

Prognostic Significance of Combined PD-L1 Expression and Chemotherapeutic Status in Cholangiocarcinoma Patients

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Abstract

Background/Aim: Cholangiocarcinoma (CCA) is a malignant tumor with high mortality and recurrence rates after treatment. The discovery of prognostic markers is, therefore, essential for improving patient outcomes after treatment. This study aimed to investigate the prognostic value of membranous and cytoplasmic PD-L1 expression, individually and in combination. In addition, the combination of PD-L1 expression and chemotherapeutic status was also explored. **Patients and Methods:** The expression of PD-L1 was examined in 200 CCA patients using immunohistological staining, and its correlation with clinicopathological data was investigated using the Chi-squared test. Overall survival (OS) and recurrence-free survival (RFS) investigations were performed using Kaplan-Meier (log-rank) and Cox-regression analysis.

Results: The results demonstrated that high PD-L1 expression in the membrane and low expression in the cytoplasm had a correlation with intrahepatic CCA and lymph node metastasis. Furthermore, these patients had shorter OS and RFS compared to other groups of patients. Moreover, when the expression of PD-L1 was combined with chemotherapeutic status, the result demonstrated that patients who had high expression of PD-L1 in the membrane with low expression of PD-L1 in the cytoplasm and did not receive adjuvant chemotherapy showed significantly shorter OS and RFS compared to other patients. This could be an independent prognostic factor for RFS and OS with HR 1.889 and 1.990, respectively.

continued



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Conclusion: PD-L1 expression levels can serve as a valuable predictor of outcomes in CCA patients, especially when evaluated alongside chemotherapeutic status.

Keywords: Cholangiocarcinoma, PD-L1, chemotherapy, prognostic marker, survival analysis.

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor that originates from the bile duct epithelium, accounting for 15% of primary hepatobiliary malignancies (1). The incidence of CCA varies geographically, but the highest incidence of CCA has been noted in Thailand, particularly in the northeastern region (2). Because CCA patients are mostly asymptomatic in the early stage, diagnosis is often made in advanced stages of disease resulting in poor outcomes for patients after treatment (2). Moreover, CCA patients also have high risk of recurrence, which can affect patients' survival (3). Taken together, CCA is therefore a cancer with a high mortality rate. Identifying the markers that can predict cancer recurrence as well as patients' survival is essential to improve patient outcomes after treatment.

Programmed death-ligand 1 (PD-L1), also known as B7-H1 (CD274), is a transmembrane glycoprotein encoded on chromosome nine (4). PD-L1 is expressed in several cell types, including hematopoietic, endothelial, and epithelial cells, and is expressed on tumor cells (5). In tumor cells, PD-L1 is believed to aid escape from immune cells by binding with PD-1 (4). Interaction between PD-L1 and PD-1 reduces the proliferation of PD-1 positive cells, inhibits cytokine secretion and induces apoptosis (6). In recent years, the PD-1/PD-L1 pathway has been a target for new anticancer therapies, and has improved patient survival in a number of cancers (7). Thus, investigation of PD-L1 expression and its correlation with clinical outcomes may be beneficial for treatment. In breast cancer, the expression of PD-L1 is correlated with the epithelial-to-mesenchymal transition (EMT) process in human breast cancer stem cells (BCSCs) (8). Additionally, an association between PD-L1 expression and

clinicopathological data has been found in lung cancer. This report indicated that PD-L1 expression was positively correlated with male sex, advanced stage, invasion, and squamous cell carcinoma histology. PD-L1 positive patients had poorer outcomes than those in the negative group (9).

The prognostic value of PD-L1 has also been explored to some extent in CCA. One study used machine learning technology to investigate the prognostic value of PD-L1, with results suggesting PD-L1 positive patients had a worse prognosis than negative patients (10). Furthermore, several other studies have also demonstrated advantageous outcomes with low PD-L1 expression compared to high expression in terms of survival (11). There are, however, some studies that show high PD-L1 expression correlates with a good prognosis. Zhu and colleagues showed that PD-L1 overexpression was significantly correlated with good overall survival (OS) and disease-free survival (DFS) in CCA (5). In 2019, one study demonstrated that PD-L1 expression correlated with a lack of local invasion as well as improved survival (12). Many other studies indicated that PD-L1 level has no association with the survival of patients (7, 13). Current research therefore presents a controversial picture of the prognostic value of PD-L1. Additionally, any benefits of examining PD-L1 expression in different CCA cell compartments are so far unknown. This study aimed to investigate the prognostic value of PD-L1 expression in cancer cell membranous and cytoplasm, individually and in combination.

