JBUON 2020; 25(3): 1423-1429
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

PD-L1 expression in colorectal cancer and its relationship with TLR-4 expression

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Summary

Purpose: *Expression of programmed death ligand-1 (PD-L1) is related to the prognosis of many solid tumors, but the prognostic value of PD-L1 expression in colorectal cancer (CRC) remains unclear. The aim of this study was to clarify the role of PD-L1 expression in predicting prognosis, and then provide further insight into the relation between PD-L1 and toll like receptor-4 (TLR-4) in CRC progression.*

Methods: *The expression of PD-L1 and TLR-4 in patients with resected CRC was analyzed using immunohistochemistry (IHC). The biological relation of PD-L1 and TLR-4 in CRC was explored using gene set enrichment analysis (GSEA).*

Results: *Positive PD-L1 and TLR-4 expression in tumor cells were observed in 12.7% and 41.2% respectively. High PD-L1 and TLR-4 expression levels were significantly correlated with poor disease-free survival. PD-L1 expression was closely associated with TLR-4 expression. Multivariate analyses further confirmed that increased expression levels of PD-L1 are unfavorable prognostic factors for operable CRC.*

Conclusion: *High PD-L1 expression can be used as a prognostic indicator for patients with operable CRC. PD-L1 expression is associated with TLR-4 expression, thereby providing a theoretical basis for the combined use of PD-1/PD-L1 inhibitors and TLR agonists.*

Key words: *programmed cell death-ligand 1, toll like receptor-4, prognosis, colorectal cancer*

Introduction

Colorectal cancer (CRC) remains one of the most frequently diagnosed cancers and the second leading cause of cancer-related death in United States [1]. Radical surgery is the optimal treatment for early CRC. Despite advances in diagnosis and treatment approaches in the past decades, 30–50% of patients who undergo potentially curative surgery relapse and die of distant metastases [2]. Avoiding immune destruction is regarded as a hallmark of cancer recurrence or metastasis [3]. Thus, to improve the prognosis of CRC patients it is of great importance to identify underlying immune biomarkers to predict the prognosis and to monitor the progression of CRC patients.

Aberrant activation of immune checkpoints leads to tumor immune escape, and one of the most important immune checkpoints is programmed cell death ligand 1 (PD-L1) [4]. PD-L1 negatively regulates T-cell proliferation through binding of programmed cell death protein 1 (PD-1) and induces activated T cells exhaustion and apoptosis [5]. The blockade of PD-1/PD-L1 pathway in cancer with monoclonal antibodies leads to restore of activated T lymphocytes, and is currently considered the most promising antitumor immunotherapy [6]. A series of phase I-II studies confirmed clinical activity of PD-1/PD-L1 inhibitors in patients with CRC [7,8]. A previous study indicated that increased PD-L1 ex-

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Received: 06/01/2020; Accepted: 03/02/2020

pression was correlation with poor prognosis in esophageal cancer [9], non-small cell lung cancer [10], head and neck cancer [11] and prostate cancer [12]. However, the correlation between PD-L1 expression and prognosis remains controversial in CRC.

TLR-4 is a member of the toll-like family. As a pattern recognition receptor, TLR-4 is primarily capable of enhancing endogenous immunity and immune presentation, as well as mediating inflammatory responses and participating in the expression of inflammatory factors. TLR-4 is highly expressed in epithelial-derived tumors, and its overexpression may be associated with poor prognosis [13]. Recent studies have suggested that toll-like signaling pathways can induce the expression of PD-L1 [14]. However, no studies have investigated the relationship between PD-L1 and TLR-4 in CRC. In this study, we aimed to investigate the expression of PD-L1 and its correlation with clinicopathological features and outcomes in operable CRC, and then provide further insight into the relation between PD-L1 and TLR-4 in CRC. To our knowledge, this is the first report to demonstrate the association of PD-L1 and TLR-4 in operable CRC.

