PD-L1 Expression in High-Risk Early-Stage Colorectal Cancer—Its Clinical and Biological Significance in Immune Microenvironment

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Abstract: Programmed death-ligand 1 (PD-L1) is an immune checkpoint molecule that can regulate immune responses in the tumor microenvironment (TME); however, the clinical applications of PD-L1 in early-stage colorectal cancer (CRC) remain unclear. In this study, we aimed to investigate the relationship between PD-L1 expression and survival outcome and explore its relevant immune responses in CRC. PD-L1 expression was evaluated by immunohistochemical staining to determine the tumor proportion score and combined positive score (CPS) in a Taiwanese CRC cohort. The oncomine immune response research assay was conducted for immune gene expression analyses. CRC datasets from the TCGA database were reappraised for PD-L1-associated gene enrichment analyses using GSEA. The high expression of PD-L1 (CPS ≥ 5) was associated with longer recurrence-free survival (p = 0.031) and was an independent prognostic factor as revealed by multivariate analysis. High PD-L1 expression was related to six immune-related gene signatures, and CXCL9 is the most significant overexpressed gene in differential analyses. High CXCL9 expression correlated with increased infiltration levels of immune cells in the TME, including CD8+ T lymphocytes and M1 macrophages. These findings suggest that high PD-L1 expression is a prognostic factor of early-stage CRC, and CXCL9 may play a key role in regulating PD-L1 expression.

Keywords: PD-L1; combined positive score; colorectal cancer; prognostic biomarker; CXCL9

1. Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers around the world. In Taiwan, nearly 16,000 cases of CRC were newly diagnosed in 2019, and about 80% of them were regional or locally advanced CRCs [1]. Approximately 6000 patients died from this disease in the same year in Taiwan. Regular screening plays an important role in CRC management because the survival outcome differs greatly between early and late-stage
CRCs. Even though early-stage CRCs have a relatively good prognosis with a 5-year survival of 72–91% [2], high-risk stage II and stage III CRCs are the subgroups with the poorest survival. Only about 59% of patients in these subgroups would achieve disease-free status by undergoing curative surgery alone [3]; so, curative surgery following adjuvant chemotherapy is the standard treatment. Although adjuvant chemotherapy improves the 3-year disease-free survival to 78.2% in these subgroups [4], a certain proportion of patients cannot receive any benefit from adjuvant chemotherapy. How to precisely detect which patient needs adjuvant chemotherapy is still an unresolved issue. Many classification methods and prognostic biomarkers have been proposed for evaluating survival in early-stage CRCs; however, they are yet to become the guidance in clinical practice because of many factors, such as feasibility, reproducibility, and accuracy.

PD-L1, also known as CD274, is a transmembrane checkpoint protein expressed on various types of immune and tumor cells. The PD-L1/ PD-1 pathway downregulates T-cell function during inflammatory states and takes part in adaptive immune resistance in cancer [5]. PD-L1 expression has a prognostic value in various types of cancer [6–14]; however, its significance in early-stage CRCs is still debated. The inconsistent results are probably from the heterogeneity of the study population, different PD-L1 staining antibodies, different definitions of PD-L1 positivity, etc. However, PD-L1 immunohistochemistry has standardized guidance for staining protocol and interpretation criteria. As a prognostic marker, PD-L1 staining is also convenient and relatively cost effective. Owing to more efforts exploring the utility of PD-L1 as a prognostic marker, studies demonstrated that PD-L1 expressed on tumor cells or tumor-infiltrating cells might result from separate mechanistic pathways and affect survival outcomes differently. [13–15]. The regulations of PD-L1 expression are complicated, including genomic, epigenetic, transcriptional, and posttranscriptional levels; however, its detailed mechanisms are yet to be elucidated in the CRC microenvironment [16]. A better understanding of mechanisms regulating PD-L1 expression in the tumor microenvironment (TME) may help us clarify the clinical utility of PD-L1 expression or even immunotherapy-based treatments in CRC. Therefore, this study aimed to evaluate the prognostic value of PD-L1 expression in high-risk, early-stage CRCs and to explore the possible underlying mechanisms.

2. Results
2.1. Clinical Characteristics of the Study Participants

A total of 100 patients, including 3 patients with high-risk stage II and 97 patients with stage III CRC, were enrolled. Until the end of 2021, the median follow-up of this CRC cohort was 5.2 years. Recurrence was detected in 32 of 100 patients and 68 patients remained in disease-free states. The clinical characteristics of these 100 patients are shown in Table 1. The median age of these patients was 56.5 years, 54% were male, and 23% had right-sided colon cancer. The majority of tumors were adenocarcinomas (91%) and moderately differentiated (91%). The RAS mutation, BRAF mutation, and deficient mismatch repair/microsatellite instability-high (dMMR/MSI-H) were detected in 42%, 6%, and 6% of tumors, respectively. PD-L1 expression in primary CRC tumor samples was determined by the tumor proportion score (TPS) and combined positive score (CPS). Figure 1 shows the representative immunostaining results of CRC with a CPS score of <1 (Figure 1A), 1–4 (Figure 1B), 5–9 (Figure 1C), and ≥10 (Figure 1D). When PD-L1 expression was evaluated by TPS, most of the tumors had no or very low PD-L1 expression (<1%), and only 5% of tumors had TPS of ≥1%. When CPS was used to assess the PD-L1 expression, 53% of tumors had positive PD-L1 expression, which was defined by CPS of ≥1, and 47% of tumors had no PD-L1 expression (CPS < 1). CPS 1–4, 5–9, and ≥10 accounted for 26%, 15%, and 12% of the entire cohort. By using the double staining technique, the main proportion of PD-L1 expression was identified on CD68+ macrophages and a small proportion was on CD3+ T lymphocytes in the tumor tissue with high CPS (>10) as shown in Figure S1.
Table 1. Clinical characteristics of 100 CRC patients.

| Characteristics                        | No. of Cases (%) |
|----------------------------------------|------------------|
| Total patients                         | 100 (100)        |
| Age, median (range)—year               | 56.5 (29–78)     |
| Gender                                 |                  |
| Male                                   | 54 (54)          |
| Female                                 | 46 (46)          |
| Primary tumor location                  |                  |
| Right-sided                            | 23 (23)          |
| Left-sided \(^a\)                      | 76 (76)          |
| Multiple                               | 1 (1)            |
| Tumor histology                        |                  |
| Adenocarcinoma                         | 91 (91)          |
| Mucinous adenocarcinoma                | 9 (9)            |
| Tumor grade                            |                  |
| Well differentiated                    | 5 (5)            |
| Moderately differentiated              | 91 (91)          |
| Poorly differentiated                  | 4 (4)            |
| TMN stage                              |                  |
| II                                     | 3 (3)            |
| III                                    | 97 (97)          |
| RAS status                             |                  |
| Mutant                                 | 42 (42)          |
| KRAS                                   | 40 (40)          |
| NRAS                                   | 2 (2)            |
| Wild type                              | 58 (58)          |
| BRAF status                            |                  |
| Mutant                                 | 6 (6)            |
| Wild type                              | 94 (94)          |
| MMR status                             |                  |
| dMMR/MSI-high                          | 6 (6)            |
| pMMR/MSI-stable and -low               | 94 (94)          |
| PD-L1 expression                       |                  |
| Tumor proportion score                 |                  |
| <1%                                    | 95 (95)          |
| ≥1%                                    | 5 (5)            |
| Combined positive score                |                  |
| <1                                    | 47 (47)          |
| 1–4                                    | 26 (26)          |
| 5–9                                    | 15 (15)          |
| ≥10                                    | 12 (12)          |
| Recurrence                             |                  |
| Yes                                    | 32 (32)          |
| No                                     | 68 (68)          |

\(^a\) The left-sided colon is defined as the splenic flexure to rectum. Abbreviations: MMR, mismatch repair; dMMR, deficient mismatch repair; MSI, microsatellite instability.

2.2. Survival Analysis by Different Levels of PD-L1 Expression

To investigate the prognostic value of PD-L1 expression, we performed a cut point analysis for RFS, overall survival (OS), and hazard ratios across different scoring methods and different levels of PD-L1 expression. When TPS was applied to determine the PD-L1 expression, there was no survival difference between patients with TPS of ≥1% and TPS of <1%, as shown in Figure 2A. It was noteworthy that when CPS of ≥3 or 5 was used to categorize the patients as high PD-L1 expression, patients with high PD-L1 expression had significantly better RFS than those with low PD-L1 expression (p = 0.038 and p = 0.031, respectively; Figure 2B,C). There was also a trend showing a better OS for the high PD-L1 expression group compared with those with low PD-L1 expression, especially for patients with CPS of ≥5. These results indicate that CPS can be a prognostic marker for patients with early-stage CRC.
There was no significant difference between patients with high and low PD-L1 expression in terms of age, gender, tumor histology, tumor grade, Tstage, TNM stage, Tumor location, Gender, Mutational status of RAS and BRAF mutation status, and different levels of PD-L1 expression.

