Clinical Significance and Therapeutic Potential of the Programmed Death Ligand-1 (PD-L1) and PD-L2 Expression in Human Colorectal Cancer

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Abstract

Purpose: The programmed death-1/programmed death ligand (PD-1/PD-L) pathway in T cell activation has been shown to play an important role in tumor evasion from host immunity. The predictive value of PD-L1 and PD-L2 expression in colorectal cancer (CRC) remains still under discussion. We analyzed whether negative signaling of infiltrating PD-1-positive T cells through PD-L1 and PD-L2 within the tumor could promote further tumor progression through downregulation of anti-tumor immunity.

Methods: We investigated PD-L1 and PD-L2 expression in tumors with CRC and analyzed its prognostic significance with respect to outcome analysis.

Results: T cell infiltration was observed in 90.5% of the tumors, with 58% of the patients demonstrating PD-1-positive T cells in their tumors. Patients who developed PD-1-positive T cell infiltration showed increased PD-L1 expression within their tumors than PD-1-negative individuals. Presence of tumor infiltrating PD-1-positive T cells was more pronounced in advanced stage cancers than in early cancers. Ligand expression (PD-L1/PD-L2) in the tumors combined with dense PD-1-positive T cell infiltration was associated with poor prognosis. Multivariate analysis demonstrated that PD-L1 expression in the tumors was an independent prognostic factor in CRC.

Conclusion: The presented results from primary tumors and CRC patient outcome analysis suggest that negative signaling of infiltrating PD-1-positive T cells through PD-L1 expression within the tumor may promote further tumor progression through downregulation of anti-tumor immunity. Co-expression of PD-1 on CD4/Foxp3-positive T cells was found indicating T regulatory cell-mediated mechanisms by which tumor cells can evade immune recognition and destruction. This study demonstrates the importance of strategies inhibiting negative PD-1/PD-L1 signaling in CRC.

Keywords: PD-1/PD-L signaling pathway; Tumor evasion; Immunotherapy; Tumor-infiltrating T lymphocytes; Colorectal cancer

Introduction

Colorectal cancer is one of the leading causes of cancer-associated mortality worldwide. Despite major advances in diagnosis and treatment of the disease, overall mortality is still high and needs further efforts to reduce cancer related death [1,2]. Currently, Dukes' or UICC classification are the most commonly used predictors of prognosis for CRC patients; indeed 30% to 40% of UICC stage III and IV patients experience relapse and die of the disease [1,2] as they often develop distant metastases in the liver or lung even after curative surgery. Although combinational pre-operative chemotherapy or radio chemotherapy with surgery show a survival benefit long-term outcome, in many patients still remains unfavorable [3-5]. Thus, novel strategies need to be developed and established in order to improve patients' prognosis.

The role of the immune system in the progression of malignancies currently gains further interest. Modulation of the anti-tumor immune response by the tumor cells is a critical mechanism implicated in evasion of the tumor from immune recognition, thus, influencing tumor progression and metastasis. It appears that an immune privileged microenvironment is formed around tumors, that protects malignant cells from immunological destruction [6-9].

Programmed death-1 (PD-1) is a co-inhibitory receptor expressed by antigen-stimulated T cells and functions as a major negative regulator of T cell immunity, to control peripheral tissue tolerance and immune homeostasis [10-12]. Two ligands for PD-1, PD-L1 (B7-H1, also known as CD274) and PD-L2 (B7-DC, also known as CD273) have been identified [13-17]. Previous studies have demonstrated that PD-1/PD-L1 signaling inhibits T cell growth and cytokine release [14]. PD-L1...
expressed on tumor cells increases apoptosis of antigen-specific T cell clones in vitro [18]. Furthermore, PD-L1 blockade with an anti-PD-L1 monoclonal antibody enhanced anti-tumor immunity and inhibited tumor growth in vivo [19]. Therefore, PD-L1 has been suggested to play an important role in immune evasion from the host immune system via PD-1-mediated inhibitory signals that give tumors advantage by selectively inhibiting CD8-positive T cells [13,20,21]. In addition, PD-L1 blockade prevents activation and expansion of CD4-positive T cells with regulatory functions. These studies have been well shown [22] conclusive results using murine tumor models and anti-PD-L1 antibody studies have previously been started to evaluate its clinical relevance in solid tumors [18,20,22,23]. Although PD-L1 expression has been reported in human cancers of the lung, ovary, colon, and in melanomas, its effect on patient's prognosis for CRC and lung cancer is still controversial [18,24]. On the other hand, the function of PD-L2 in tumors remains largely unknown and only a few reports have suggested that PD-L2 may also play a role in tumor immunity [25,26]. It was shown that PD-L2 expression on the tumor cells promotes CD8 T cell-mediated rejection at both the induction and effector phase of antitumor immunity [25]. However, there is little information of PD-L2 expression in tumors and its relevance in the clinical setting. In this study, we investigated the expression of PD-L1 and PD-L2 in human colorectal cancer to define their prognostic relevance in the context of PD-L1 expression profiles on T cells.