Methods

Patients and tissue samples

Tumor samples were obtained from 236 consecutive patients who had undergone surgical resection for CRC at the Second Affiliated Hospital of Harbin Medical University between April 2010 and March 2013. As of December 2018, the median follow-up time was 75.8 months. TNM was used, based on the 7th edition of the CRC staging system. No patient received preoperative chemotherapy or radiotherapy. Clinical data, including age, gender, primary site, tumor differentiation, and tumor stage, were obtained from medical records. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. Signed informed consents were obtained from all participants before the study entry.

Immunohistochemical (IHC) staining

The relative protein expressions from CRC samples were analyzed by IHC. IHC were performed mainly according to a previous study [15]. Primary antibodies used were anti-TLR-4(ab22048, Abcam, Cambridge, UK) and anti-PD-L1 (ab205921, Abcam, Cambridge, UK) at 1:100 dilutions. IHC analysis of every section was evaluated by two pathologists. Immunohistochemical expression of PD-L1 and TLR-4 was assessed by determining staining intensity and the percentage of tumor cell positivity. All cases in which more than 5% of tumor cells displayed moderate or strong staining were considered. Patients with weak staining or less than 5% of tumor cells were considered negative.

Measurement of the neutrophil to lymphocyte ratio (NLR)

The neutrophil count divided by the lymphocyte count was defined as the NLR. Preoperative peripheral blood samples were obtained routinely before surgery. The cut-off value of NLR was set at 3, which was used in

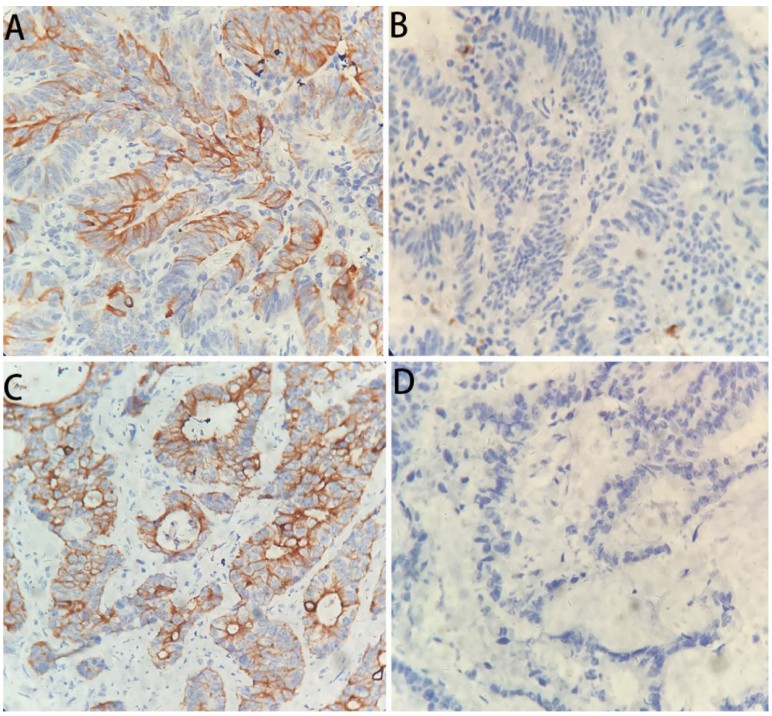

Figure 1. Representative immunohistochemical staining of PD-L1 and TLR-4 in colorectal cancer patients. **A:** Positive PD-L1 expression (magnification 400×). **B:** Negative PD-L1 expression (magnification 400×). **C:** Positive TLR-4 expression (magnification 400×). **D:** Negative TLR-4 expression (magnification 400×).

a previous study [16]. The samples were categorized into two groups based on an NLR ratio of >3 and that of ≤3.

Gene set enrichment analysis (GSEA)

GSEA is a computational method that determines whether an *a priori* defined set of genes shows statistically significant, concordant differences between two biological states [17]. In this study, the TCGA database was used to derive the expression characteristics of CRC genes. PD-L1 expression was categorized as high or low according to the median value of PD-L1 expression in CRC. Signaling pathways associated with high expression of PD-L1 were analyzed by GSEA. The normalized enrichment score (NES) and nominal p value were used to sort the pathways enriched.