Patients and Methods

Patients. Formalin-fixed paraffin-embedded tissues (FFPE) were obtained from CCA patients who had

undergone surgery at the Srinagarind Hospital, Khon Kaen University, Thailand, between 2007 and 2016. Clinicopathological data were provided by the Cholangiocarcinoma Research Institute (CARI). Recurrence was defined as detection of a new tumor. Patients were excluded from the study if they received chemotherapy or radiotherapy before surgery to avoid potential effects on PD-L1 expression (14). The present study was approved by the Institutional Review Board of the Khon Kaen University Ethics Committee based on the Declaration of Helsinki (HE611412).

Immunohistochemistry (IHC). Tissue microarray (TMA) was prepared as 4 µm thick sections, with two independent punctures prepared for each patient. IHC staining was used to examine protein levels. Briefly, the sections were deparaffinized in xylene and rehydrated with a series of concentrations of ethanol. Antigen retrieval was performed using Tris-EDTA buffer in a pressure cooker. Endogenous hydrogen peroxide and nonspecific binding were blocked with 0.3% hydrogen peroxide and 10% skimmed milk. Primary antibody (PD-L1 rabbit monoclonal antibody, #13684, Cell Signaling Technology, Danvers, MA, USA) was incubated for 1 h at room temperature followed by 4°C overnight. After washing, secondary antibody (Dako EnVision, Carpinteria, CA, USA) was added for 1 h. 3, 3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Laboratories, Inc., Newark, CA, USA) was added to develop the signal, then counterstained with Mayer's hematoxylin. Sections were dehydrated and mounted with Permout solution and observed under a light microscope (Nikon Ni-U, Tokyo, Japan).

IHC scoring system. Protein levels were recorded as frequency and intensity scores. Frequency was semi-qualitatively scored as the percentage of positive staining cells: 0%=negative, 1-25%=1+, 26-50%=2+, and >50%=3+. The intensity scores were defined as 0 for negative staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The total score was calculated by multiplying the intensity and frequency. The results were

classified as low expression if the score was less than the median and high expression if the score was more than the median.

Statistical analysis. The Statistical Package for the Social Sciences (SPSS) software was used for statistical analysis. The correlation between protein expression and clinicopathological features was determined using the Chi-square test. Overall survival (OS) and recurrence-free survival (RFS) were performed using Kaplan-Meier (log-rank) and Cox-regression analysis. A *p*-value less than 0.05 was considered statistically significant.

Results

Patient characteristics and the correlation with PD-L1 expression. Table I shows the patient characteristics and histopathology including sex, age, tumor location, histology, metastasis status, stage, and adjuvant chemotherapy status of patients. PD-L1 expression is shown in Figure 1. The correlation of PD-L1 expression with patients' characteristics was analyzed based on location, either in cancer cell cytoplasm or membrane. Among 200 patients, 34.5% and 41.0% demonstrated high expression in the membrane or cytoplasm, respectively. Correlation between protein expression levels and clinicopathological features was also explored. In the membrane, high expression of PD-L1 was significantly associated with intrahepatic CCA (*p*=0.045), while high expression in the cytoplasm was significantly associated with extrahepatic CCA (*p*=0.014). However, when considering the expression in each part individually, there was no correlation between PD-L1 expression levels and other clinicopathological features, including sex, age, histology type, TNM stage, and recurrence. The combination of membranous and cytoplasmic expression was further examined. The results demonstrated that patients with high expression of PD-L1 in membrane and low expression in cytoplasm showed a correlation with intrahepatic CCA (*p*=0.010) and lymph node metastasis (*p*=0.033) (Table I).

Table I. Correlation between PD-L1 expression and clinicopathological features.