Materials and Methods

Patients

This study prospectively enrolled consecutive patients that were undergoing elective surgery for primary CRC (n=670) at the University Hospital of Wuerzburg from July 2003 to June 2008. Of these, 116 patients with a mean age of 66.1 ± 5.6 years were included that underwent curative R0 resections. Patients with secondary carcinoma were excluded. All patients were followed-up regularly at 3 months, 6 months and 12-month intervals in accordance with the guidelines of the German tumor centers (completeness index of 0.96) [4]. The follow-up of all patients comprised 60 months. The study was approved by the regional Ethics committee. Patients were assigned to UICC stages as followed: stage I, n=20; stage II, n=27; stage III, n=40, and stage IV, n=29. Tumors were evaluated for location, stage, and differentiation grade. Data concerning age, gender, postoperative course with follow up for the analysis of tumor recurrence and tumor related survival were collected in our database (oral resection margin). Tumor tissue samples as well as normal colon tissues from the patients related survival were collected in our database (oral resection margin).

Antibodies

The PD-1 (Programmed death-1) monoclonal antibody (mAb) was purchased from MBL (Naka-ku, Nagoya, Japan). PD-L1 and PD-L2 mAb were from eBioscience (Kranenburg, Germany). T cell subpopulations were identified by positive staining for CD4+ (T helper) and CD8+ (T cytotoxic) cells (DAKO, Glostrup, Denmark). For analysis of regulatory T cells Foxp3 (forkhead transcription factor) (Abcam, Cambridge, UK) was used. Isotype control mAbs were obtained from Pharmingen (Heidelberg, Germany). The secondary antibodies were hors eradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG purchased from DAKO (Glostrup, Denmark). Secondary antibodies used for immunofluorescence were Cy5-conjugated AffiniPure Donkey anti-goat IgG and FITC (Fluorescein-5-isothiocyanate)-conjugated AffiniPure Donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., Suffolk, England).

Immunohistochemistry

To analyze the expression of PD-1, PD-L1, PD-L2, CD4, CD8, and Foxp3 in tumor specimens, we performed staining of these factors. Serial cryostat sections were performed followed by incubation of the sections with primary mAb or control mAb and with a secondary HRP-conjugated antibody. The sections were then developed by incubation with 3’-diaminobenzidine (DAB) substrate (Biogenex, San Ramon, CA). PD-1 was analyzed on infiltrating T cells using sequential HRP/AP immunoenzymatic double staining. Antibodies bound during the first staining step were blocked using EnVision K1395 double-stain Block (DAKO, Glostrup, Denmark) according to the manufacturer’s instructions. The sections were subsequently incubated with the second primary mAb followed by incubation with an AP-conjugated secondary antibody and development by incubation with K1395 Fast Red (DAKO, Glostrup, Denmark) followed by counterstaining with hemalum. The intensity of each immunoenzymatic staining on tumor cells in six individual magnified fields (× 400) for each staining was scored using a four-scale system. Intense staining in more than 50% of cells was considered as +++; staining present in a smaller proportion of the cells (10% to 50%) as +; faint or light brown/red membranous or cytoplasmic staining in a few tumor cells (<10%) was scored as +; and no staining was scored as 0. Analysis was performed independently by two observers in a blinded fashion. The sequential immunofluorescent double labelling was done by incubation with a Cy5-conjugated AffiniPure Donkey anti-goat IgG against the host species of the first primary antibody. The following second primary antibody was incubated with a FITC-conjugated AffiniPure Donkey anti-mouse IgG. After incubation with normal mouse serum (Biomaed, Burlingame, CA) diluted in TBS a PE (Phycocerythrin) labelled CD4 antibody was added. The sections were counterstained with DAPI (4′,6-diamidino-2-phenylindol, dihydrochloride, Invitrogen, Eugene, Oregon, USA) and mounted with Fluoromont (SouthernBiotech, Birmingham, USA).