Statistics

The correlation of PD-L1 and TLR-4 expression with clinicopathological parameter was evaluated using chi-square (x^2) test or Fisher's exact test. Spearman's correlation coefficient was used for rank correlation. Kaplan-Meier method was used to plot survival curves and log-rank test was performed to evaluate significant differences between groups. All significance tests were

Table 1. Correlation of PD-L1 and TLR4 expression in tumor cells with clinicopathologic features

Clinicopathologic characteristics	*All patients n (%)*	*PD-L1*			*TLR-4*		
		Negative	*Positive*	*p value*	*Negative*	*Positive*	*p value*
Age, years	236	206	30	0.631	138	98	0.397
≤65	132	114	18		74	58	
>65	104	92	12		64	40	
Gender				0.064			0.069
Male	128	107	21		68	60	
Female	108	99	9		70	38	
Primary site				0.131			0.167
Colon	140	126	14		87	53	
Rectum	96	80	16		51	45	
Tumor differentiation				0.016			0.063
Well/Moderate	200	179	21		122	78	
Poor	36	27	9		16	20	
T stage				0.074			0.234
T1-2	90	83	7		57	33	
T3-4	146	123	23		81	65	
Lymoph node metastasis				0.218			0.209
Negative	127	114	13		79	48	
Positive	109	92	17		59	50	
pTNM stage				0.61			0.011
I-II	136	120	16		89	47	
III-IV	100	86	14		49	51	
Vascular invasion				0.965			0.83
Absent	196	171	25		114	82	
Present	40	35	5		24	16	
Perineural invasion				0.595			0.509
Absent	204	179	25		121	83	
Present	32	27	5		17	15	
MMR status				0.012			0.359
MMR-proficient	201	180	21		120	81	
MMR-deficient	35	26	9		18	17	
CEA (ng/ml)				0.862			0.157
≤ 5	145	127	18		90	55	
>5	91	79	12		48	43	
NLR				0.433			0.143
≤3	164	145	19		101	63	
>3	72	61	11		37	35	

2-tailed and p values less than 0.05 were considered significant. All analyses were performed using SPSS, version 23 (SPSS Inc., Chicago, IL, USA).

Results

PD-L1 and TLR-4 expression in CRC tissues

To determine the prevalence of PD-L1 and TLR-4 expression in CRC, the PD-L1 and TLR-4 protein levels were evaluated by immunohistochemistry. In 238 CRC patients, 30 patients (12.7%) showed positive PD-L1 expression in the cell membrane. TLR-4 protein was expressed in the cytoplasm and nucleus. Positive TLR-4 expression of protein was detected in 98 patients (41.2%) (Figure 1).

Correlations of PD-L1 and TLR-4 expression with clinicopathological features

Evaluation of the correlation of PD-L1 and TLR-4 expression with clinicopathological characteristics showed significant correlations between PD-L1 expression and tumor differentiation (0.016) and mismatch repair (MMR) status (p=0.012). TLR-4 expression was correlated with TNM stage (p=0.011) (Table 1). The relationship between PD-L1 and TLR-4 expression was investigated and Spearman's test demonstrated that there was a significant correlation between PD-L1 and TLR-4 (p=0.028) (Table 2).

Prognostic value of PD-L1 and TLR-4 expression

To determine the prognostic value of PD-L1 and TLR-4 expression in CRC, we analyzed the correlation between PD-L1 or TLR-4 expression and clinical outcome. Compared with the PD-L1-negative group, the PD-L1-positive group had significantly shorter DFS (p=0.003). The TLR-4-positive group had shorter DFS (p=0.009) than the TLR-4-negative group (Figure 2).

The univariate Cox regression model indicated that TNM stage (p=0.021), NLR (p=0.005), PD-L1 expression (p=0.003) and TLR-4 expression (p=0.01) were correlated with DFS, whereas age (p=0.259), gender (p=0.362), tumor differentiation (p=0.461), T stage (p=0.069), lymph node metastasis (p=0.055), vascular invasion (p=0.187), perineural invasion (p=0.117) and CEA (p=0.232) were not. Multivariate analysis demonstrated that NLR (p=0.048) and PD-L1 expression (0.019) were significant independent predictors of DFS (Table 3).