Clinicopathological features N (%)	PD-L1 (Membrane)			PD-L1 (Cytoplasm)			Combined PD-L1 (M and C)		
	Low (65.5%)	High (34.5%)	<i>p</i> -Value	Low (59.0%)	High (41.0%)	<i>p</i> -Value	Others (75.5%)	M ^H and C ^L (24.5%)	<i>p</i> -Value
Sex									
Female	42 (60.9)	27 (39.1)	0.317	39 (56.5)	30 (43.5)	0.605	53 (76.8)	16 (23.2)	0.754
Male	89 (67.9)	42 (32.1)		79 (60.3)	52 (39.7)		98 (74.8)	33 (25.2)	
Age (years)									
<61	65 (68.4)	30 (31.6)	0.408	56 (58.9)	39 (41.1)	0.989	75 (78.9)	20 (21.1)	0.281
≥61	66 (62.9)	39 (37.1)		62 (59.0)	43 (41.0)		76 (72.4)	29 (27.6)	
Tumor location									
Intrahepatic	66 (59.5)	45 (40.5)	0.045	74 (66.7)	37 (33.3)	0.014	76 (68.5)	35 (31.5)	0.010
Extrahepatic	65 (73.0)	24 (27.0)		44 (49.4)	45 (50.6)		75 (84.3)	14 (15.7)	
Histology									
Papillary	57 (67.1)	28 (32.9)	0.690	51 (60.0)	34 (40.0)	0.805	65 (76.5)	20 (23.5)	0.784
Others	74 (64.3)	41 (35.7)		67 (58.3)	48 (41.7)		86 (74.8)	29 (25.2)	
Primary tumor (T)									
I, II	91 (67.9)	43 (32.1)	0.307	76 (56.7)	58 (43.3)	0.349	105 (78.4)	29 (21.6)	0.181
III, IV	40 (60.6)	26 (39.4)		42 (63.6)	24 (36.4)		46 (69.7)	20 (30.3)	
Lymph nodes metastasis (N)									
No	77 (71.3)	31 (28.7)	0.062	62 (57.4)	46 (42.6)	0.620	88 (81.5)	20 (18.5)	0.033
Yes	54 (58.7)	38 (41.3)		56 (60.9)	36 (39.1)		63 (68.5)	29 (31.5)	
Distant metastasis (M)									
No	124 (66.0)	64 (34.0)	0.755	109 (58.0)	79 (42.0)	0.366	144 (76.6)	44 (23.4)	0.171
Yes	7 (58.3)	5 (41.7)		9 (75.0)	3 (25.0)		7 (58.3)	5 (41.7)	
TNM Stage									
I, II	55 (70.5)	23 (29.5)	0.233	46 (59.0)	32 (41.0)	0.995	64 (82.1)	14 (17.9)	0.085
III, IV	76 (62.3)	46 (37.7)		72 (59.0)	50 (41.0)		87 (71.3)	35 (28.7)	
Adjuvant CMT status									
No	87 (65.9)	45 (34.1)	0.865	75 (56.8)	57 (43.2)	0.382	102 (77.3)	30 (22.7)	0.417
Yes	44 (64.7)	24 (35.3)		43 (63.2)	25 (36.8)		49 (72.1)	19 (27.9)	
Recurrence									
No	89 (65.0)	48 (35.0)	0.814	80 (58.4)	57 (41.6)	0.797	103 (75.2)	34 (24.8)	0.878
Yes	42 (66.7)	21 (33.3)		38 (60.3)	25 (39.7)		48 (76.2)	15 (23.8)	

PD-L1: Programmed death-ligand 1; TNM: size of primary tumor-node metastasis-distant metastasis; Adjuvant CMT; adjuvant chemotherapy; C: cytoplasm; M: membrane. Statistically significant *p*-values are shown in bold.

The correlation between PD-L1 expression in the membrane and the cytoplasm was also examined. The results indicated that patients with high PD-L1 expression in the membrane had low expression of PD-L1 in the cytoplasm ($p=0.012$).

PD-L1 expression and survival analysis. Survival rate was analyzed according to the expression levels of PD-L1. The results showed that patients with low expression of PD-L1 in the cytoplasm had significantly shorter overall survival (OS) ($p=0.021$) and recurrence-free survival (RFS) ($p=0.005$) compared to those with high expression (Figure 2A and B).

In contrast, patients with high expression of PD-L1 in the membrane tend to have shorter OS and RFS compared with those with low expression (Figure 2C and D). Additionally, the combination of membranous and cytoplasmic expression was also investigated. The results showed that patients with high expression of PD-L1 in the membrane and low expression of PD-L1 in the cytoplasm showed significantly shorter OS ($p=0.009$) and RFS ($p=0.009$) compared with other groups of patients (Figure 2E and F).

