



Association of Programmed Death Ligand 1 and C-X-C Chemokine Receptor Type 4 Immunoexpression with Pelvic Lymph Node Metastasis in Cervical Squamous Cell Carcinoma

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Abstract

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BACKGROUND: Squamous cell carcinoma (SCC) is the most common type of cervical cancer. Pelvic lymph node metastasis in cervical SCC is common. Programmed death ligand 1 (PD-L1) on tumor cells has been reported to impede anti-tumor immunity, resulting in immune evasion. C-X-C chemokine receptor type 4 (CXCR4) plays an important role in proliferation, survival, and migration (chemotaxis) of tumor cells.

AIM: This study aimed to analyze the association of PD-L1 and CXCR4 immunoexpression with pelvic lymph node metastasis in cervical SCC.

MATERIALS AND METHODS: Forty cases of cervical SCC in the Department of Anatomical Pathology, Faculty of Medicine, Padjadjaran University, Dr. Hasan Sadikin Hospital, Bandung, during 2013–2018 were collected and divided into two groups; (1) cervical SCC metastasize to pelvic lymph node and (2) cervical SCC non-metastasize to pelvic lymph node, of 20 cases, respectively. The expression of PD-L1 and CXCR4 was detected using immunohistochemistry.

RESULTS: High immunoexpression of PD-L1 and CXCR4 in cervical SCC showed significant association with pelvic lymph node metastasis ($p < 0.05$). The stepwise logistic regression analysis revealed that both PD-L1 and CXCR4 immunoexpression influenced pelvic lymph node metastasis simultaneously.

CONCLUSION: It could be concluded that the higher PD-L1 and CXCR4 immunoexpression showed the higher ability of tumor cells to metastasize to the pelvic lymph node.

Introduction

Cervical cancer is one of the most common forms of cancer in women and a leading cause of death among gynecological malignancies worldwide. In Indonesia, cervical cancer is the second most common women cancer in terms of incidence and mortality [1]. Squamous cell carcinoma is the most common type of cervical cancer [2], [3]. Squamous cell carcinoma is defined as an invasive epithelial tumor composed of squamous cells of varying degrees of differentiation [2]. Squamous cell carcinoma is an aggressive cancer and pelvic lymph node metastasis in cervical SCC is common [4].

Pelvic lymph node metastasis (based on imaging or pathological findings, where available) is one of the parameters to assign the stage based on new 2018 International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical cancer [5], [6]. PD-L1 and CXCR4 immunohistochemistry on cervical SCC are expected to be an alternative method to predict pelvic lymph node metastasis in cervical SCC.

PD-L1, also known as B7 homolog 1 (B7-H1) or cluster of differentiation (CD) 274, is a ligand of programmed death protein 1 (PD-1), encoded by the CD274 gene, which is located in chromosome 9p24.1 [7], [8], [9], [10], [11], [12]. It is expressed in different tissues, but mainly in activated T and B cells, dendritic cells, monocytes, and various types of tumor cells [10], [13]. The PD-1/PD-L1 interaction (axis) inhibits T-cells activation and increases inhibitory cytokines secretion, results in immune evasion [14], [15], [16], [17], [18]. PD-L1 overexpression in tumor cells is an indicator of tumor progression [19], [20].

CXCR4, also known as fusin or CD184, is a specific receptor for the CXC chemokine stromal cell-derived factor-1 α (SDF-1 α , also known as C-X-C motif chemokine ligand 12 [CXCL12]). CXCR4 is a G-protein-coupled chemokine receptor encoded on chromosome 2 [21], [22], [23], [24]. Similar to CXCL12, the expression of CXCR4 is low or absent in many healthy tissues, but it is demonstrated that CXCR4 is highly expressed in various different tumor types [23], [25]. The CXCR4/CXCL12 axis in cancer is associated with chemotaxis,

migration, proliferation, and cell tumor survival, also being an indicator of higher tumor progression [24], [26].

The association of PD-L1 immunoexpression with pelvic lymph node metastasis in cervical SCC has been investigated in various studies. However, the results are still conflicting [27], [28], [15]. The same association conflict also seen in various studies of CXCR4 immunoexpression with pelvic lymph node metastasis in cervical SCC [29], [30], [31].

Therefore, this study aimed to analyze the association of PD-L1 and CXCR4 immunoexpression with pelvic lymph node metastasis in cervical SCC.