When CPS of ≥5 was used to define the high expression of PD-L1, patients with high PD-L1 expression were significantly more likely to have right-sided (p = 0.046) and dMMR/MSI-H CRC (p = 0.024) compared to patients with low PD-L1 expression (Table 2). There was no significant difference between patients with high and low PD-L1 expression in terms of age, gender, tumor histology, tumor grade, RAS mutation status, and BRAF mutation status. The univariate analysis showed that mutant RAS and low PD-L1 expression were associated with worse RFS (Table 3). In the multivariate analysis, PD-L1 expression was still an independent prognostic factor for RFS.

**Figure 1.** Representative images of PD-L1 staining in CRC tumor cells (arrow head) and immune cells (arrow). CRCs with CPS of <1 (A), 1–4 (B), 5–9 (C), and ≥10 (D) are shown at 100× and 400× magnification.

**Figure 2.** Survival difference of CRC patients at different cutoffs of PD-L1 expression levels. Kaplan–Meier analyses of recurrence-free survival and overall survival in CRC patients with high and low PD-L1 expression using a TPS cutoff value of 1 (A), CPS cutoff values of 3 (B), and 5 (C) are shown. RFS, recurrence-free survival; OS, overall survival; CI, 95% confidence interval.

2.3. Clinicopathological Features of Patients with High and Low PD-L1 Expression

When CPS of ≥5 was used to define the high expression of PD-L1, patients with high PD-L1 expression were significantly more likely to have right-sided (p = 0.046) and dMMR/MSI-H CRC (p = 0.024) compared to patients with low PD-L1 expression (Table 2). There was no significant difference between patients with high and low PD-L1 expression in terms of age, gender, tumor histology, tumor grade, RAS mutation status, and BRAF mutation status. The univariate analysis showed that mutant RAS and low PD-L1 expression were associated with worse RFS (Table 3). In the multivariate analysis, PD-L1 expression was still an independent prognostic factor for RFS.
Table 2. Clinical characteristics of patients with high and low PD-L1 expression.

| Characteristics                      | PD-L1 Expression | p-Value |
|--------------------------------------|------------------|---------|
|                                      | CPS ≥ 5          | CPS < 5 | p-Value |
| Age—no. (%)                          |                  |         |         |
| ≥65 years                            | 7 (7)            | 20 (20) | 0.883   |
| <65 years                            | 20 (20)          | 53 (53) |         |
| Gender—no. (%)                       |                  |         |         |
| Male                                 | 16 (16)          | 38 (38) | 0.521   |
| Female                               | 11 (11)          | 35 (35) |         |
| Tumor histology—no. (%)              |                  |         |         |
| Adenocarcinoma                       | 25 (25)          | 66 (66) | 0.735   |
| Mucinous adenocarcinoma              | 2 (2)            | 7 (7)   |         |
| Tumor grade—no. (%)                  |                  |         |         |
| Well differentiated                  | 0 (0)            | 5 (5)   | 0.231   |
| Moderately differentiated            | 25 (25)          | 66 (66) |         |
| Poorly differentiated                | 2 (2)            | 2 (2)   |         |
| Tumor location                       |                  |         |         |
| Primary tumor location—no. (%)       |                  |         |         |
| Right-sided                          | 10 (10)          | 13 (13) | 0.046 * |
| Left-sided                           | 17 (17)          | 59 (60) |         |
| Stage—no. (%)                        |                  |         |         |
| II                                   | 2 (2)            | 1 (1)   | 0.116   |
| III                                  | 25 (25)          | 72 (72) |         |
| RAS status—no. (%)                   |                  |         |         |
| Mutant                               | 12 (12)          | 30 (30) | 0.763   |
| Wild type                            | 15 (15)          | 43 (43) |         |
| BRAF status—no. (%)                  |                  |         |         |
| Mutant                               | 1 (1)            | 5 (5)   | 0.557   |
| Wild type                            | 26 (26)          | 68 (68) |         |
| MMR status—no. (%)                   |                  |         |         |
| dMMR/MSI-high                        | 4 (4)            | 2 (2)   | 0.024 * |
| pMMR/MSI-stable or -low              | 23 (23)          | 71 (71) |         |

* Abbreviation: MMR, mismatch repair; dMMR, deficient mismatch repair; MSI, microsatellite instability. * p < 0.05.
* One patient was diagnosed of simultaneous right- and left-sided CRC, and this case was not included in the comparison study.

Table 3. Univariate and multivariate analysis for RFS.

| Characteristics                  | Univariate Analysis | p-Value | Multivariate Analysis | p-Value |
|----------------------------------|---------------------|---------|-----------------------|---------|
|                                  | Hazard Ratio | 95% CI     |                      | Hazard Ratio | 95% CI     |                      |
| Age—no. (%)                      |            |            |                      |            |            |                      |
| ≥65 years                        | 1.703      | 0.832–3.487| 0.145                | 1.540      | 0.751–3.157| 0.238                |
| <65 years                        | -          |            |                      | -          |            |                      |
| Gender—no. (%)                   |            |            |                      |            |            |                      |
| Male                             | 0.818      | 0.409–1.636| 0.57                 |            |            |                      |
| Female                           |            |            |                      |            |            |                      |
| Tumor histology—no. (%)          |            |            |                      |            |            |                      |
| Adenocarcinoma                   | 0.592      | 0.207–1.690| 0.327                |            |            |                      |
| Mucinous adenocarcinoma          |            |            |                      |            |            |                      |
| Tumor grade—no. (%)              |            |            |                      |            |            |                      |
| Well differentiated              | 1.544      | 0.210–11.341| 0.669               |            |            |                      |
| Moderately differentiated        | 2.266      | 0.205–25.008| 0.504               |            |            |                      |
| Poorly differentiated            |            |            |                      |            |            |                      |
| Tumor location—no. (%)           |            |            |                      |            |            |                      |
| Right-sided                      | 0.649      | 0.249–1.690| 0.376                |            |            |                      |
| Left-sided                       |            |            |                      |            |            |                      |
| Stage—no. (%)                    |            |            |                      |            |            |                      |
| II                               | 0.047      | 0.000–173.959| 0.466              |            |            |                      |
Table 3. Cont.

| Characteristics | Univariate Analysis | Multivariate Analysis |
|-----------------|---------------------|----------------------|
|                 | Hazard Ratio | 95% CI | p-Value | Hazard Ratio | 95% CI | p-Value |
| III RAS status  |           |       |         |           |       |         |
| Mutant          | 2.338      | 1.154–4.737 | 0.018 * | 2.307      | 1.136–4.682 | 0.021 * |
| Wild type       | 0.044      | 0.000–15.846 | 0.299    |           |       |         |
| BRAF status     |           |       |         |           |       |         |
| Mutant          | 0.044      | 0.000–15.846 | 0.299    |           |       |         |
| Wild type       |           |       |         |           |       |         |
| MMR status      |           |       |         |           |       |         |
| dMMR/MSI-high   | 0.484      | 0.066–3.544 | 0.475    |           |       |         |
| pMMR/MSI-stable or -low PD-L1 expression | | | | |
| CPS ≥ 5         | 0.330      | 0.116–0.942 | 0.038 * | 0.327      | 0.115–0.934 | 0.037 * |
| CPS < 5         |           |       |         |           |       |         |

Abbreviation: CPS, combined positive score; dMMR, deficient mismatch repair; pMMR, proficient mismatch repair; MMR, mismatch repair; MSI, microsatellite instability. *p < 0.05.

2.4. Immune Pathways Associated with High PD-L1 Expression

To explore the potential pathways associated with PD-L1 expression, mRNA expression data were downloaded from the TCGA PanCancer Atlas database. After data cleaning, a total of 184 CRCs, including 99 stage II and 85 stage III CRCs, were enrolled for analysis. Detailed clinical characteristics provided by the TCGA database are listed in Table S1. When hallmark gene sets were applied for the GSEA analysis, 15 gene signatures were statistically enriched in the high CD274 expression group (Table S2). Among these gene signatures, interferon (IFN)-gamma response, IL-2-STAT5 signaling, IL-6-STAT3 signaling, inflammatory response, TNFA signaling via NFKB, and IFN-alpha response are immune-related pathways (Figure 3). Moreover, CD274 expression was positively correlated with CD3G (r = 0.720; *p < 0.001) and CD68 (r = 0.555; *p < 0.001) expression, respectively, using the TCGA database (Table S3). These findings suggest that PD-L1-associated immune responses may be involved in the clinical outcomes of patients with early CRC.