To identify an independent prognostic factor for OS and RFS, univariate and multivariate analyses were explored. The results from univariate analysis showed that the

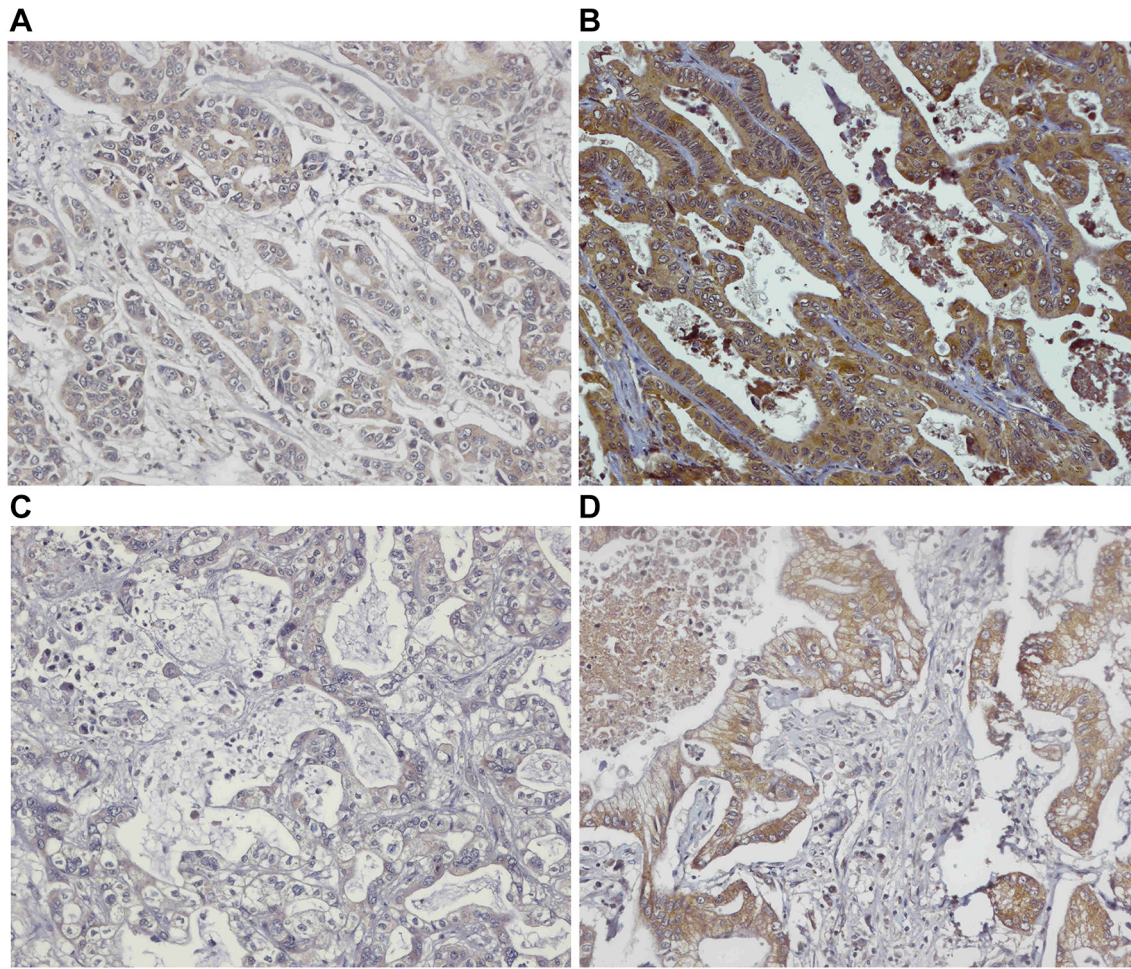


Figure 1. Representative figure of PD-L1 expression. (A) Low expression in the cytoplasm, (B) high expression in the cytoplasm, (C) low expression in the membrane, and (D) high expression in the membrane.

patients with high T stage (Stage III, IV), lymph node metastasis, and late stage showed unfavorable outcomes for OS and RFS. The hazard ratio (HR) of high T stage was 2.414 ($p < 0.001$; RFS) and 2.480 ($p < 0.001$; OS), lymph node metastasis was 2.001 ($p < 0.001$; RFS) and 2.238 ($p < 0.001$; OS), and late stage was 2.438 ($p < 0.001$; RFS) and 2.639 ($p < 0.001$; OS) compared with their references. While the HR of patients with high PD-L1 expression in the cytoplasm was 0.660 ($p = 0.005$; RFS) and 0.714 ($p = 0.022$; OS), for those with high expression of PD-L1 in membrane and low expression of PD-L1 in cytoplasm it was 1.546 ($p = 0.010$; RFS) and 1.544 ($p = 0.010$; OS) compared with their

references (Table II). The results from multivariate analysis indicated that T stage and lymph node metastasis were independent prognostic factors of RFS and OS. The HR of the T stage was 1.679 ($p = 0.011$; RFS) and 1.727 ($p = 0.006$; OS) compared with their references. While the HR of lymph node metastasis was 1.546 ($p = 0.043$; OS) compared with their references. However, there was no statistical significance in the PD-L1 expression on multivariate analysis (Table III).

