# PROGRAMMED DEATH-LIGAND 1 (PD-L1) EXPRESSION AND TUMOR-INFILTRATING LYMPHOCYTES (TILs) IN COLORECTAL ADENOCARCINOMA

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**Abstract**

BACKGROUND : Programmed death-ligand 1 (PD-L1) expression and tumor-infiltrating lymphocytes (TILs) are considered have a prognostic value in several malignancies. This study investigated the correlation between PD-L1 expression of tumor cells with the degree of stromal TILs in colorectal adenocarcinoma.

METHODS : A cross sectional study design performed by taking 52 colorectal adenocarcinoma samples in the period of 2014-2016. The specimens were stained by immunohistochemical procedure using PD-L1 rabbit monoclonal antibody and the degrees of TILs were assessed base on hematoxylin and eosin (H&E) staining.

RESULTS : From all of 52 samples, the positive PD-L1 expression of tumor cells were 44 (84.6%) samples with 22 (50.0%), 18 (40.9%) and 4 (9.1%) samples had low-, moderate-, and high-degree TILs, respectively. While the negative PD-L1 expression were 8 (15.4%) samples with 1 (12.5%), 3 (37.5%) and 4 (50.0 %) samples had low-, moderate-, and high-degree TILs, respectively. A value of p = 0.017 ($p < 0.05$) was obtained by the Chi-square test.

CONCLUSIONS : This study concluded that there was a significant correlation between PD-L1 expression of tumor cells and the degree of TILs in colorectal adenocarcinoma. This result indicated that the degree of TILs had the potential to be used as a predictive factor for PD-L1 expression of tumor cells in colorectal adenocarcinoma.

**Key words** : PD-L1 expression, stromal TILs, colorectal adenocarcinoma.

## INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world. Globally, 1.4 million new cases of CRC and almost 700,000 deaths were recorded worldwide in 2012, which was the fourth largest contributor to cancer deaths.[1,2] They are expected to increase in 2030 by 60% to more than 2.2 million new cases and 1.1 million deaths of CRC.[2]

Based on the histological type, the most common CRC is adenocarcinoma, which is more than 90% of cases from various studies that has been conducted on CRC.[3,4]

The most prominent development of immunotherapy in the last decade is the presence of checkpoint inhibitors, which are monoclonal antibodies that modify the

major histocompatibility complex (MHC) –T cell receptor (TCR) signaling pathway by targeting co-inhibitor molecules such as programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1).[5] At present, a number of phase III clinical trials are being conducted to determine the useful of anti-PD-1 or anti-PD-L1 in mismatch repair-deficient CRC. Two clinical trials explored the use of pembrolizumab or atezolizumab on the first line with metastasis, and one clinical trial explored the use of atezolizumab in combination with folinic acid, fluorouracil, and oxaliplatin as adjuvant therapy for stage III mismatch repair-deficient CRC.[6]

Tumor-infiltrating lymphocytes are often abbreviated as TILs, that are lymphocytes in the area of the tumor, either directly in the tumor nest (intratumoral TILs) or between the tumor nests (stromal TILs).[7,8] At present, TILs can be assessed base on hematoxylin and eosin (H&E) staining.[9] TILs are considered as a reflection of the primary immune response of the host against tumor cells.[10,11]Infiltration of inflammatory cells is a part of the tumor microenvironment, and the infiltration can be a marker of a good prognostic in CRC.[12] CD8+ cytotoxic T cells (CTLs) are one of the subpopulations of TILs that have an important role in the immune response to tumor, that can directly kill tumor cells after recognizing antigens derived from the tumor.[8,13]

The previous study has reported that lymphocytes infiltration has an association with a better survival in several types of cancer.[10] Several studies have also shown that the presence of TILs, both intratumoral TILs and stromal TILs, have contributed to a better prognosis in CRC.[14] Study by Huh et al has proposed that there is a significant correlation between the degree of TILs and the degree of tumor differentiation in CRC, i.e the low degree of TILs is associated with the poor differentiation of tumor.[15]

In the early stage of carcinogenesis, immune cells that infiltrate tumor especially CTLs and natural killer (NK) cells have the potential to limit the tumor growth. However, tumors can also develop several mechanisms to avoid the immune response of host.[16,17]