2.5. Immune-Related Genes Differentially Expressed between PD-L1 High and Low CRCs

To further understand the tumor immune microenvironment contributing to the difference in outcome between CRC patients with high and low PD-L1 expression, the oncomine immune response research assay, a targeted NGS assay analyzing the expression of 395 genes associated with the immune response, was applied on the primary CRC tumor samples. The differential gene expressions were compared between patients with high (CPS ≥ 5) and low PD-L1 expression (CPS < 5). In this CRC cohort, the gene expression levels were available in 70 patients, including 18 patients with high PD-L1 expression and 52 patients with low PD-L1 expression. As shown in Figure 4A,B, a total of 12 genes were found to be differentially expressed between the two groups (adjusted *p < 0.1). The expression levels of CXCL9, PRF1, PDCD1, SIT1, BST2, and ISG15 were higher in CRCs with high PD-L1 expression compared to those with low PD-L1 expression. In contrast, CRCs with low PD-L1 expression had higher expression levels of CDKN3, CD44, ABCF1, MAPK1, RPS6, and LAMP1 than those with high PD-L1 expression. The prognostic value of these genes reported in the literature and relevant references are listed in Figure 4B. Among these genes, CXCL9 was the top gene differentially expressed between these two groups of CRCs and can encode the chemokine, CXCL9, known to be regulated by IFN-gamma [17]. When the gene expression level of PD-L1 was analyzed, we also observed a positive correlation between CXCL9 expression and CD274 expression (Figure 4C) in our CRC cohort, which was supported by the data of colon cancer (Figure 4D) and rectal cancer cohort from TCGA (Figure 4E).
CXCL9 plays an important role in regulating immune cell migration, differentiation, and activation of the TME [17]. Since CXCL9 was differentially expressed between CRCs with high and low PD-L1 expression, we further analyzed the impact of CXCL9 on immune cell infiltrations. CRCs from the TCGA database were categorized into the group of high and low CXCL9 expression according to the median expression value of CXCL9, and immune cell infiltrations were compared between these two groups by using the TIMER 2.0. As shown in Figure 5, B-cell infiltration was lower in CRCs with high CXCL9 expression than in those with low CXCL9 expression. CRCs with high CXCL9 expression had more CD8+ T cells and less regulatory T-cell infiltration than those with low CXCL9 expression. The infiltrations of M1 macrophages, M2 macrophages, and cancer-associated fibroblasts were also higher in CRCs with high CXCL9 expression than in those with low CXCL9 expression. No significant difference in CD4+ T-cell and neutrophil infiltration was found between these two groups.

2.6. Correlation between CXCL9 Expression and Immune Cell Infiltrations

CXCL9 plays an important role in regulating immune cell migration, differentiation, and activation of the TME [17]. Since CXCL9 was differentially expressed between CRCs with high and low PD-L1 expression, we further analyzed the impact of CXCL9 on immune cell infiltrations. CRCs from the TCGA database were categorized into the group of high and low CXCL9 expression according to the median expression value of CXCL9, and immune cell infiltrations were compared between these two groups by using the TIMER 2.0. As shown in Figure 5, B-cell infiltration was lower in CRCs with high CXCL9 expression than in those with low CXCL9 expression. CRCs with high CXCL9 expression had more CD8+ T cells and less regulatory T-cell infiltration than those with low CXCL9 expression. The infiltrations of M1 macrophages, M2 macrophages, and cancer-associated fibroblasts were also higher in CRCs with high CXCL9 expression than in those with low CXCL9 expression. No significant difference in CD4+ T-cell and neutrophil infiltration was found between these two groups.

Figure 3. Gene set enrichment analysis in CRCs with high and low CD274 expression. Hallmark gene sets were applied for gene set enrichment analysis in two phenotypes: CRCs with high and low CD274 expression according to the median expression level of CD274. The colored band at the bottom represents the degree of correlation of the expression of these genes (red for a positive correlation and blue for a negative correlation) in each gene signature. The immune-related gene sets enriched in CRCs with high CD274 expression are shown, including (A) the IFN-gamma response, (B) IL-2-STAT5-signaling, (C) IL-6-JAK-STAT-signaling, (D) the inflammatory response, (E) TNFA-signaling-via NFKB, and (F) the IFN-alpha response. IFN, interferon; TNFA, tumor necrosis factor-alpha; NFKB, nuclear factor kappa-B.
genes with significantly higher and lower expression levels in CRCs with high PD-L1 expression. (A) The MA plot shows the log2 fold of change against the average expression levels of 395 immune-related genes between CRCs with high (CPS ≥ 5) and low PD-L1 expression (CPS < 5). The y-axis represents the log2 fold of change for each gene and the x-axis represents the mean value of normalized reads. Points in red and blue indicate genes with significantly higher and lower expression levels in CRCs with high PD-L1 expression (adjusted p < 0.1). (B) The list of differentially expressed genes with statistical significance (p < 0.1) between the groups of high and low PD-L1 expressions, and the prognostic value of those genes in CRC [7,18–32]. Genes in red and blue indicate genes with significantly higher and lower expression levels in CRCs with high PD-L1 expression (adjusted p < 0.1). The correlation between CD274 and CXCL9 was analyzed using the Spearman correlation coefficient in CRCs from NCKUH (C), and the colon (D) and rectal cancer (E) cohort from the TCGA PanCancer Atlas. r, Spearman’s rank correlation coefficient.

Figure 5. Immune cell infiltrations in CRCs with high and low CXCL9 expression. Immune cells infiltrations, including naïve B cells (A), plasma cells (B), CD8+ T cells (C), CD4+ memory T cells (D),
T follicular helper cells (E), regulatory T cells (F), neutrophils (G), M1 macrophages (H), M2 macrophages (I), and cancer associated fibroblasts (J), were estimated using TIMER 2.0 and the \( t \)-test was used to determine the difference between CRCs with high and low \( \text{CXCL9} \) expression. The central line, bottom, and top of the box indicate the median, 25th percentile, and 75th percentiles of the data. ** \( p < 0.001 \); * \( p < 0.05 \); NS, not significant.

3. Discussion

In this study, we analyzed the prognostic impact of PD-L1 expression in patients with stage III and high-risk stage II CRC, and the potential interactions in the tumor immune microenvironment. Our data showed that CPS of \( \geq 5 \) was associated with better RFS in early-stage CRC patients undergoing the standard surgery and receiving the adjuvant FOLFOX chemotherapy. CRCs with high PD-L1 expression had a higher expression level of \( \text{CXCL9} \). The data from the TCGA PanCancer Atlas showed that IFN-gamma signaling was one of the immune-related gene signatures enriched in CRCs with high PD-L1 expression, and high \( \text{CXCL9} \) expression was associated with more CD8+ T cells and M1 macrophages but less regulatory T-cell infiltration.

The role of PD-L1 expression as a prognostic marker for survival in early-stage CRC is controversial. This controversy may result from several factors, including the heterogeneity of the study populations, various IHC staining methods, different cutoff values for defining PD-L1 positivity, and treatment modalities. Although the prognostic value of PD-L1 expression remains inconclusive, several studies have reported that PD-L1 expression is an important prognostic factor of CRC. Recent studies have demonstrated that higher PD-L1 expression on immune cells in the TME is correlated with better survival outcomes in early-stage CRC [6,8,9,33]. On the contrary, some clinical studies have reported that prominent PD-L1 expression on tumor cells indicates poorer survival outcomes of CRC. [7,8,11,12]. Notably, a few reports have shown that PD-L1 expression is a prognostic factor for CRC in a site-dependent manner [8,13]. PD-L1 expression on tumor or immune cells can have distinct survival outcomes for patients with CRC. Because of these divergent findings, the clinical utility of PD-L1 expression as a prognostic marker remains uncertain in CRC.