Materials and Methods

This was an observational analytic study with a cross-sectional design. The research has received ethical approval from the Ethical Committee of Universitas Padjadjaran with certificate number 1214/UN6.KEP/EC/2019.

Samples selection

The samples were paraffin blocks from cervical cancer patients who underwent radical hysterectomy and pelvic lymphadenectomy, had been diagnosed histopathologically as cervical SCC in the Department of Anatomical Pathology, Faculty of Medicine, Padjadjaran University, Dr. Hasan Sadikin Hospital, Bandung, during the period of 2013–2018. Samples were divided into two groups: (1) Cervical SCC metastasize to pelvic lymph node and (2) cervical SCC non-metastasize to pelvic lymph node, of 20 cases, respectively.

Inclusion criteria include: (1) Cases of cervical cancer that had undergone radical hysterectomy and lymphadenectomy, had been diagnosed histopathologically as cervical SCC (cervical SCC metastasize to pelvic lymph node and cervical SCC non-metastasize to pelvic lymph node); (2) paraffin block was available; (3) patients have not received radiotherapy or chemotherapy for the treatment of cervical SCC; and (4) patient clinical data were available.

Exclusion criteria include: (1) The paraffin block of tumor mass was empty, damaged, or paraffin ribbons cannot be stained with PD-L1 and CXCR4 immunohistochemistry and (2) cases of cervical SCC non-metastasize to pelvic lymph node with <10 lymph nodes removed.

Data extraction

Characteristic data, including age, stage, histopathological subtype, histopathological grade,

tumor size, and status of pelvic lymph node metastasis were extracted from pathological, cancer registry reports, and medical record databases.

Immunohistochemistry staining and scoring

PD-L1 and CXCR4 immunohistochemistry were performed on the whole samples. Each paraffin block was sectioned at 4 μ m and deparaffinized using xylene. After rehydration and washing, the sections were incubated by ethylenediaminetetraacetic acid (EDTA) buffer pH 6.0 for antigen retrieval. After blocking, the specimens were incubated for 1 h using primary antibodies rabbit monoclonal anti-PD-L1 (clone 28-8, cat No. ab205921, Abcam, Inc., Cambridge, USA; 1:200 dilution) and rabbit polyclonal anti-CXCR4 (cat No. GTX13854, GeneTex, Inc., California, USA; 1:300 dilution). The samples were labeled using labeled streptavidin-biotin immunoperoxidase complex by One Step Neopoly Detection Kit (Biogear Scientific, BioVentures, Inc., Iowa, USA), visualized using 3,3'-diaminobenzidine (DAB), and counterstained using Harris hematoxylin. After dehydration, the samples were mounted and analyzed. The positive control for each marker was normal tonsil tissue (according to Abcam and GeneTex recommendation).

The evaluation of immunoexpression from both biomarkers was analyzed using histoscore, a combined scoring system which is the analysis of intensity and staining distribution. Positive result was shown by brown color in different areas of the tumor cells for each biomarker. PD-L1 positivity was in the cell membrane (with or without cytoplasm) of tumor cell; meanwhile, CXCR4 was positive if cell membrane and/or cytoplasm of tumor cell was also colored brown. The staining intensity was scored as 0 (negative), +1 (weak), +2 (moderate), or 3+ (strong). Positivity of PD-L1 in the tumor cell was scored as 0 (<5%) or 1 (\geq 5%), meanwhile, positivity of CXCR4 was scored as 0 (<5%), 1 (5–10%), 2 (11–50%), 3 (51–75%), or 4 (>75%). Histoscore was determined from the multiplication of intensity and staining distribution scoring, which was 0–3 for PD-L1 and 0–12 for CXCR4. The immunoexpression histoscore was later categorized as low (\leq 1 for PD-L1 and \leq 6 for CXCR4) and high (>1 for PD-L1 and >6 for CXCR4). These analyses were performed independently by two experienced pathologists who had no prior knowledge of characteristic data. The few different scoring from both pathologists was resolved by consensus using a conference microscope.

Statistical analysis

Statistical analysis using regression logistic test. The significance of the data was obtained when $p < 0.05$. Data processing using the Statistical Package for the Social Sciences (SPSS) version 24.0 for Windows.

Results

Patients characteristic

Forty patients between 32 and 66 years old were included in this study, with mean and median 43.375 and 44 years old, respectively. Patients ≤ 45 years old were more common than >45 years old. Stage IB1 was the most encountered stadium, which was 16 cases (40%). The majority histopathological subtypes were non-keratinized, with 27 cases (67.5%). The most common histological grade were moderately differentiated, in 24 cases (60%). Tumor size <4 cm was the most common tumor size, which was 23 cases or 57.5% (Table 1).