Programmed death-1 (PD-1) is a part of regulatory T cells (Tregs) that is expressed on the surface of active T cells, B cells and NK.[18,19] PD-1 expression in T cells is considered as one of the markers of exhausted T cells, where PD-1 can undergo selective upregulation due to the persistent exposure to antigens.[20]Ligands of PD-1, called PD-L1 are expressed in tumor cells, T cells and B cells, macrophages, and a number of specific cell types. The bond between PD-L1 and PD-1 will deliver the inhibitor signals that will reduce the production of cytokines and T cell proliferation and it will ultimately lead to an increase in apoptosis of T cells. Expression of PD-L1 in tumors has been described as a predictive marker for tumor response to anti-PD-1 or anti-PD-L1 immunotherapy in several types of malignancies.[19]

The previous study with CRC samples by Rosenbaum et al in the United States has reported that histologically, tumors with positive PD-L1 expression are more likely to have poor differentiation. However, the study also finds that tumors with positive PD-L1 expression contain large amounts of CD8+ TILs, and TILs are significantly more likely to have tumors with positive PD-L1 expression.[21]Another study from Masugi et al has reported that PD-L1 expression of colorectal tumors is inversely related to TILs level in univariable analysis, but it does not have a significant correlation in multivariable analysis.[22] To date, there are still many things that need to be explored about complexity between PD-L1 and TILs.

This study was conducted by taking samples of CRC patients in Makassar to assess the degree of stromal TILs and PD-L1 expression of tumor cells in CRC of adenocarcinoma type, to find out whether there are differences in the degree of TILs in colorectal adenocarcinomas with negative and positive PD-L1 expression of tumor cells, and further to determine whether the degree of TILs has a correlation with the expression of PD-L1, that is important to be used as a predictive and a prognostic factor in CRC patients.

## MATERIALS AND METHODS

This study was conducted by taking data and formaline-fixed paraffin embedded of patients with colorectal adenocarcinoma from 2014-2016 period at the Anatomical Pathology Laboratory Dr. Wahidin Sudirohusodo and Hasanuddin University Hospital Makassar. All of 52 samples were obtained by simple random sampling. Each histological grade and TILs scoring was assessed base on H&E staining, and immunohistochemical staining carried out by using PD-L1 rabbit monoclonal antibody, clone 28-8 (CELL MARQUE) with dilution of 1:50.

The histological grade of colorectal adenocarcinoma has been evaluated base on the degree of differentiation of the tumor. The well-differentiated is recognizable glandular/tubular formation in tumor is greater than 95%; the moderately differentiated is the glandular/tubular formation in tumor around 50%-95%; while the poor differentiated is the glandular/tubular formation in tumor is less than 50%.[23]

The TILs scoring method was similar to the method in breast cancer based on the recommendation of the International TILs Working Group, 2014 on H&E staining that was assessed by two Anatomical Pathology specialists. TILs were reported in

the stromal compartment, that was an area infiltrating by mononuclear inflammatory cells in all tumor stroma areas.[9] The TILs percentage is determined base on the average value of TILs density from 5 stromal areas (using microscopic objective magnification (obj.) 10x) with varying TILs density (not focusing on hot spot areas), then be able to assess stromal TILs more clearly (using stronger microscopic magnification). The samples were grouped based on scoring values as the study of Jakubowska et al, i.e score 1 = low-degree (0-10% TILs), score 2 = moderate-degree (20%-40% TILs), and score 3 = high-degree (50%-90% TILs).[7] The representative samples of each degree of stromal TILs were shown in Figure 1.

Immunohistochemical staining procedure was performed to determine PD-L1 expression of tumor cells in each sample using a light microscope by two Anatomical Pathologi specialists. PD-L1 expression value was determined based on the large percentage of tumor cells at any stained intensity based on immunohistochemical staining. Score 0 = <5%, score 1 = 5%-49%, and score 2 = ≥50%. Furthermore, it was considered as negative if it had score 0, and as positive if it had score 1-2.[21] The representative samples of PD-L1 expression of each score were shown in Figure 2.

Data from the TILs scoring and the immunohistochemical staining scoring were analyzed using the Chi-square test to assess the a correlation between PD-L1 expression of tumor cells with the degree of TILs of the samples.

**RESULTS**

From all samples collected (total 52 samples), 15 samples were obtained with well-differentiated, 33 samples with moderately differentiated, and as many as 4 samples with poorly differentiated.