In the past decades, scientists have been eager to find practical prognostic factors for early-stage CRC patients. Several efforts have been made to determine which patients should receive adjuvant chemotherapy or which should be closely monitored. Although many classification methods have been introduced, such as CMS, MSI, and the immune score, none of them have been applied in real-world clinical practice. The circulating tumor DNA (ctDNA)-guided approach is a promising method that has been proven to prevent unnecessary adjuvant chemotherapy [34]. However, the financial burden of applying ctDNA to daily practice would still be a potential hurdle to overcome in the future. On the contrary, our findings showed that CPS, a long-established biomarker with standardized methodology, has good prognostic value in early-stage CRCs. In the present study, early-stage CRCs with high expression of PD-L1 (especially CPS \( \geq 5 \)) were significantly associated with better RFS. CPS itself was also an independent prognostic factor of RFS using univariate and multivariate analyses. In current clinical practice, the CPS score, which is defined as the number of PD-L1-positive cells, including tumor and immune cells, divided by the total number of tumor cells \( \times 100 \), has been an approved biomarker for predicting the efficacy of anti-PD-1/PDL-1 antibodies (the so-called immune-checkpoint inhibitor (ICI)) in various types of cancer [35,36]. This method of evaluating PD-L1 expression in the TME is a standardized and easily-accessible approach for clinical physicians. Collectively, our findings suggest the clinical utility of PD-L1 expression as a prognostic marker for patients with early CRC.

For further investigation of possible associated mechanisms of the regulation of PD-L1 expression, we used the GSEA to analyze different enriched pathways between early-stage CRCs with high and low \( \text{CD274} \) expression from the TCGA database. Fifteen gene signatures were enriched in the higher \( \text{CD274} \) expression group, and several enriched pathways are cancer-associated immune responses. The role of these immune-related
pathways in the carcinogenesis in CRC have been extensively studied. Generally, IL-2 [37,38], IFN-gamma [39], IFN-alpha [40], and TNF-alpha signaling [41] can elicit a pro-inflammatory response and demonstrate potent anti-tumor effects in CRC through a direct and indirect mechanism. In parallel with the anti-tumor immunities, clinical studies have showed the positive correlation between enrichment of these immune-related pathways and better clinical outcomes of CRC [42–44]. These results can support our findings that high-CPS is a prognostic factor for longer survivals of patients with CRC. In contrast, IL-6 is a cytokine well known to be involved in the development and progression of many cancer types, including CRC [45]. In addition to the direct promoting effects on tumor cells, IL-6 could induce CRC progression by modulating the tumor immune microenvironment [37,38]. Prior studies have showed that high IL-6 levels are associated with an advanced stage, high risk of relapse, and worse survival outcomes of CRC [46–48]. Several studies have explored the PD-L1 expression mediated by IL-6 in certain cancer types [49,50]. Understanding the interaction between PD-L1 and IL-6 in the tumor immune microenvironment would provide novel insights of therapeutic strategies against CRC.

These results were consistent with findings from immune gene profiling from our National Cheng Kung University Hospital (NCKUH) cohort, in which CXCL9, a chemokine regulating immune cell migration, differentiation, multiplication, and activation, was also upregulated in the high CPS expression group. According to our GSEA analysis results, CXCL9 took part in three of the immune response-enriched gene signatures, including IFN-gamma, IL-6, and inflammatory response pathways. CXCL9 secretion can be induced by IFN-gamma stimulation, and this IFN-gamma-CXCL9 pathway has an essential role in regulating tumor growth [17,51]. In general, IFN-gamma-JAK-STAT1 signaling is the most well-known pathway that regulates PD-L1 expression in the TME. However, several studies have shown that PD-L1 expression can be upregulated on various types of tumor cells by the activation of the STAT3 and PI3K-Akt pathways when CXCL9 is stimulated by IFN-gamma signaling [52,53]. While CXCL9 upregulates and recruits immune cells in the TME to inhibit tumor growth, it also induces PD-L1 expression to help tumors escape immune cell surveillance. Tokunaga et al. proposed the idea that CXCL9 has two types of signaling methods in regulating the TME. One is through paracrine signaling from monocytes, endothelial cells, and fibroblasts, and the other is through autocrine signaling from cancer cells [17]. These two signaling interactions have totally different impacts on tumor growth: CXCL9 with paracrine signaling mainly has tumor-suppression effects, and CXCL9 with autocrine signaling causes cancer cell proliferation and metastasis. These complicated and contradictory roles of CXCL9 on tumor growth may be related to the intricate spatial and temporal interaction of immune responses in the TME, not simply the location where CXCL9 is secreted.

According to our results, $CD274$ expression is positively correlated with $CXCL9$ expression. Accumulating studies have demonstrated that high expression of $CXCL9$ is associated with better survival outcomes of CRC [54,55]. These results can support the rationale of PD-L1 expression as a potential prognostic factor of CRC. Additionally, CXCL9 is a crucial cytokine regulating immune cell migration, differentiation, multiplication, and activation, and the lack of CXCL9 can cause the failure of effector T-cell trafficking to TME [56,57]. CD103+ dendritic cells (DCs) are the primary source of $CXCL9$ secretion in the TME. After stimulation by IFN-gamma, DC-derived $CXCL9$ increases and reactivates CD8+ T cells. Subsequently, more IFN-gamma can be produced by CD8+ T cells and a positive feedback loop intensifying antitumor immune response can be formed [58]. In addition to T-cell migration and activation, DCs are associated with the efficacy of anti-PD1 therapies through intratumoral CD8+ T-cell proliferation [18]. Therefore, the TME of early-stage CRC is notably different between the high and low $CXCL9$ expression groups. The high $CXCL9$ group has increased infiltration levels of CD8+ T cells and macrophages. Because CD8+ T-cell infiltration in TME is a well-established prognostic factor across various types of cancer, it may partly explain why patients with early-stage CRC showing high CPS expression have better survival benefits compared to their low CPS expression counterparts [59–63].
In addition to prognostic significance, this evidence may suggest a potential therapeutic approach to CRC through the combination of ICIs and CXCL9-based therapy. The efficacy of anti-PD-1/PD-L1 antibodies has been well demonstrated in various types of cancer, including MSI-H CRC [64,65]. However, dMMR/MSI-H CRC comprises approximately 5% of metastatic CRC, and the efficacy of ICIs has been unsatisfactory in the majority of CRC which is mismatch repair proficient (pMMR) or microsatellite stable (MSS) [66,67]. Because MSS metastatic CRC is usually classified as a typical “cold” cancer that presents a lack of the activation of immune responses, the studies investigating how to generate a “hot” TME are critical to the treatment improvement of MSS CRC [68,69]. The production of CXCL9 in the TME can induce T-cell infiltration and may contribute to the orchestration of the “hot” TME of MSS CRC. Several preclinical studies have shown that the efficacy of immunotherapeutic strategies can be enhanced through the manipulation of CXCL9-involving immune interactions [70,71]. In clinical studies, therapeutic interventions targeting toll-like receptors (TLRs), which are involved in the innate immune system, can induce CXCL9 production in the immune microenvironment [55,72]. Some early-phase clinical trials using a combination strategy with TLR agonists and pembrolizumab (anti-PD-1 antibody) in various types of cancer are ongoing, including pMMR CRC (NCT02834052). The results of these clinical trials are notable because of their potential for advancing clinical applications of immunotherapy in CRC.

This present study combined a Taiwanese cohort and the TCGA database for the analysis. The population of the NCKUH cohort is homogenous, which is purely high-risk stage II and stage III patients who underwent curative surgery and received adjuvant FOLFOX chemotherapy in a single medical center. The survival analysis would be more reliable than pure online database research. However, our study had some limitations. First, this is a retrospective study, which means there might have been selection bias. Second, we only used one IHC staining method (Dako 22C3) to evaluate PD-L1 expression. Potential discordance between different antibodies may affect subsequent analytic findings. Moreover, different cutoff values are applied to determine PD-L1 positivity in different studies, which would also lead to inconsistent findings. Third, the analysis of possible pathways and the TME behind high PD-L1 or CXCL9 expression is mainly based on in silico studies. These studies identified a few immunosuppressive cell types correlated with CXCL9 expression in the TME, such as M2 macrophages and cancer-associated fibroblasts. Although accumulating evidence has demonstrated that CXCL9 expression in response to IFN-gamma signaling can exert antitumor activity through increased infiltration of effector T lymphocytes, the subsequent CXCR3 activation may be involved in macrophage or stroma cell polarization of the TME [73,74]. The biological processes in regulation of tissue inflammation and wound healing may contribute to versatile functions of these immune molecules. Cancer cells may take advantage of biological processes maintaining physical function to facilitate tumor growth. For advanced understanding the CXCL9-PD-L1 immune interactions in TME of CRC, additional biological experiments and mechanistic studies are warranted.