Survival outcomes based on PD-L1 expression and adjuvant chemotherapy status. Patients' survival was also analyzed

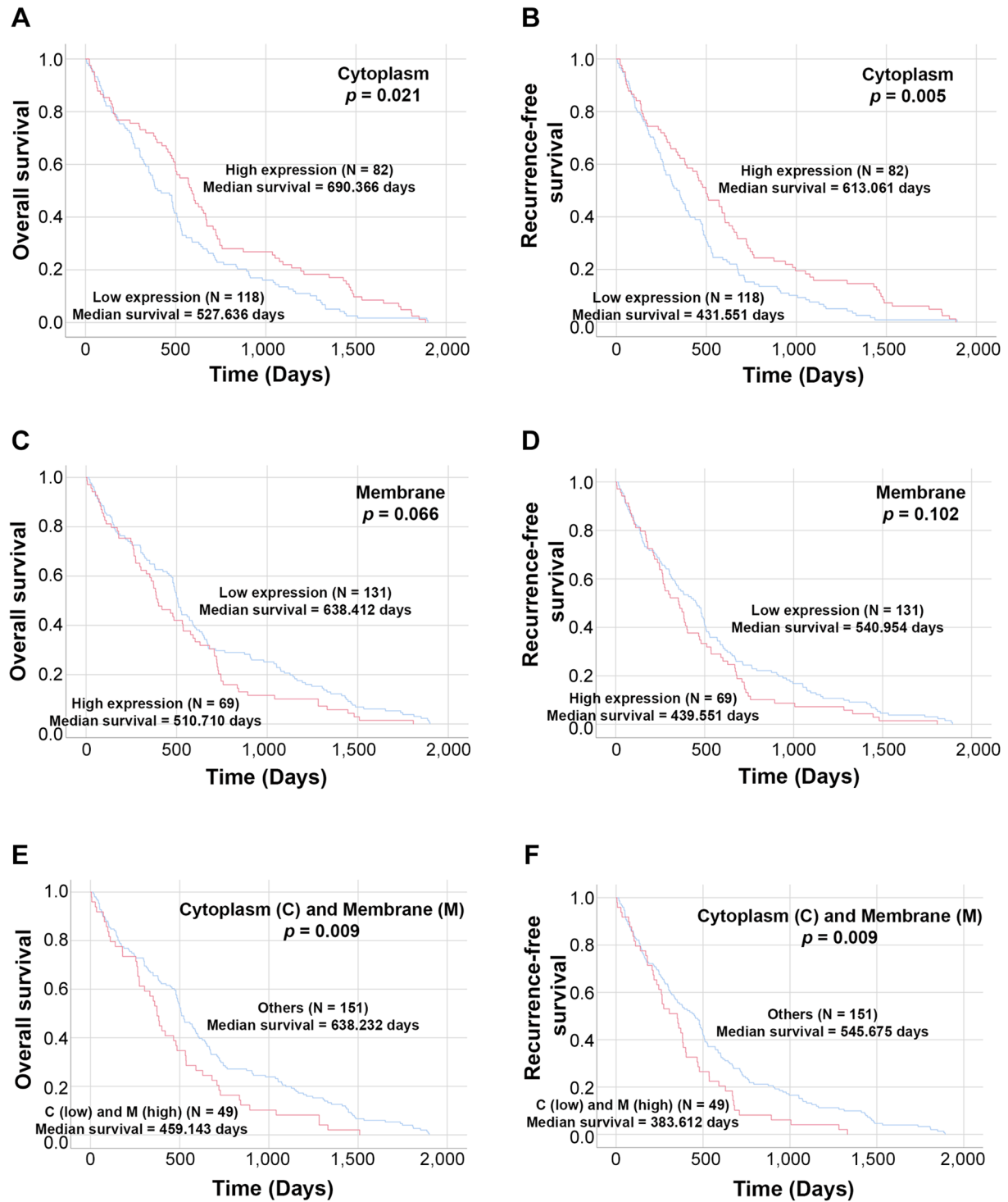


Figure 2. Survival analysis of PD-L1. (A) Overall survival and (B) Recurrence-free survival of CCA patients according to PD-L1 expression in the cytoplasm. (C) Overall survival and (D) Recurrence-free survival of CCA patients according to PD-L1 expression in the membrane, and (E) Overall survival and (F) Recurrence-free survival of CCA patients according to the combination of PD-L1 expression in the cytoplasm and membrane. CCA: Cholangiocarcinoma.