Table 1.The characteristics of samples

Table 1 shows that from all of 52 samples, the age category <40 years were 4 (7.7%) samples and the age category ≥40 years were 48 (92.3%) samples with a mean age was 55.83 years. The number of male samples were 27 (51.9%) samples and female were 25 (48.1%) samples. Based on the histological grade, the samples that classified as well-differentiated were 15 (28.8%), moderately differentiated were 33 (63.5%) samples, and poorly differentiated were 4 (7.7%) samples. The samples with low-degree of TILs was obtained as 23 (44.2%) samples, the moderate-degree were 21 (40.4%) samples, and the high-degree were 8 (15.4%) samples. The positive PD-L1 expression of tumor cells were 44 (84.6%) samples, and the negative expression of PD-L1 were obtained in 8 (15.4%) samples.

Figure 1. H&E staining of representative sample of low-degree TILs with stromal area showing 5% TILs, obj.10x (A); moderate-degree TILs with stromal area showing 30% TILs, obj.10x (B); and high-degree TILs with a stromal area showing 60% TILs, obj.10x (C) and obj.40x(D)

Table 2. TILs degree according to the histological grade of samples

Table 2 shows from all of 52 samples, there were 23 (44.2%) samples with low-degree TILs, 21 (40.4%) samples with moderate-degree TILs, and 8 (15.4%) samples with high-degree TILs . In the group of well-differentiated, from whole of 15 samples, 7 (46.7%) samples had low-degree TILs, 6 (40.0%) samples had moderate-degree TILs, and 2 (13.3%) samples had high-degree TILs. In the group of moderately differentiated, from whole of 33 samples, those are 13 (39.4%) samples had low-degree TILs, 14 (42.4%) had moderate-degree TILs, and 6 (18.2%) samples had high-degree TILs. As for the poorly differentiated group, from whole of 4 samples, 3 (75.0%) samples with low-degree TILs, 1 (25.0%) samples with moderate-degree TILs, and no (0.0%) sample with high-degree TILs. Based on the Chi-square test, the value of $p = 0.619$ ($p > 0.05$) was obtained so that it was concluded that there was no significant difference in the degree of TILs in colorectal adenocarcinoma of well-, moderately, and poorly differentiated.

Figure 2. PD-L1 Immunohistochemistry staining of representative sample showing PD-L1 expression of tumor cells: positive expression with stained tumor cells ≥ 50% / score 2, obj.4x (A) and obj.40x (B); positive expression with stained tumor cells 5%-49% / score 1), obj.4x (C); negative expression with <5% stained tumor cells / score 0, obj.20x (D).

Table 3. PD-L1 expression of tumor cells according to the histological grade of samples

Table 3 shows from all of 52 samples, there were 44 (84.6%) samples with positive PD-L1 expression of tumor cells and 8 (15.4%) samples with negative PD-L1 expression. From whole of 15 samples included in the group of well-differentiated, 12 (80.0%) samples were positive PD-L1 expression of tumor cells and 3 (20.0%) samples were negative PD-L1 expression. In moderately differented samples group, from whole of 33 samples, 29 (87.9%) samples were positive PD-L1 expression of tumor cells and 4 (12.1%) samples were negative PD-L1 expression. While in the poorly differentiated samples group, from whole of 4 samples, 3 (75.0%) samples were positive PD-L1 expression of tumor cells and 1 sample (25.0%) was negative PD-L1 expression. Based on the Chi-square test, $p = 0.683$ ($p > 0.05$) was obtained, that its concluded that there was no significant difference of PD-L1 expression of tumor cells in colorectal adenocarcinoma samples of well-, moderately, and poorly differentiated.

Table 4. PD-L1 expression of tumor cells and TILs degree of samples

Table 4 shows that in the samples group of the positive PD-L1 expression of tumor cells, from whole of 44 samples, 22 (50.0%) samples had low-degree TILs, 18 (40.9%) samples had moderate-degree TILs, and 4 (9.1%) samples had high-degree TILs. While in the negative PD-L1 expression group, total 8 samples, 1 (12.5%) sample showed low-degree TILs, 3 (37.5%) samples had moderate-degree TILs, and

4 (50.0 %) samples had high-degree TILs. By using the Chi-square test, p = 0.017 (p <0.05) was obtained, that means there was a statistically significant correlation between PD-L1 expression of tumor cells and the degree of TILs of this study.