In summary, our data revealed that PD-L1 expression is a practical prognostic marker for RFS in patients with high-risk early-stage CRC. In the TME of CRC, high PD-L1 expression is associated with crucial cancer-associated immune responses, including IFN-gamma response, IL-2-STAT5 signaling, and the IL-6-STAT3 signaling pathway. Moreover, CXCL9 is an important mediator involved in immune interactions upregulating PD-L1 expression and the activation of various types of immune cells, such as CD8+ T cells and M1 macrophages. These findings may provide novel insights into prognostic evaluations and therapeutic strategies for CRC.

4. Materials and Methods
4.1. The Study Population and Data Collection

Patients with CRC enrolled in two clinical studies investigating chemotherapy-induced peripheral neuropathy at NCKUH were used for the analysis. The information of these
two studies was described in detail in the previous study [75]. All patients in these two studies were at stage III or high-risk stage II and underwent standard surgery followed by adjuvant chemotherapy with mFOLFOX6. After the treatment, patients were regularly followed up with computed tomography (CT) scanning to detect recurrence according to the routine daily practice of NCKUH. Clinical information, including the age, sex, primary tumor location, tumor histology, tumor grade, status of the RAS mutation, BRAF mutation, mismatch repair/microsatellite instability (MMR/MSI), recurrence or not, and survival, were obtained from the medical records. The primary tumor samples of CRC were used for immunohistochemical (IHC) staining and immune-related gene expression analyses. Informed consent was provided by each patient before they were included in these studies and the studies were conducted per the principles of the Declaration of Helsinki. The protocols were approved by the Institutional Review Board of NCKUH (A-ER-103-395 and A-ER-104-0153).

4.2. Immunohistochemical Staining of PD-L1 and Immunofluorescent Double Staining

The formalin-fixed and paraffin-embedded (FFPE) primary tumor samples of CRC were used for IHC staining. The monoclonal mouse anti-human PD-L1 antibody (Clone 22C3, Dako, 1:50) was used as the primary antibody, and the procedures were performed with the Bond-Max Automated IHC Stainer (Leica Biosystems Newcastle Ltd., Victoria, Australia) per the following protocol. Four-micrometer sections were cut from the paraffin blocks followed by deparaffinized with xylene and pre-treated with the Epitope Retrieval Solution 2 (EDTA buffer, pH 9.0) at 100 °C for 40 min. Subsequently, the sections were incubated with the primary antibody at room temperature for 90 min. After the staining with the primary antibody, sections were incubated with the polymer at room temperature for 8 min using the Bond Polymer Refine Detection Kit (Leica Biosystems, Newcastle Ltd., Newcastle Upon Tyné, UK) and then developed with 3,3′-diaminobenzidine chromogens for 10 min. Counterstaining was carried out with hematoxylin. For immunofluorescent double staining, Rat anti-CD68 antibody (Novus, Centennial, CO, USA, NBP2-33337) and mouse anti-PD-L1 antibody (Invitrogen, Waltham, MA, USA, #14-5983-82) were used to detect PD-L1 expression on macrophages. Alexa Fluor 594-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA, #712-585-150) and Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA, #715-545-150) was used for the staining of CD68 and PD-L1, respectively. To determine PD-L1 expression on T lymphocytes, mouse anti-CD3 antibody (Invitrogen, Waltham, MA, USA, #14-0037-82) and rabbit anti-PD-L1 (GeneTex, Irvine, CA, USA, GTX104763) were applied. Alexa Fluor 594-conjugated secondary antibody (Invitrogen, Waltham, MA, USA, A21207) and Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA, #715-545-150) was used for the staining of CD3 and PD-L1, respectively. Tissue sections were co-stained with 4′,6′-diamidino-2-phenylindole (DAPI) to detect nucleus. The stained slides were excited by laser at 405, 488, and 594 nm, respectively, and immunofluorescent images were captured by the confocal microscope (FV-3000, Olympus, Japan).

4.3. Scoring of PD-L1 Expression

The level of PD-L1 expression was assessed by an experienced pathologist who was well-trained in the scoring of PD-L1 expression. According to the PD-L1 IHC 22C3 pharmDx package insert [76], the TPS and CPS were calculated to determine the level of PD-L1 expression. In brief, the TPS was defined as the number of viable tumor cells showing partial or complete membrane staining of PD-L1 divided by the total number of viable tumor cells and then multiplied by 100%. The CPS was defined as the number of PD-L1-positive cells (tumor cells, macrophages, and lymphocytes) divided by the total number of viable tumor cells and then multiplied by 100 [77,78]. At least 100 viable tumor cells are required to evaluate the PD-L1 expression in each testing.
4.4. Analysis of Immune-Related Gene Expression

The oncomine immune response research assay was performed on the primary CRC tumor samples to evaluate the immune-related gene expression as previously described [75]. Briefly, the RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract the RNA from FFPE primary CRC tumor tissues followed by reverse transcription with 20 ng RNA using the SuperScript IV VILO Master Mix kit. After cDNA synthesis, the Ion AmpliSeq Kit™ for Chef DL8 (Thermo Fisher Scientific, Waltham, MA, USA) was used for the preparation of libraries. Template preparation, chip loading, and sequencing were carried out on the Ion Chef™ System and the Ion S5 XL sequencing system per the manufacturer’s instructions. Torrent Suite (Thermo Fisher Scientific, Waltham, MA, USA) was used to process the raw gene expression data and the DESeq2 package in R was used for the analysis. After data normalization by the DESeq function, the expression levels of genes were compared between the high and low PD-L1 expression groups to identify the differentially expressed genes.

4.5. Gene Set Enrichment Analyses

RNA-Seq data of CRC from the TCGA PanCancer Atlas database were downloaded from cBioportal (https://www.cbioportal.org/ (accessed on 11 May 2022)). The data of stage II and III CRCs were extracted to explore the potential pathways related to CD274 expression. The median expression value of CD274 was used to divide CRCs into two groups: the high and low CD274 expression groups. GSEA with the HALLMARK gene set analysis (50 gene sets) was performed using the GSEA software (GSEA v4.23 for windows). The gene sets with nominal p-value of <0.05 and a false discovery rate (FDR) q-value of <0.25 were considered the significant enrichment gene sets.

4.6. Analysis of Immune Cell Infiltration

Stage II and III CRCs from the TCGA PanCancer Atlas database were divided into the high and low CXCL9 expression groups per the median CXCL9 expression levels. RNA expression data were used for the analysis of immune cell infiltration by using the Tumor Immune Estimation Resource 2.0 (TIMER2.0) web server (http://timer.cistrome.org/ (accessed on 20 August 2022)). The immune composition, including the naïve B cells, plasma B cells, CD8+ T cells, CD4+ T cells, follicular helper T cells, regulatory T cells, neutrophils, M1 macrophages, and M2 macrophages, was estimated by the CIBERSORT cellular composition estimation algorithm. The EPIC algorithm was used for characterizing cancer-associated fibroblasts. The infiltration levels of immune cells and cancer-associated fibroblasts were compared between CRCs with high and low CXCL9 expression.

4.7. Statistical Analysis

Statistical analyses were conducted using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA). Categorical data were analyzed using the Chi-squared test or Fischer’s exact test. Spearman’s correlation coefficient was used to determine the correlation of expression levels between different genes. Recurrence-free survival (RFS) was defined as the time from curative surgery to the time of recurrence detected by CT scanning, and OS was defined as the time from curative surgery to death. RFS and OS were illustrated by Kaplan–Meier curves and the log-rank test was used to determine the differences between groups. Kaplan–Meier survival plots were generated by using GraphPad Prism 9. The Cox proportional hazards regression model was performed for univariate and multivariate survival analyses. A p-value of <0.05 was considered statistically significant. When comparing the expression of immune-related genes between groups, an adjusted p-value of <0.1 was considered statistically significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232113277/s1.
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Institutional Review Board Statement: The study was conducted per the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of National Cheng Kung University Hospital (A-ER-103-395 and A-ER-104-0153).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Publicly available datasets were analyzed in this study. These data can be found on this website: https://www.cbioportal.org/study/summary?id=coaread_tcgapancan_atlas_2018 (accessed on 14 September 2022). Our own data presented in this study are available from the corresponding author upon reasonable request.