GSEA identifies a PD-L1-related signaling pathway

To identify the potential mechanism of PD-L1 expression in CRC, we conducted GSEA between high and low PD-L1 expression data sets from TCGA data-

Table 2. Correlation between PD-L1 expression and the TLR-4

PD-L1	*n*	*TLR-4*		*rho*	*p*
		Positive	*Negative*		
Positive	30	18	12	0.143	0.028
Negative	206	80	126		

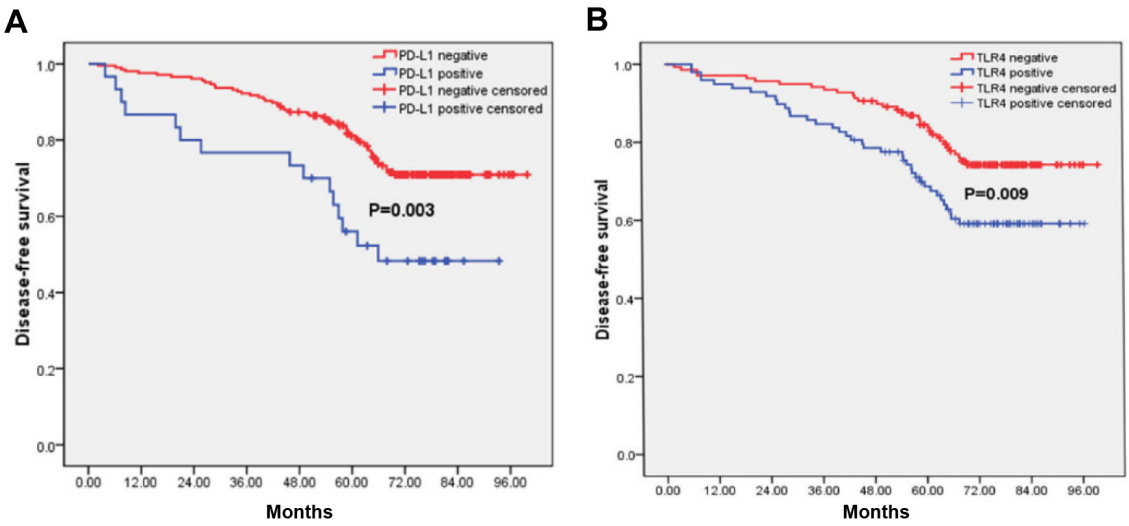

Figure 2. Prognostic significance of PD-L1 and TLR-4 expression in colorectal cancer patients. **A:** Disease-free survival for patients with positive PD-L1 expression and negative PD-L1 expression (p=0.003). **B:** Disease-free survival for patients with positive TLR-4 expression and negative TLR-4 expression (p=0.009).

Table 3. Univariate and multivariate analyses of prognostic factors for overall survival

Factor	*Univariate analysis*		*Multivariate analysis*	
	HR (95% CI)	*p value*	*HR (95% CI)*	*p value*
Age, years (>65 *vs.* ≤65)	1.307 (0.821–2.081)	0.259		
Gender (Male *vs.* Female)	0.805 (0.506–1.283)	0.362		
Tumor differentiation (Poor *vs.* Moderate/Well)	1.263 (0.679-2.350)	0.461		
T stage (T3-4 *vs.* T1-2)	1.595 (0.964–2.639)	0.069		
Lymph node metastasis (Positive *vs.* Negative)	1.580 (0.990-2.519)	0.055		
TNM stage (III-IV *vs.* I-II)	1.729 (1.084-2.756)	0.021	1.486(0.888-2.487)	0.132
Vascular invasion (Absent *vs.* Present)	1.468 (0.830–2.596)	0.187		
Perineural invasion (Absent *vs.* Present)	1.618 (0.887-2.954)	0.117		
CEA (ng/ml) (>5 *vs.* ≤5)	1.331 (0.833–2.128)	0.232		
NLR (>3 *vs.* ≤3)	1.949 (1.224-3.106)	0.005	1.093 (0.655-1.823)	0.048
PD-L1 (Positive *vs.* Negative)	2.345 (1.325–4.149)	0.003	2.008(1.120-3.602)	0.019
TLR4 (Positive *vs.* Negative)	1.852 (1.161–2.954)	0.010	1.636(0.995-2.689)	0.052

