead to new treatment options for these patients in the future.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KL and CS designed the study and wrote the manuscript. SC and NP performed the statistical analysis and revised the manuscript. SV and JT performed the retrospective analyses and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Medical Ethics Committee of Navamindradhiraj University (Bangkok, Thailand). Informed consent was obtained from all patients prior to enrollment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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