## DISCUSSION

This study only assessed stromal TILs based on H&E staining, without assessing the subpopulation of TILs. The assessment of stromal TILs in this study also included TILs in the stromal area at the invasive margin of the tumor, as in the previous study by Iseki et al which assesses the stromal TILS in the area of the invasive front of the tumor, where is considered as the optimal area for assessing TILs.[24]

In Table 1, which illustrates the clinicopathological characteristics of the samples, it is seen in the age category, the sample of colorectal adenocarcinoma sufferers aged ≥40 years, which were far more than those aged <40 years (48 vs 4 samples). The mechanism by which CRC occurs is thought to be a heterogeneous molecular event including genetic and epigenetic factors.[25] The transformation from a normal mucosa of colon to an invasive cancer can develop through an accumulation of genetic and epigenetic changes. Most CRC are thought to develop from a pre-existing adenoma condition that has a genetic malignant lesion, where this transformation can last for 10-15 years.[26] The existence of this long enough time span might be one of the underlying factors, then more cases of CRC were found at an older age.

In this study, as shown in Table 2, the poorly differentiated tumors were more often found to have low-degree of TILs. In tumor conditions, due to the

continuous exposure of tumor antigens that can cause exhausted T cells, this can trigger upregulation of PD-1 expression in T cells.[20] PD-1 that expressed in T cells if bound to PD-L1 expressed by tumor cells will inhibit TCR signal transduction, thereby causing inhibition of cytotoxic activity of T cells and ultimately can increase apoptosis in T cells.[27,28]Previous studies on several types of cancer have reported a significant correlation between PD-1 expression and the degree of tumour differentiation, that PD-1 overexpression is more likely in tumors with poor degree of differentiation.[29,30] This may be associated with an increase in exhausted T cells with increasing histological grade of tumor. However, in this study based on the Chi-square test, the value of p = 0.619 (p> 0.05) was obtained so that it was concluded that there was no significant difference in the degree of TILs based on the histological grade of the sample. A small number of samples with uneven distribution of sample groups might cause no meaningful results to be obtained.

Some studies suggest that high lymphocytes infiltration in CRC is linked to specific molecular features to this cancer, in this case microsatellite instability-high (MSI-h).[22] MSI-h is more immunogenic due to the presence of large numbers of abnormal peptides due to frameshift mutations compared to tumors that are microsatellite stable.[14] In addition, as with other types of tumors, in CRC there are also known to be a large number of Tregs, which may be triggered by proliferating tumor cells and also dead tumor cells that provide a large amount of self-antigens that will recognized by Tregs. It was also mentioned, the increase in Tregs might also be triggered by inflammatory conditions in tumor that recruit Tregs. It is believed, Tregs can suppress the anti-tumor immune response in the tumor microenvironment. An increase in Tregs in the tumor area, will affect the ratio of CD8+ T cells to Tregs, and is reported to be associated with a poor prognosis in a number of

malignancies.[31] This might be related to the presence of samples with the poor-degree of differentiation, but have the high-degree of TILs. However, in this study, there was no assessment of the molecular profile or subpopulation of TILs of the samples. Further study is needed that confirms the degree of TILs by examining subpopulations of TILs, especially CD8+ T cells, Tregs, and assessing the MSI-h status of samples.

Based on Table 3, the results were obtained that samples with positive PD-L1 expression of tumor cells were found both in the sample group of well-differentiated, moderately differentiated and also in the poorly differentiated samples. In this study, PD-L1 was expressed not only in the tumor cell membrane, but also in part in the cytoplasm. This was also obtained from previous studies of various types of cancer that assess the expression of PD-L1 in cell membrane and cytoplasm.[32,33,34,35] It is known that PD-L1 is a transmembrane protein consisting of one transmembrane region and two extracellular domains, immunoglobulin V (IgV)-like domain and IgC-like domain. In addition, PD-L1 also has a cytoplasmic domain that is short and transmits intracellular signals.[36]

A study by Rosenbaum et al in the United States has reported that histologically, tumors with positive PD-L1 expression are more likely to have poor differentiation.[21] While Lin et al in their study using univariate analysis find that PD-L1 expression has a significant association with the higher tumor stage.[37] Based on the Chi-square test, there was no significant difference in PD-L1 expression of tumor cells between the sample groups of well-, moderately, and poorly differentiated. The different result with the previous studies may be due to differences in sample size, unbalanced distribution of sample groups, and the use of PD-L1 antibody of different clone.