Table 4. Gene sets enriched in phenotype high

Location	*Gene set name*	*NES*	*NOM p value*	*FDR q value*
Colon cancer	KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	2.65	<0.001	<0.001
	KEGG_JAK_STAT_SIGNALING_PATHWAY	2.64	<0.001	<0.001
	KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	2.61	<0.001	<0.001
	KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	2.58	<0.001	<0.001
	KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	2.53	<0.001	<0.001
Rectum cancer	KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.97	<0.001	<0.001
	KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	1.94	<0.001	<0.001
	KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	1.92	<0.001	<0.001
	KEGG_JAK_STAT_SIGNALING_PATHWAY	1.92	<0.001	<0.001
	KEGG_LEISHMANIA_INFECTION	1.88	<0.001	<0.001

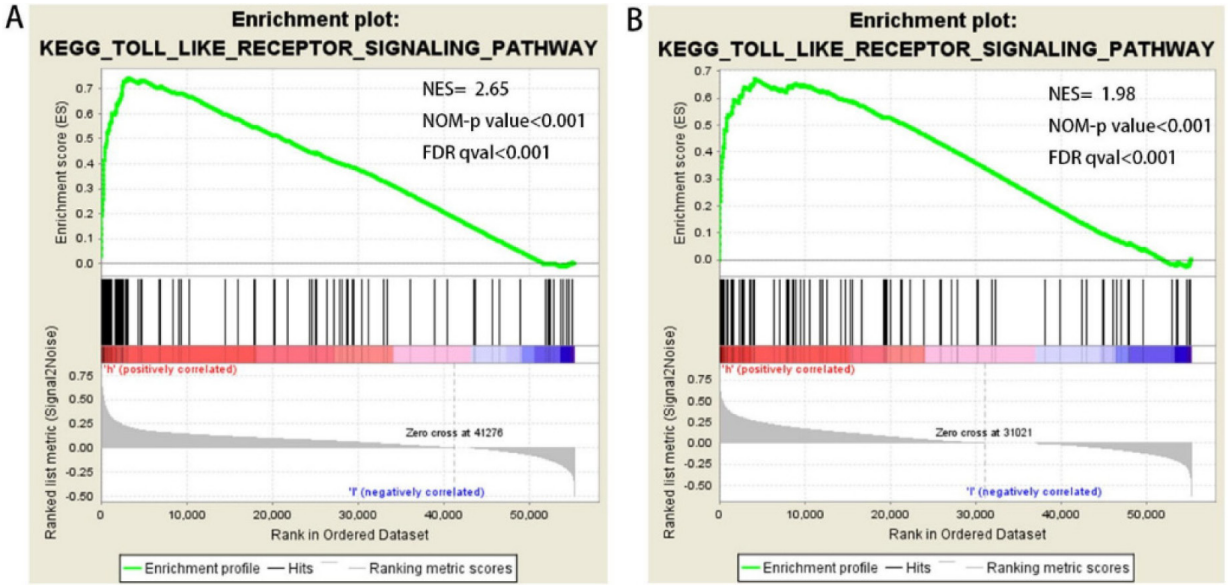

Figure 3. Enrichment plots from gene set enrichment analysis (GSEA). GSEA results showing Toll-like receptor signaling pathway is differentially enriched in PD-L1-related colon **(A)** and rectal cancer **(B)**. ES: enrichment score; NES: normalized ES; NOM: p-val, normalized p-value.

set. Selected were the most significantly enriched signaling pathways based on their NES (Table 4). The most significant difference between PD-L1 high expression phenotype and the toll-like receptor signaling pathway was observed (p<0.001) (Figure 3).