Table 1: Patients characteristic

| Variable | n (%) |
|---------------------------|--------------------|
| Age (year) | |
| Mean \pm Std | 45.375 \pm 9.086 |
| Median | 44 |
| Range (min-max) | 32-66 |
| ≤ 45 | 25 (62.5) |
| >45 | 15 (37.5) |
| Stage ^a | |
| IB1 | 16 (40) |
| IB2 | 8 (20) |
| IIA1 | 10 (25) |
| IIA2 | 5 (12.5) |
| IIB | 1 (2.5) |
| Histopathological subtype | |
| Non-keratinized | 27 (67.5) |
| Keratinized | 9 (22.5) |
| Basaloid | 4 (10) |
| Histopathological grade | |
| Well differentiated | 3 (7.5) |
| Moderately differentiated | 24 (60) |
| Poorly differentiated | 13 (32.5) |
| Tumor size | |
| <4 cm | 23 (57.5) |
| ≥ 4 cm | 17 (42.5) |

^aAccording to 2009, FIGO cervical cancer staging system

The association between patients characteristic and pelvic lymph node metastasis in cervical squamous cell carcinoma is shown in Table 2. There was no significant association between patients characteristic (age, stage, histopathological subtype, histopathological grade, or tumor size) and pelvic lymph node metastasis (Table 2).

PD-L1 and CXCR4 immunoeexpression

Immunohistochemistry staining on PD-L1 and CXCR4 was performed on each sample. The staining results were evaluated according to intensity and staining distribution of positive tumor cells (Figures 1 and 2). There were no negative CXCR4 immunoeexpression findings in this study.

Association of PD-L1 immunoeexpression with pelvic lymph node metastasis in cervical squamous cell carcinoma

p value in PD-L1 immunoeexpression variable was less than 0.05 ($p = 0.006$), showed a statistically significant difference between PD-L1 immunoeexpression variable in cervical SCC metastasize to pelvic lymph

Table 2: Association between patients characteristic and pelvic lymph node metastasis in cervical squamous cell carcinoma

| Variable | Cervical squamous cell carcinoma group | | OR | p value ^a |
|---------------------------|---|---|-------|----------------------|
| | Metastasize to pelvic lymph node n (%) | Non-metastasize to pelvic lymph node n (%) | | |
| Age (years) | | | | |
| ≤ 45 | 14 (56) | 11 (44) | Ref | |
| >45 | 6 (40) | 9 (60) | 0.524 | 0.330 |
| Stage ^b | | | | |
| IB1 | 6 (37,5) | 10 (62,5) | Ref | |
| IB2 | 5 (62,5) | 3 (37,5) | 2.778 | 0.253 |
| IIA1 | 5 (50) | 5 (50) | 1.667 | 0.532 |
| IIA2 | 3 (60) | 2 (40) | 2.550 | 0.382 |
| IIB | 1 (100) | 0 (0) | NA | |
| Histopathological subtype | | | | |
| Non-keratinized | 14 (51,9) | 13 (48,1) | Ref | |
| Keratinized | 3 (33,3) | 6 (66,7) | 0.464 | 0.341 |
| Basaloid | 3 (75) | 1 (25) | 2.786 | 0.400 |
| Histopathological grade | | | | |
| Well differentiated | 1 (33,3) | 2 (66,7) | Ref | 0.591 |
| Moderately differentiated | 12 (50) | 12 (50) | 2.000 | 0.529 |
| Poorly differentiated | 7 (53,8) | 6 (46,2) | 2.333 | |
| Tumor size | | | | |
| <4 cm | 9 (39,1) | 14 (60,9) | Ref | |
| ≥ 4 cm | 11(64,7) | 6 (35,3) | 2.852 | 0.114 |

OR: Odds ratio, Ref: Reference, NA: Not applicable. ^aSimple regression logistic test, ^bAccording to 2009, FIGO cervical cancer staging system

node group and cervical SCC non-metastasize to pelvic lymph node group. Based on odds ratio, it could be concluded that the risk of cervical SCC patients with high PD-L1 immunoeexpression for sustaining pelvic lymph node metastasis was 8.5 times higher than cervical SCC patients with low PD-L1 immunoeexpression (confidence interval of 1.861-38.817) (Table 3).