Table II. *Univariate analysis of PD-L1 expression.*

Characteristics	Recurrence-free survival			Overall survival		
	HR	95% CI	p-Value	HR	95% CI	p-Value
Sex (Female vs. Male)	1.223	0.909-1.646	0.183	1.249	0.927-1.684	0.143
Age (<61 vs. ≥61)	1.129	0.853-1.495	0.395	1.318	0.993-1.750	0.056
Tumor location (Intra. vs. Extra)	0.860	0.650-1.139	0.293	0.871	0.657-1.155	0.339
Histology (Papillary vs. Others)	1.249	0.942-1.657	0.123	1.102	0.831-1.462	0.499
Primary tumor (T) (I/II vs. III/IV)	2.414	1.770-3.293	<0.001	2.480	1.819-3.381	<0.001
Lymph nodes metastasis (N) (No vs. Yes)	2.001	1.495-2.679	<0.001	2.238	1.667-3.005	<0.001
Distant metastasis (M) (No vs. Yes)	1.337	0.744-2.404	0.332	1.606	0.891-1.894	0.115
TNM Stage (I/II vs. III/IV)	2.438	1.788-3.323	<0.001	2.639	1.020-3.611	<0.001
Adjuvant CMT (No vs. Yes)	0.873	0.651-1.171	0.366	0.801	0.597-1.076	0.140
Membrane PD-L1 (Low vs. High)	1.279	0.952-1.718	0.103	1.319	0.981-1.774	0.067
Cytoplasm PD-L1 (Low vs. High)	0.660	0.494-0.883	0.005	0.714	0.535-0.952	0.022
Combined PD-L1 (M and C) (Others vs. M ^H and C ^L)	1.546	1.111-2.152	0.010	1.544	1.111-2.145	0.010
Combined PD-L1 and Adjuvant CMT (Others vs. M ^H , C ^L and Adjuvant CMT ^{No})	2.022	1.351-3.026	0.001	2.142	1.434-3.199	<0.001

PD-L1: Programmed death-ligand 1; TNM: size of primary tumor-node metastasis-distant metastasis; Adjuvant CMT: adjuvant chemotherapy; HR: hazard ratio; C: cytoplasm; M: membrane. Statistically significant *p*-values are shown in bold.

Table III. *Multivariate analysis of PD-L1 expression.*

Characteristics	Recurrence-free survival			Overall survival		
	HR	95% CI	p-Value	HR	95% CI	p-Value
Primary tumor (T) (I/II vs. III/IV)	1.679	1.126-2.503	0.011	1.727	1.169-2.553	0.006
Lymph nodes metastasis (N) (No vs. Yes)	1.430	0.938-2.180	0.097	1.546	1.014-2.355	0.043
TNM Stage (I/II vs. III/IV)	1.495	0.888-2.519	0.131	1.502	0.893-2.527	0.125
Cytoplasm PD-L1 (Low vs. High)	0.740	0.533-1.027	0.072	0.782	0.562-1.087	0.143
Combined PD-L1 (M and C) (Others vs. M ^H and C ^L)	0.789	0.464-1.342	0.382	0.813	0.479-1.382	0.445
Combined PD-L1 and Adjuvant CMT (Others vs. M ^H , C ^L and Adjuvant CMT ^{No})	1.889	1.035-3.449	0.038	1.990	1.103-3.591	0.022

PD-L1: Programmed death-ligand 1; TNM: size of primary/tumor-node metastasis/distant metastasis; Adjuvant CMT: adjuvant chemotherapy; HR: hazard ratio; C: cytoplasm; M: membrane. Statistically significant *p*-values are shown in bold.

according to adjuvant chemotherapy status. In patients who did not receive adjuvant chemotherapy, the result showed that the patients with high expression of PD-L1 in the membrane and low expression of PD-L1 in the cytoplasm showed significantly shorter OS ($p=0.001$) and RFS ($p=0.002$) compared with other groups of patients (Figure 3). In contrast, there was no statistical significance in patients who received adjuvant chemotherapy (data not shown).