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References

1. Health Promotion Administration, Ministry of Health and Welfare Taiwan Cancer Registration Report. 2019. Available online: https://www.hpa.gov.tw/Pages/Detail.aspx?nodeid=269&pid=14913 (accessed on 1 September 2022).
2. American Cancer Society. Cancer Facts & Figures 2022; American Cancer Society: Atlanta, GA, USA, 2022.
3. O’Connell, M.J.; Mailliard, J.A.; Kahn, M.J.; Macdonald, J.S.; Haller, D.G.; Mayer, R.J.; Wieand, H.S. Controlled trial of fluorouracil and low-dose leucovorin given for 6 months as postoperative adjuvant therapy for colon cancer. J. Clin. Oncol. 1997, 15, 246–250. [CrossRef] [PubMed]
4. André, T.; Boni, C.; Mounedji-Boudiaf, L.; Navarro, M.; Tabernero, J.; Hickish, T.; Topham, C.; Zaninelli, M.; Clingan, P.; Bridgewater, J.; et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N. Engl. J. Med. 2004, 350, 2343–2351. [CrossRef] [PubMed]
5. Pardoll, D.M. The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer 2012, 12, 252–264. [CrossRef] [PubMed]
6. Azcue, P.; Encio, I.; Guerrero Setas, D.; Suarez Alecha, J.; Galbete, A.; Mercado, M.; Vera, R.; Gomez-Dorronsoro, M.L. PD-L1 as a prognostic factor in early-stage colon carcinoma within the immunohistochemical molecular subtype classification. Cancers 2021, 13, 1943. [CrossRef]
7. Enkhbat, T.; Nishi, M.; Takasu, C.; Yoshikawa, K.; Jun, H.; Tokunaga, T.; Kashiwara, H.; Ishikawa, D.; Shimada, M. Pro-grammed cell death ligand 1 expression is an independent prognostic factor in colorectal cancer. Anticancer Res. 2018, 38, 3367–3373. [CrossRef]
8. Lee, K.S.; Kim, B.H.; Oh, H.K.; Kim, D.W.; Kang, S.B.; Kim, H.; Shin, E. Programmed cell death ligand-1 protein expression and CD274/PD-L1 gene amplification in colorectal cancer: Implications for prognosis. Cancer Sci. 2018, 109, 2957–2969. [CrossRef]
9. Kuo, Y.T.; Liao, C.K.; Chen, T.C.; Lai, C.C.; Chiang, S.F.; Chiang, J.M. A high density of PD-L1-expressing immune cells is significantly correlated with favorable disease free survival in nonmetastatic colorectal cancer. Medicine 2022, 101, e28573. [CrossRef]
10. Huemer, F.; Kliers, E.; Neureiter, D.; Schlintl, V.; Rinnerthaler, G.; Pagès, F.; Kirilovsky, A.; El Sissy, C.; Iglseder, W.; Singhartinger, F.; et al. Impact of PD-L1 scores and changes on clinical outcome in rectal cancer patients undergoing neoadjuvant chemoradiotherapy. J. Clin. Med. 2020, 9, 2775. [CrossRef]
11. Shao, L.; Peng, Q.; Du, K.; He, J.; Dong, Y.; Liu, X.; Li, J.; Wu, J. Tumor cell PD-L1 predicts poor local control for rectal cancer patients following neoadjuvant radiotherapy. Cancer Manag. Res. 2017, 9, 249–258. [CrossRef]
12. Shan, T.; Chen, S.; Wu, T.; Yang, Y.; Li, S.; Chen, X. PD-L1 expression in colon cancer and its relationship with clinical prognosis. Int. J. Clin. Exp. Pathol. 2019, 12, 1764–1769.
13. Koganemaru, S.; Inoshita, N.; Miura, Y.; Miyama, Y.; Fukui, Y.; Ozaki, Y.; Tomizawa, K.; Hanaoka, Y.; Toda, S.; Suyama, K.; et al. Prognostic value of programmed death-ligand 1 expression in patients with stage III colorectal cancer. *Cancer Sci.* 2017, 108, 853–858. [CrossRef] [PubMed]

14. Calik, I.; Calik, M.; Turken, G.; Ozercan, I.H.; Dagli, A.F.; Artas, G.; Sarikaya, B. Intrasurgical cytotoxic T-lymphocyte density and PD-L1 expression are predictive biomarkers for patients with colorectal cancer. *Medicina* 2019, 55, 223. [CrossRef] [PubMed]

15. Kowanetz, M.; Zhou, W.; Gettinger, S.N.; Koeppen, H.; Kockx, M.; Schmid, P.; Kadel, E.E., 3rd; Wistuba, I.; Chaft, J.; Rizvi, N.A.; et al. Differential regulation of PD-L1 expression by immune and tumor cells in NSCLC and the response to treatment with atezolizumab (anti-PD-L1). *Proc. Natl. Acad. Sci. USA* 2018, 115, E10119–E10126. [CrossRef] [PubMed]

16. Yi, M.; Niu, M.; Xu, L.; Luo, S.; Wu, K. Regulation of PD-L1 expression in the tumor microenvironment. *J. Hematol. Oncol.* 2021, 14, 10. [CrossRef] [PubMed]

17. Tokunaga, R.; Zhang, W.; Naseem, M.; Puccini, A.; Berger, M.D.; Soni, S.; McSkane, M.; Baba, H.; Lenz, H.J. CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation—A target for novel cancer therapy. *Cancer Treat. Rev.* 2018, 63, 40–47. [CrossRef] [PubMed]

18. Wu, Z.; Huang, X.; Han, X.; Li, Z.; Zhu, Q.; Yan, J.; Yu, S.; Jin, Z.; Wang, Z.; Zheng, Q.; et al. The chemokine CXC9 expression is associated with better prognosis for colorectal carcinoma patients. *Biomed. Pharmacother.* 2016, 78, 8–13. [CrossRef] [PubMed]

19. Agostini, M.; Janssen, K.P.; Kim, J.; D’Angelo, B.; Pizzini, S.; Zanagando, A.; Zanon, C.; Pastrello, C.; Maretto, I.; Digito, M.; et al. An integrative approach for the identification of prognostic and predictive biomarkers in rectal cancer. *Oncotarget* 2015, 6, 32561–32574. [CrossRef] [PubMed]

20. Li, X.; Zhong, Q.; Luo, D.; Du, Q.; Liu, W. The prognostic value of CXC subfamily ligands in stage I-III patients with colorectal cancer. *PLoS One* 2019, 14, e0214611. [CrossRef] [PubMed]

21. Li, W.H.; Zhang, L.; Wu, Y.H. CDKN3 regulates cisplatin resistance to colorectal cancer through TIE1. *Eur. Rev. Med. Pharmacol. Sci.* 2020, 24, 3614–3623. [PubMed]

22. Wang, Z.; Tang, Y.; Xie, L.; Huang, A.; Xue, C.; Gu, Z.; Wang, K.; Zong, S. The Prognostic and Clinical Value of CD44 in Colorectal Cancer: A Meta-Analysis. *Front. Oncol.* 2019, 9, 309. [CrossRef] [PubMed]

23. Wang, J.L.; Su, W.Y.; Lin, Y.W.; Xiong, H.; Chen, Y.X.; Xu, J.; Fang, Y.J. CD44v6 overexpression related to metastasis and poor prognosis of colorectal cancer: A meta-analysis. *Oncotarget* 2017, 8, 12866–12876. [CrossRef] [PubMed]

24. Xia, P.; Xu, X.Y. Prognostic significance of CD44 in human colon cancer and gastric cancer: Evidence from bioinformatic analyses. *Oncotarget* 2016, 7, 45538–45546. [CrossRef] [PubMed]

25. Hlavata, I.; Mohelnikova-Duchonova, B.; Vaclavikova, R.; Liska, V.; Pitule, P.; Novak, P.; Bruha, J.; Vcytal, O.; Holubec, L.; Treska, V.; et al. The role of ABC transporters in progression and clinical outcome of colorectal cancer. *Mutagenesis* 2012, 27, 187–196. [CrossRef] [PubMed]

26. Al-Badran, S.S.; Grant, L.; Campo, M.V.; Intaghagard, J.; Pennel, K.; Quinn, J.; Konanahalli, P.; Hayman, L.; Horgan, P.G.; McMllan, D.C.; et al. Relationship between immune checkpoint proteins, tumour microenvironment characteristics, and prognosis in primary operable colorectal cancer. *J. Pathol. Clin. Res.* 2021, 7, 121–134. [CrossRef] [PubMed]

27. Kuai, W.; Xu, X.; Yan, J.; Zhao, W.; Li, Y.; Wang, B.; Yuan, N.; Li, Z.; Jia, Y. Prognostic Impact of PD-1 and Tim-3 Expression in Tumor Tissue in Stage I-III Colorectal Cancer. *BioMed Res. Int.* 2020, 2020, 5294043. [CrossRef] [PubMed]