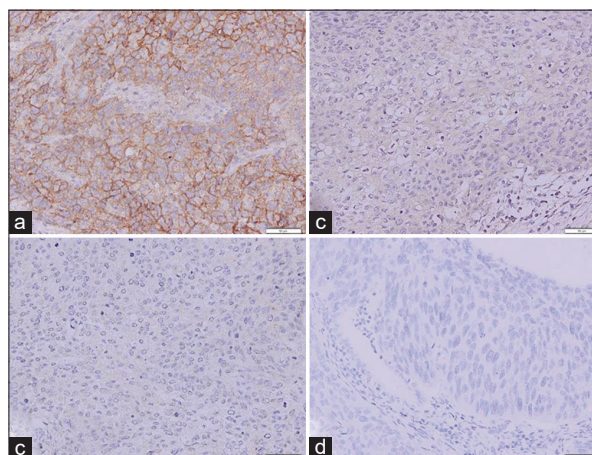


Figure 1: PD-L1 immunoeexpression. (a) Strong (+3); (b) moderate (+2); (c) weak (+1); (d) negative (0) ($\times 200$)

Association of CXCR4 Immunoeexpression with Pelvic Lymph Node Metastasis in Cervical Squamous Cell Carcinoma

p value in CXCR4 immunoeexpression variable was less than 0.05 ($p = 0.001$), showed a statistically significant difference between CXCR4

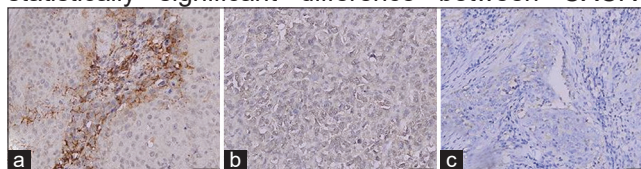


Figure 2: CXCR4 immunoeexpression. (a) Strong (+3); (b) moderate (+2); (c) weak (+1). ($\times 200$)

Table 3: Association of PD-L1 immunoexpression with pelvic lymph node metastasis in cervical squamous cell carcinoma

| Variable | Cervical squamous cell carcinoma group | | OR (CI 95%) | p value ^a |
|----------|--|--|--------------------|----------------------|
| | Metastasize to pelvic lymph node n (%) | Non-metastasize to pelvic lymph node n (%) | | |
| PD-L1 | | | | |
| Low | 3 (20) | 12 (80) | Ref | 0.006* |
| High | 17 (68) | 8 (32) | 8.5 (1.861–38.817) | |

OR: Odds ratio, CI: Confidence interval, Ref: Reference. ^aSimple regression logistic test. *p < 0.05, means significant or statistically significant

immunoexpression variable in cervical SCC metastasize to pelvic lymph node group and cervical SCC non-metastasize to pelvic lymph node group. Based on odds ratio, it could be concluded that the risk of cervical SCC patients with high CXCR4 immunoexpression for sustaining pelvic lymph node metastasis was 12 times higher than cervical SCC patients with low CXCR4 immunoexpression (confidence interval of 2.7–53.33) (Table 4).

Table 4: Association of CXCR4 immunoexpression with pelvic lymph node metastasis in cervical squamous cell carcinoma

| Variable | Cervical squamous cell carcinoma group | | OR (CI 95%) | p value ^a |
|----------|--|--|----------------|----------------------|
| | Metastasize to pelvic lymph node n (%) | Non-metastasize to pelvic lymph node n (%) | | |
| CXCR4 | | | | |
| Low | 5 (23.8) | 16 (76.2) | Ref | 0.001* |
| High | 15 (78.9) | 4 (21.1) | 12 (2.7–53.33) | |

OR: Odds ratio, CI: Confidence interval, Ref: Reference. ^aSimple regression logistic test. *p < 0.05, means significant or statistically significant

Multivariate analysis of association of PD-L1 and CXCR4 immunoexpression with pelvic lymph node metastasis in cervical squamous cell carcinoma

The results of multivariate analysis show that all p values of all variables were less than 0.05 (p < 0.05), showed PD-L1 and CXCR4 immunoexpression associated with pelvic lymph node metastasis in cervical SCC simultaneously. The variable that simultaneously had most influence on pelvic lymph node metastasis was CXCR4 immunoexpression (OR: 13.797), then PD-L1 immunoexpression (OR: 10.042) (Table 5).