Discussion

The tumor immune microenvironment and inflammation are two important factors inducing CRC, while PD-L1 and TLR-4 are the key molecules involved in tumor immunity and inflammatory response, respectively. Therefore, it was important to investigate the roles of PD-L1 and TLR-4 expressions in CRC. We observed that the expressions of PD-L1 and TLR-4 were associated with poor prognosis. In addition, PD-L1 expression was correlated with TLR-4 protein expression. We also analyzed the role of NLR in operable CRC and observed that NLR was closely associated with poor prognosis of the disease. NLR could be an independent indicator to predict the prognosis of CRC.

PD-1/PD-L1 pathway inhibitors have demonstrated good results in treating CRC [18]. Additionally, PD-L1 expression is very important in predicting the efficacy of PD-1/PD-L1 [19]. Therefore, in-depth studies on the expression of PD-L1 protein in colorectal cancer are needed. Presently, the positive rate of PD-L1 expression was 12%. Similarly, Wang et al reported a positive rate of PD-L1 expression in tumor cells of 20.6% [20]. Mismatch-repair deficiency predicts response of solid tumors to PD-1 blockade [21]. Furthermore, we have demonstrated that dMMR is closely related to PD-L1 expression. As a result, PD-1/PD-L1 pathway inhibitors may exhibit higher efficacy in patients with high PD-L1 expression accompanied with dMMR.

Previous studies have shown that high PD-L1 expression is associated with the poor prognosis of various tumor types. However, the relationship between PD-L1 expression and prognosis remains controversial. Liu et al believed that high PD-L1 expression was associated with good prognosis [22], while other authors suggested otherwise [23,24]. Presently, high PD-L1 expression was related with poor prognosis, which is consistent with a recent meta-analysis conducted by Li et al [25], which demonstrated the association of PD-L1 expression with worse DFS. In addition, this study analyzed the correlations between PD-L1 expression and clinical parameters and the data suggested that increasingly worse tumor differentiation is associated with increasingly greater PD-L1 expression. This result further illustrates that the expression of PD-L1 in tumor cells is associated with their grade of malignancy.

TLRs play an important role in the innate and acquired immune responses toward tumors [26]. Previous studies have verified that as a critical member of the TLR family, TLR-4 plays a decisive role in tumorigenesis and development. In bladder cancer, the TLR-4 signaling pathway upregulates PD-L1 expression by activating the MAPK pathway [27]. However, the relationship between PD-L1 and TLR-4 in CRC remains unclear. Our study demonstrated that PD-L1 expression is closely associated with TLR-4. It has also been demonstrated that in operable lung cancer, PD-L1 expression is associated with TLR-4 expression, and the high expression of TLR-4 is related to the poor prognosis of lung cancer [28]. A recent study also suggested that the adoption of TLR agonists can successfully enhance the sensitivity to anti-PD-L1 antibody [29]. Therefore, it is likely that the use of PD-1/PD-L1 pathway inhibitors in combination with TLR-4 agonists will become a new strategy for cancer treatment.

Our study has certain limitations. First, the study focused on patients with early CRC and did not analyse any patients with advanced disease. This is mainly because the number of available advanced CRC specimens was limited, while these specimens are important in guiding the follow-up treatment of patients. Second, all histological specimens were retrospectively analyzed without considering the effects of patients choosing different treatment options and having different baseline statuses, which could lead to a selection bias. Third, the TLR signaling pathway is complicated. However, the investigation of the relationship between PD-L1 and TLR signaling pathways conducted in this study was relatively simple, and therefore further research will be performed in subsequent studies. Fourth, at the end of the follow-up, death events do not meet the analysis of overall survival, so overall survival was not carried out in our study.

Conclusions

In conclusion, high PD-L1 expression can be used as a prognostic indicator for patients with operable CRC. PD-L1 expression is associated with TLR-4, thereby providing a theoretical basis for the combined use of PD-1/PD-L1 inhibitors and TLR agonists.

Acknowledgements

This study was supported by the Natural Science Foundation (Grant No. YQ2019H007).

Conflict of interests

The authors declare no conflict of interests.

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