Furthermore, survival analysis regarding the combination of PD-L1 expression and adjuvant chemotherapy status was also examined. Patients who had high expression of PD-L1 in

the membrane and low expression of PD-L1 in the cytoplasm and did not receive adjuvant chemotherapy showed significantly shorter OS ($p<0.001$) and RFS ($p<0.001$) compared to other patients (Figure 4). To examine whether the combination of PD-L1 expression and adjuvant chemotherapy status could be used as a prognostic factor, univariate and multivariate analyses were examined. Univariate analysis showed the HR of patients who had high expression of PD-L1 in the membrane and low expression of PD-L1 in the cytoplasm and did not receive adjuvant chemotherapy was 2.022 ($p=0.001$; RFS) and 2.142 ($p<0.001$;

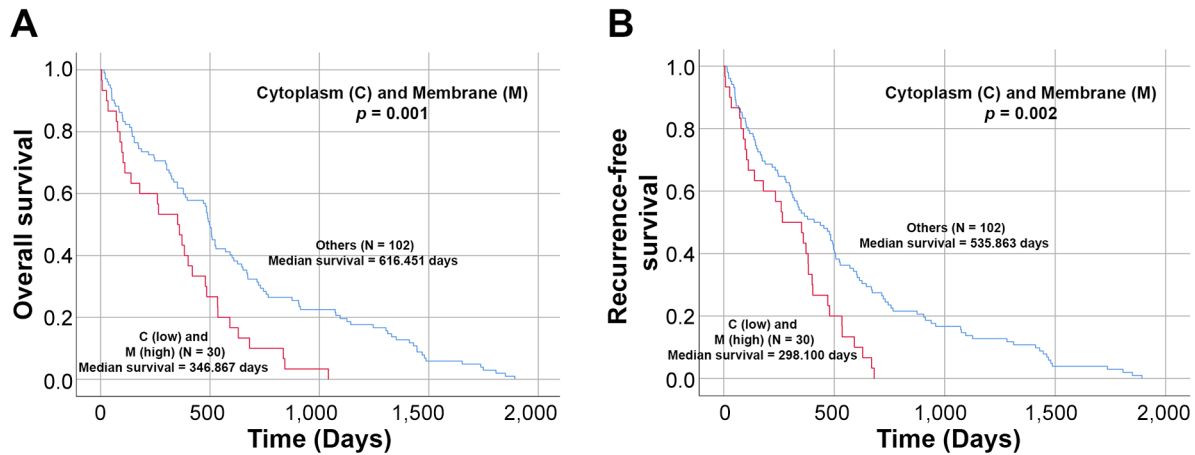


Figure 3. Survival analysis of CCA patients who did not receive chemotherapy. (A) Overall survival (B) recurrence-free survival of CCA patients according to the combination of PD-L1 expression in the cytoplasm and membrane. CCA: Cholangiocarcinoma.

OS) compared with their reference (Table II). Moreover, it could be used as an independent prognostic factor for RFS and OS with HR 1.889 ($p=0.038$; RFS) and 1.990 ($p=0.022$; OS), respectively (Table III).

Discussion

Cholangiocarcinoma is a cancer that is mostly asymptomatic in the early stages; thus, most patients are diagnosed when the disease becomes advanced (15). This results in a high mortality rate among these patients. Moreover, many patients experience recurrence after treatment, resulting in a poor prognosis associated with the disease (16). To solve this problem, identification of methods for early diagnosis as well as markers related to the patient's prognosis after treatment are essential.

Programmed death-ligand 1 (PD-L1) is a transmembrane glycoprotein expressed in several cell types as well as in tumor cells (17). Interactions between PD-L1 and its receptor, PD-1, attenuate T cell-activating signals, such as inhibiting cytokine production, T cell proliferation, survival, etc. PD-L1 is thus believed to help cancer cells evade immune responses (18). Because of its role in T cell suppression, antibody-based cancer therapeutics have been developed to target PD-L1 (19). However, treatment with a PD-1/PD-L1 inhibitor can trigger immune-related

adverse events (20). Knowing its role and its importance to patients' prognoses is therefore beneficial before using it as a drug target. In 2019, the prognostic value of PD-L1 was examined in colorectal cancer using a systematic review and meta-analysis from ten studies. The results indicated that high expression of PD-L1 was significantly correlated with poor overall and disease-free survival. This suggests that the expression of PD-L1 might be a biomarker for a poor prognosis in patients with colorectal cancer (21). PD-L1 shows a broad distribution in many cellular compartments, including the cell membrane, cytoplasm, and nucleus, and can also be secreted into the circulation (22). Recently, there was a study that highlighted the importance of investigating PD-L1 expression in different compartments of endometrial cancer (EC). The results demonstrated that soluble PD-L1 is easily detectable and is associated with aggressive clinical features in EC patients (23). In our studies, we thus investigate the expression of PD-L1 in CCA tissues. The prognostic value of PD-L1 was examined based on its location, in either the cytoplasm or membrane. We found that high expression of PD-L1 in the membrane was associated with intrahepatic CCA. On the other hand, the high expression of PD-L1 in the cytoplasm was associated with extrahepatic CCA. Furthermore, the combination of membranous and cytoplasmic expression was also explored. The results showed that the combining