Table 5: Multivariate analysis of association of PD-L1 and CXCR4 immunoexpression with pelvic lymph node metastasis in cervical squamous cell carcinoma

| Variable | Coefficient | p value ^a | OR | OR CI 95% | |
|----------|-------------|----------------------|--------|-----------|--------|
| | | | | Lower | Upper |
| PD-L1 | 2.307 | 0.016* | 10.042 | 1.548 | 65.151 |
| CXCR4 | 2.624 | 0.003* | 13.797 | 2.376 | 80.102 |
| Constant | -2.719 | | | | |

OR: Odds ratio, CI: Confidence interval. ^aMultivariate analysis with multiple logistic regression. The independent variable included in this logistic regression is the independent variable which in the simple regression logistic test has p < 0.25. *p < 0.05, means significant or statistically significant.

Discussion

There are various studies analyzing the association of PD-L1 or CXCR4 immunoexpression with pelvic lymph node metastasis in cervical SCC, but

in this study, we analyzed the association of both PD-L1 and CXCR4 immunoexpression. We demonstrated that PD-L1 and CXCR4 immunoexpression were significantly higher in patients with cervical SCC displaying pelvic lymph node metastasis. The stepwise logistic regression analysis revealed that both PD-L1 and CXCR4 immunoexpression influenced pelvic lymph node metastasis simultaneously. This result indicates that both PD-L1 and CXCR4 expression are upregulated in cervical SCC metastasize to pelvic lymph node cases and may contribute to the pelvic lymph node metastasis occurring in cervical SCC. The result of this study is consistent with the study performed by Meng *et al.* [27] that describe a significant correlation of PD-L1 overexpression with lymph node metastasis in cervical cancer. Similarly, the result of this study was also observed in the study performed by Dai *et al.* [29] and Kodama *et al.* [30] that describe a significant correlation of CXCR4 overexpression with lymph node metastasis in cervical cancer.

In many cancer, including cervical SCC, PD-L1, and CXCR4, upregulation is caused by intrinsic and extrinsic factors, such as genomic alterations and hypoxia [13], [32]. PD-L1 upregulation will induce T cell anergy, functional exhaustion, and apoptosis; increase inhibitory cytokines secretion (such as IL-10 and TGF- β); and favor the conversion of T cells into Tregs which creates a local immunosuppressive condition in tumor microenvironment that inhibits anti-tumor immune response, results in immune evasion [20], [33]. Meanwhile, CXCR4 upregulation will activate pathways of the MEK/ERK, STAT3, and P13K/AKT which activates intracellular signals that play role in proliferation, survival, migration, and chemotaxis. In addition, PD-L1 upregulation will also cause the same pathways activation of the MEK/ERK, STAT3, and P13K/AKT that cause CXCR4 overexpression [13], [34]. This theory supports our result that PD-L1 and CXCR4 immunoexpression influenced pelvic lymph node metastasis in cervical SCC simultaneously.

This study showed that the risk of cervical SCC patients with high PD-L1 and CXCR4 immunoexpression for sustaining pelvic lymph node metastasis was 8.5 and 12 times higher than patients with low PD-L1 and CXCR4 immunoexpression, respectively. Interestingly, the risk of cervical SCC patients with both high PD-L1 and CXCR4 immunoexpression for sustaining pelvic lymph node metastasis was increased to 10.042 and 13.797 times higher, respectively. The variable that simultaneously had most influence on pelvic lymph node metastasis was CXCR4 immunoexpression (OR: 13.797), then PD-L1 immunoexpression (OR: 10.042)

We need similar studies for the association of PD-L1 and CXCR4 immunoexpression with pelvic lymph node metastasis in cervical SCC to elucidate

more about mechanism of PD-L1 and CXCR4 affect pelvic lymph node metastasis simultaneously.

Conclusion

Our study showed that high immunoeexpression of PD-L1 and CXCR4 in cervical SCC showed significant association with pelvic lymph node metastasis. It could be concluded that the higher PD-L1 and CXCR4 immunoeexpression showed the higher ability of tumor cells to metastasize to the pelvic lymph node. The results of this study should be considered to perform routine PD-L1 and CXCR4 immunoeexpression of cervical SCC biopsy samples to predict pelvic lymph node metastasis in cervical SCC.

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Ethical Approval

The research has received ethical approval from the Ethical Committee of Universitas Padjadjaran with certificate number 1214/UN6.KEP/EC/2019.

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