28. Zhang, Y.; Kang, S.; Shen, J.; He, J.; Jiang, L.; Wang, W.; Guo, Z.; Peng, G.; Chen, G.; He, J.; et al. Prognostic significance of programmed cell death 1 (PD-1) or PD-1 ligand 1 (PD-L1) Expression in epithelial-originated cancer: A meta-analysis. *Medicine* 2015, 94, e515. [CrossRef] [PubMed]

29. Chen, X.; Sun, K.; Jiao, S.; Cai, N.; Zhao, X.; Hou, X.; Xie, Y.; Wang, X.; Zhong, M.; Wei, L. High levels of SIRT1 expression enhance tumorigenesis and associate with a poor prognosis of colorectal carcinoma patients. *Sci. Rep.* 2014, 4, 7481. [CrossRef] [PubMed]

30. Zu, G.; Ji, A.; Zhou, T.; Che, N. Clinicopathological significance of SIRT1 expression in colorectal cancer: A systematic review and meta-analysis. *Int. J. Surg.* 2016, 26, 32–37. [CrossRef] [PubMed]

31. Chang, S.F.; Kan, C.Y.; Hsiao, Y.C.; Tang, R.; Hsieh, L.L.; Chiang, J.M.; Tsai, W.S.; Yeh, C.Y.; Hsieh, P.S.; Liang, Y.; et al. Bone Marrow Stromal Antigen 2 Is a Novel Plasma Biomarker and Prognosticator for Colorectal Carcinoma: A Secretome-Based Verification Study. *Dis. Markers* 2015, 39, 874054. [CrossRef] [PubMed]

32. Mukai, S.; Oue, N.; Oshima, T.; Mukai, R.; Tatsumoto, Y.; Sakamoto, N.; Sentani, K.; Tanabe, K.; Egli, H.; Hinoi, T.; et al. Overexpression of Transmembrane Protein BST2 Is Associated with Poor Survival of Patients with Esophageal, Gastric, or Colorectal Cancer. *Ann. Surg. Oncol.* 2017, 24, 594–602. [CrossRef] [PubMed]

33. Berntsson, J.; Eberhard, J.; Nordin, B.; Leandersson, K.; Larsson, A.; Jirstrom, K. Expression of programmed cell death protein 1 (PD-1) and its ligand PD-L1 in colorectal cancer: Relationship with sidedness and prognosis. *Oncoimmunology* 2018, 7, e1465165. [CrossRef] [PubMed]

34. Tie, J.; Cohen, J.D.; Lahouel, K.; Lo, S.N.; Wang, Y.; Kosmider, S.; Wong, R.; Shapiro, J.; Lee, M.; Harris, S.; et al. Circulating tumor DNA analysis guiding adjuvant therapy in Stage II colon cancer. *N. Engl. J. Med.* 2022, 386, 2261–2272. [CrossRef] [PubMed]

35. Yi, M.; Jiao, D.; Xu, H.; Liu, Q.; Zhao, W.; Han, X.; Wu, K. Biomarkers for predicting efficacy of PD-1/PD-L1 inhibitors. *Mol. Cancer* 2018, 17, 129. [CrossRef] [PubMed]

36. Davis, A.A.; Patel, V.G. The role of PD-L1 expression as a predictive biomarker: An analysis of all US Food and Drug Administration (FDA) approvals of immune checkpoint inhibitors. *J. Immunother. Cancer* 2019, 7, 278. [CrossRef] [PubMed]

37. Briukhovetska, D.; Dörr, J.; Endres, S.; Libby, P.; Dinarello, C.A.; Kobold, S. Interleukins in cancer: From biology to therapy. *Nat. Rev. Cancer* 2021, 21, 481–499. [CrossRef] [PubMed]
38. Li, J.; Huang, L.; Zhao, H.; Yan, Y.; Lu, J. The Role of Interleukins in Colorectal Cancer. *Int. J. Biol. Sci.* 2020, 16, 2323–2339. [CrossRef]

39. Du, W.; Frankel, T.L.; Green, M.; Zou, W. IFNy signaling integrity in colorectal cancer immunity and immunotherapy. *Cell. Mol. Immunol.* 2022, 19, 23–32. [CrossRef]

40. Buoncervello, M.; Romagnoli, G.; Buccarelli, M.; Fragale, A.; Toschi, E.; Parlato, S.; Lucchetti, D.; Macchia, D.; Spada, M.; Canini, I.; et al. IFN-α potentiates the direct and immune-mediated antitumor effects of epigenetic drugs on both metastatic and stem cells of colorectal cancer. *Oncotarget* 2016, 7, 26361–26373. [CrossRef]

41. Lasry, A.; Zinger, A.; Ben-Neriah, Y. Inflammatory networks underlying colorectal cancer. *Nat. Immunol.* 2016, 17, 230–240. [CrossRef]

42. Slattery, M.L.; Lundgreen, A.; Bondurant, K.L.; Wolff, R.K. Interferon-signaling pathway: Associations with colon and rectal cancer risk and subsequent survival. *Carcinogenesis* 2011, 32, 1660–1667. [CrossRef]

43. Dimberg, J.; Shamoun, L.; Landerholm, K.; Andersson, R.E.; Kolodziej, B.; Wågsäter, D. Genetic Variants of the IL2 Gene Related to Risk and Survival in Patients With Colorectal Cancer. *Anticancer Res.* 2019, 39, 4933–4940. [CrossRef] [PubMed]

44. Reissfelder, C.; Stamova, S.; Goßmann, C.; Braun, M.; Bonertz, A.; Walliczek, U.; Grimm, M.; Rahbari, N.N.; Koch, M.; Saadati, M.; et al. Tumor-specific cytotoxic T lymphocyte activity determines colorectal cancer patient prognosis. *J. Clin. Investig.* 2015, 125, 739–751. [CrossRef] [PubMed]

45. Lin, Y.; Ye, Z.; Ye, J.; Liu, Z.; She, X.; Gao, X.; Liang, R. Progress in Understanding the IL-6/STAT3 Pathway in Colorectal Cancer. *OncoTargets Ther.* 2020, 13, 10302–10302. [CrossRef] [PubMed]

46. Olsen, J.; Kirkeby, L.T.; Olsen, J.; Eiholm, S.; Jess, P.; Gøgenfur, I.; Troelsen, J.T. High interleukin-6 mRNA expression is a predictor of relapse in colon cancer. *Anticancer Res.* 2015, 35, 2235–2240. [PubMed]

47. Knüppfer, H.; Preiss, R. Serum interleukin-6 levels in colorectal cancer patients—A summary of published results. *Int. J. Color. Dis.* 2010, 25, 135–140. [CrossRef]

48. Xu, J.; Ye, Y.; Zhang, H.; Szmikowski, M.; Mäkinen, M.J.; Li, P.; Xia, D.; Yang, J.; Wu, Y.; Wu, H. Diagnostic and Prognostic Value of Serum Interleukin-6 in Colorectal Cancer. *Medicine* 2016, 95, e2502. [CrossRef]

49. Tsukamoto, H.; Fujieda, K.; Miyashita, A.; Fukushima, S.; Ikeda, T.; Kubo, Y.; Senju, S.; Ihn, H.; Nishimura, Y.; Oshiumi, H. Combined Blockade of IL6 and PD-1/PD-L1 Signaling Abrogates Mutual Regulation of Their Immunosuppressive Effects in the Tumor Microenvironment. *Cancer Res.* 2018, 78, 5011–5022. [CrossRef]

50. Zhang, W.; Liu, Y.; Yan, Z.; Yang, H.; Sun, W.; Yao, Y.; Chen, Y.; Jiang, R. IL-6 promotes PD-L1 expression in monocytes and macrophages by decreasing protein tyrosine phosphatase receptor type O expression in human hepatocellular carcinoma. *J. Immunother. Cancer* 2020, 8, e000285. [CrossRef]

51. Marcovecchio, P.M.; Thomas, G.; Salek-Ardakani, S. CXCL9-expressing tumor-associated macrophages: New players in the fight against cancer. *J. Immunother. Cancer* 2021, 9, e002045. [CrossRef]

52. Xiou, W.; Luo, J. CXCL9 secreted by tumor-associated dendritic cells up-regulates PD-L1 expression in bladder cancer cells by activating the CXCR3 signaling. *BMC Immunol.* 2021, 22, 3. [CrossRef]

53. Zhang, C.; Li, Z.; Xu, L.; Che, X.; Wen, T.; Fan, Y.; Li, C.; Wang, S.; Cheng, Y.; Wang, X.; et al. CXCL9/10/11, a regulator of PD-L1 expression in gastric cancer. *BMC Cancer* 2016, 16, 3246–3259. [CrossRef] [PubMed]