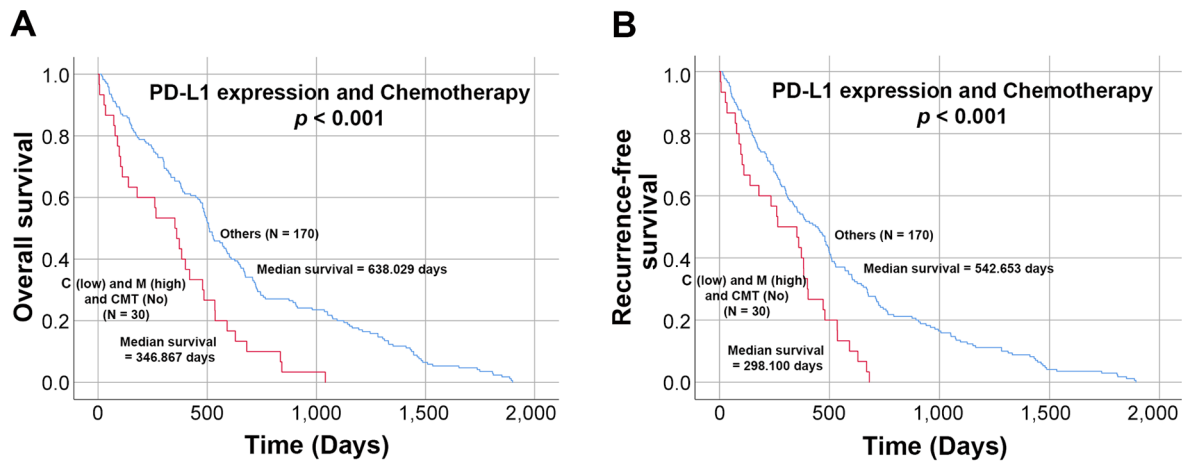


Figure 4. Survival analysis regarding the combination of PD-L1 expression and adjuvant chemotherapy status. (A) Overall survival (B) recurrence-free survival of CCA patients according to the combination of PD-L1 expression and adjuvant chemotherapy status. CCA: Cholangiocarcinoma.

of high expression of PD-L1 in the membranous part with low expression of PD-L1 in the cytoplasmic part was associated with intrahepatic CCA and lymph node metastasis. This result indicated that high expression of PD-L1 on the membrane was associated with more aggressive cancer. This may be associated with its function as a ligand of PD-1, as binding of PD-L1 and PD-1 leads to the suppression of T-cell-mediated immunity (18). Several studies have reported that the expression of PD-L1 is associated with the survival of cancer patients (7, 24-27). Similarly in our study, we found CCA patients with low expression of PD-L1 in cytoplasm had significantly shorter OS and RFS than other groups. In contrast, patients with high expression of PD-L1 in the membrane tend to have shorter OS and RFS compared with those with low expression. These results indicate the importance of the location of PD-L1 expression, which can affect its correlation with survival (24, 26).

Surgery can nowadays be a potentially curative treatment for CCA patients. However, most patients are diagnosed with CCA when the disease is in a late stage. Surgery is therefore not suitable for all patients (28). In addition to surgery, adjuvant chemotherapy or radiotherapy is beneficial and can improve a patient's survival. Therefore, to avoid the effect of chemotherapy on patients' outcomes, we analyzed the prognostic value of

PD-L1 according to chemotherapy status (29). In patients who did not receive chemotherapy, we found that patients with high expression of PD-L1 in the membrane combined with low expression in the cytoplasm had shorter OS and RFS compared with other groups of patients, which we did not find in patients who received adjuvant chemotherapy. This indicates that the expression of PD-L1 might not affect the response to chemotherapy. Moreover, if the expression of PD-L1 levels was combined with chemotherapeutic status, it could serve as an independent prognostic marker for OS and RFS of CCA patients.

Conclusion

PD-L1 expression levels serve as a valuable predictor of outcomes in cholangiocarcinoma patients, especially when evaluated alongside chemotherapeutic status.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization, S.P., M.T., W.L.; analysis and interpretation of data, S.P., M.T.; project administration, W.L.; writing and

editing manuscript, S.P., M.T., H.D., T.C., W.L. All Authors have read and agreed to the published version of the manuscript.

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Artificial Intelligence (AI) Disclosure

No artificial intelligence (AI) tools, including large language models or machine learning software, were used in the preparation, analysis, or presentation of this manuscript.

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