According to Kim et al, the fundamental mechanism of PD-L1 upregulation in CIMP-H (CpG island methylator phenotype-high) CRC cells and MSI-h especially in those with the poor differentiation remains unclear. Several studies of PD-L1 on a number of malignancies, such as non-small cell lung cancer, renal cell carcinoma, and breast cancer more or less provide evidence that an increase in epithelial-mesenchymal transition (EMT) may be related to the expression of PD-L1 in tumor cells , where the poor degree of differentiation is considered as one part of EMT.[38]

At present, there have been many studies reported that the expression of PD-L1 has a significant correlation with the infiltration of TILs in some cancers.[37] Based on the Chi-square test, a significant correlation was found between PD-L1 expression of tumor cells with the degree of TILs in this study, i.e the positive PD-L1 expression was more likely found in colorectal adenocarcinomas with the lower degree of TILs.

Table 4 shows that samples with positive PD-L1 expression of tumor cells were more likely to have lower degree of TILs. The interaction of PD-L1 on effector T cells with PD-1 will inhibit TCR signal transduction, causing inhibition of CTLs activity. Finally, the blockade of TCR signal transduction causes inhibition of PI3K (phosphoinositide 3-kinase) / Akt and MAPK (mitogen-activated protein kinase) signaling. And most importantly, inhibition of PI3K activation suppresses expression of B-cell lymphoma-extra large (Bcl-xl) and activation of Akt (Protein kinase B), which will eventually lead to an increase in T cell apoptosis.[27] This ultimately affects the number of T cells , and might explain the acquisition of lower degrees of TILs. On the other hand, the infiltration of T cells is one of the factors that can trigger the expression of PD-L1 of tumor cells due to interferon-γ released by activated T cells.[38]

This might be related to the presence of samples with positive PD-L1 expression of tumor cells, but still had the high-degree of TILs.

## CONCLUSIONS

This study concluded that there was a significant correlation between PD-L1 expression of tumor cells with the degree of stromal TILs in colorectal adenocarcinoma, i.e the positive PD-L1 expression was associated with the lower degree of TILs. This indicated that the degree of TILs can be used as a predictive factor for PD-L1 expression of tumor cells in colorectal adenocarcinoma.

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Table 1.The characteristics of samples

| Characteristic | n (%) |
|---|---|
| **Age** | |
| < 40 years | 4 (7,7) |
| ≥ 40 years | 48 (92,3) |
| **Sex** | |
| Male | 27 (51,9) |
| Female | 25 (48,1) |
| **Histological grade** | |
| Well-differentiated | 15 (28,8) |
| Moderately differentiated | 33 (63,5) |
| Poorly differentiated | 4 (7,7) |
| **TILs degree** | |
| Low | 23 (44,2) |
| Moderate | 21 (40,4) |
| High | 8 (15,4) |
| **PD-L1 expression** | |
| Positive | 44 (84,6) |

| | |
|---|---|
| Negative | 8 (15,4) |

Table 2. TILs degree according to the histological grade of samples

| Histological grade | TILs degree | | | Total (%) | |
|---|---|---|---|---|---|
| | Low (%) | Moderate (%) | High(%) | | |
| Well-differentiated | 7 (46,7) | 6 (40,0) | 2 (13,3) | 15 (100) | ***p = 0,619*** |
| Moderately differentiated | 13 (39,4) | 14 (42,4) | 6 (18,2) | 33 (100) | |
| Poorly differentiated | 3 (75,0) | 1 (25,0) | 0 (0,0) | 4 (100) | |
| Total | 23 (44,2) | 21 (40,4) | 8 (15,4) | 52 (100) | |

***Chi-square test***

Table 3. PD-L1 expression of tumor cells according to the histological grade of samples

| Histological grade | PD-L1 expression | | Total | |
|---|---|---|---|---|
| | Positive(%) | Negative(%) | (%) | |
| Well-differentiated | 12 (80,0) | 3 (20,0) | 15 (100) | ***p=0,683*** |
| Moderately differentiated | 29 (87,9) | 4 (12,1) | 33 (100) | |
| Poorly differentiated | 3 (75,0) | 1 (25,0) | 4 (100) | |
| Total | 44 (84,6) | 8 (15,4) | 52 (100) | |

***Chi- Square test***

Table 4. PD-L1 expression of tumor cells and TILs degree of samples

| PD-L1 expression | TILs degree | | | Total (%) | |
|---|---|---|---|---|---|
| | Low(%) | Moderate (%) | High (%) | | |
| Positive | 22 (50,0) | 18 (40,9) | 4 (9,1) | 44 (100) | ***p = 0,017*** |
| Negative | 1 (12,5) | 3 (37,5) | 4 (50,0) | 8 (100) | |
| Total | 23 (44,2) | 21 (40,4) | 8 (15,4) | 52 (100) | |

***Chi- Square test***

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