54. Ding, Q.; Lu, P.; Xia, Y.; Ding, S.; Fan, Y.; Li, X.; Han, P.; Liu, J.; Tian, D.; Liu, M. CXCL9: Evidence and contradictions for its role in tumor progression. *Cancer Med.* 2016, 5, 3246–3259. [CrossRef] [PubMed]

55. Mikucki, M.E.; Fisher, D.T.; Matsuzaki, J.; Skitzki, J.J.; Gaulin, N.B.; Muhitch, J.B.; Ku, A.W.; Frelinger, J.G.; Odunsi, K.; Gajewski, T.F.; et al. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nat. Commun.* 2015, 6, 7458. [CrossRef] [PubMed]

56. Spranger, S.; Dai, D.; Horton, B.; Gajewski, T.F. Tumor-Residing Bat3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell* 2017, 31, 711–723.e4. [CrossRef] [PubMed]

57. Humblin, E.; Kamphorst, A.O. CXCR3-CXCL9: It’s All in the Tumor. *Immunity* 2019, 50, 1347–1349. [CrossRef]

58. Chow, M.T.; Ozga, A.J.; Servis, R.L.; Frederick, D.T.; Lo, J.A.; Fisher, D.E.; Freeman, G.J.; Boland, G.M.; Luster, A.D. Intratumoral Activity of the CXCR3 Chemokine System Is Required for the Efficacy of Anti-PD-1 Therapy. *Immunity* 2019, 50, 1498–1512.e5. [CrossRef]

59. Sharma, P.; Shen, Y.; Wen, S.; Yamada, S.; Jungbluth, A.A.; Gnajatic, S.; Bajorin, D.F.; Reuter, V.E.; Herr, H.; Old, L.J.; et al. CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. *Proc. Natl. Acad. Sci. USA* 2007, 104, 3967–3972. [CrossRef]

60. Liu, S.; Lachapelle, J.; Leung, S.; Gao, D.; Foulkes, W.D.; Nielsen, T.O. CD8+ lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer. *Breast Cancer Res.* 2012, 14, R48. [CrossRef]

61. Glaire, M.A.; Domingo, E.; Sveen, A.; Bruun, J.; Nesbakken, A.; Nicholson, G.; Novelli, M.; Lawson, K.; Oukrif, D.; Kildal, W.; et al. Tumour-infiltrating CD8(+) lymphocytes and colorectal cancer recurrence by tumour and nodal stage. *Br. J. Cancer* 2019, 121, 474–482. [CrossRef]

62. Shimizu, S.; Hiratsuka, H.; Koike, K.; Tsuchihashi, K.; Sonoda, T.; Ogi, K.; Miyakawa, A.; Kobayashi, J.; Kaneko, T.; Igarashi, T.; et al. Tumor-infiltrating CD8(+) T-cell density is an independent prognostic marker for oral squamous cell carcinoma. *Cancer Med.* 2019, 8, 80–93. [CrossRef]
63. Li, F.; Li, C.; Cai, X.; Xie, Z.; Zhou, L.; Cheng, B.; Zhong, R.; Xiong, S.; Li, J.; Chen, Z.; et al. The association between CD8+ tumor-infiltrating lymphocytes and the clinical outcome of cancer immunotherapy: A systematic review and meta-analysis. EClinicalmedicine 2021, 41, 101134. [CrossRef] [PubMed]

64. André, T.; Shiou, K.K.; Kim, T.W.; Jensen, B.V.; Jensen, L.H.; Punt, C.; Smith, D.; Garcia-Carbonero, R.; Benavides, M.; Gibbs, P.; et al. Pembrolizumab in microsatellite-instability-high advanced colorectal cancer. N. Engl. J. Med. 2020, 383, 2207–2218. [CrossRef] [PubMed]

65. Bui, Q.L.; Mas, L.; Hollebecque, A.; Tougeron, D.; de la Fouchardière, C.; Pudlarz, T.; Alouani, E.; Guimbaud, R.; Taieb, J.; André, T.; et al. Treatments after immune checkpoint inhibitors in patients with dMMR/MSI metastatic colorectal cancer. Cancers 2022, 14, 406. [CrossRef] [PubMed]

66. Germani, M.M.; Moreto, R. Immune checkpoint inhibitors in mismatch repair proficient/microsatellite stable metastatic colorectal cancer patients: Insights from the AtezoTRIBE and MAYA trials. Cancers 2021, 1, 52. [CrossRef]

67. Boukouris, A.E.; Theochari, M.; Stefanou, D.; Papalambros, A.; Felekouras, E.; Gogas, H.; Ziogas, D.C. Latest evidence on immune checkpoint inhibitors in metastatic colorectal cancer: A 2022 update. Crit. Rev. Oncol. Hematol. 2022, 173, 103663. [CrossRef]

68. Wang, D.; Zhang, H.; Xiang, T.; Wang, G. Clinical application of adaptive immune therapy in MSS colorectal cancer patients. Front. Immunol. 2021, 12, 762341. [CrossRef]

69. Weng, J.; Li, S.; Zhu, Z.; Liu, Q.; Zhang, R.; Yang, Y.; Li, X. Exploring immunotherapy in colorectal cancer. J. Hematol. Oncol. 2022, 15, 95. [CrossRef]

70. Andersson, A.; Srivastava, M.K.; Harris-White, M.; Huang, M.; Zhu, L.; Elashoff, D.; Strieter, R.M.; Dubinett, S.M.; Sharma, S. Role of CXCR3 ligands in IL-7/IL-7R alpha-Fc-mediated antitumor activity in lung cancer. Clin. Cancer 2011, 17, 3660–3672. [CrossRef]

71. Zhang, R.; Tian, L.; Chen, L.J.; Xiao, F.; Hou, J.M.; Zhao, X.; Li, G.; Yao, B.; Wen, Y.J.; Li, J.; et al. Combination of MIG (CXCL9) chemokine gene therapy with low-dose cisplatin improves therapeutic efficacy against murine carcinoma. Gene Ther. 2006, 13, 1263–1271. [CrossRef]

72. Loos, T.; Dekeyzer, L.; Struyf, S.; Schutyser, E.; Gijsbers, K.; Gouwy, M.; Fraeyman, A.; Put, W.; Ronse, I.; Grillet, B.; et al. TLR ligands and cytokines induce CXCR3 ligands in endothelial cells: Enhanced CXCL9 in autoimmune arthritis. Lab. Investig. 2006, 86, 902–916. [CrossRef]

73. Russo, E.; Santoni, A.; Bernardini, G. Tumor inhibition or tumor promotion? The duplicity of CXCR3 in cancer. J. Leukoc. Biol. 2020, 108, 673–685. [CrossRef] [PubMed]

74. Porta, C.; Rimoldi, M.; Raes, G.; Brys, L.; Ghezzi, P.; Di Liberto, D.; Dieli, F.; Ghisletti, S.; Natoli, G.; De Baetselier, P.; et al. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. Proc. Natl. Acad. Sci. USA 2009, 106, 14978–14983. [CrossRef] [PubMed]

75. Yeh, Y.M.; Lin, P.C.; Su, W.C.; Shen, M.R. CD40 pathway and IL-2 expression mediate the differential outcome of colorectal cancer patients with different CSF1R c.1085 Genotypes. Int. J. Mol. Sci. 2021, 22, 12565. [CrossRef]

76. Agilent Technologies Inc. PD-L1 IHC 22C3 pharmDx (Package Insert). Available online: https://www.accessdata.fda.gov/cdrh_docs/pdf15/P150013c.pdf (accessed on 20 October 2022).

77. Roach, C.; Zhang, N.; Corigliano, E.; Jansson, M.; Toland, G.; Ponto, G.; Dolled-Filhart, M.; Emancipator, K.; Stanforth, D.; Kulangara, K. Development of a companion diagnostic PD-L1 immunohistochemistry assay for pembrolizumab therapy in non-small-cell lung cancer. Appl. Immunohistochem. Mol. Morphol. 2016, 24, 392–397. [CrossRef] [PubMed]

78. Kulangara, K.; Zhang, N.; Corigliano, E.; Guerrero, L.; Waldroup, S.; Jaiswal, D.; Ms, M.J.; Shah, S.; Hanks, D.; Wang, J. Clinical Utility of the Combined Positive Score for Programmed Death Ligand-1 Expression and the Approval of Pembrolizumab for Treatment of Gastric Cancer. Arch. Pathol. Lab. Med. 2019, 143, 330–337. [CrossRef]