Real Time Polymerase Chain Reaction (Real Time PCR) for PD-1, PD-L1, PD-L2, and tumor-infiltrating T cell subpopulation genes in colorectal tumor specimens

mRNA expression of PD-1, PD-L1, PD-L2, CD4, CD8, Foxp3 was analyzed in colorectal cancer specimens by real time PCR. The primer sequences used were purchased from Sigma Genosys, Woodlands, TX. PCR thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 secs and 60°C for 60 secs. All samples were assayed in duplicate and normalized during data analysis. The average threshold cycle (Ct) value was calculated as the cycle number at which fluorescence of reporter reaches a fixed threshold. The difference (ACt) between the average Ct values of the samples in the target wells and those of the housekeeping gene, GAPDH, was assessed, followed by the calculation of the difference between the average ACt values of the samples for each target and the ACt value of the control sample (normal colon tissue, n=85) for that target (ΔΔACt). The relative quantification value, fold difference, is expressed as 2-ΔΔACt. Results were normalized to colon normal tissue (1.0) and expressed as fold difference.
Statistical Analysis

The chi-square test was used for univariate analysis between PD-L1 and PD-L2 expression and clinicopathological data. The Kaplan-Meier method was performed to calculate the cumulative survival rates and to plot survival curves. The log rank test was used to find statistical differences between the curves. Tumor related survival was calculated from the time of surgery to the last contact or death. Relapse free time was calculated from the date of surgery to the date of first tumor relapse. Multivariate analysis by Cox regression was then performed to determine the most important predictors of survival among all of the possible variables and the associations of T cell PD-1 expression with outcome. P values <0.05 were considered to be statistically significant, values <0.01 highly significant. All statistical analysis was conducted using the SPSS 12.0 for Windows statistical software package. The Mann-Whitney-U-Test was performed to compare the PD-1, PD-L1, and PD-L2 immunostaining, gene expression, and the development of postoperative metastases/recurrence in the 116 patients.

Results

PD-L1 and PD-L2 expression in the tumor

Of the 116 evaluated cancer tissues, 49 (42.2%) were positive for PD-L1 expression at the protein level while 54 (46.6%) showed significant gene expression; comparably 48 (41.4%) tumors were positive for PD-L2 protein expression with 53 of the tumors (45.7%).

Figure 1: Quantification of PD-L gene expression by real-time quantitative PCR and immunohistochemical staining for human colorectal cancer. PD-L gene expression was significantly elevated at late stage of disease (UICC III/IV vs. I/II, p<0.0001). Relative quantification value, fold difference, is expressed as 2-\(\Delta\Delta C_t\) (a). Representative example of PD-L1 and PD-L2 protein expression at UICC stage II (top and bottom left, respectively) and at UICC stage III (top and bottom right, respectively) (b). PD-L1 and PD-L2 expression increased at the tumor site in patients of colorectal cancer at advanced stages as compared to early stages (UICC III/IV vs UICC I/II, p=0.0001). Original magnification × 400. Staining: 3,3’-diaminobenzidine (DAB), brown colour.
demonstrating increased PD-L2 gene expression. In addition, 28 tumors (24.1%) were positive for both PD-L1 and PD-L2 expression with significantly upregulated gene expression of the two ligands in advanced stage cancers (UICC III/IV vs. I/II, p<0.0001) (Figure 1a). Immunohistochemical analysis showed that both ligands, PD-L1 and PD-L2, were expressed mainly at the cell membrane but also stained positive in the cytoplasm of tumor cells (Figure 1b). Increased mRNA and protein expression in advanced but also in early stage cancers was positively correlated with each other for both PD-L1 (p=0.005) and PD-L2 (p=0.003). For the following data analysis, we employed the quantified data of real-time PCR.

**Prognostic value of PD-L expression**

Overall survival of PD-L1- and PD-L2-overexpressing cancer patient’s independent of their tumor stage (UICC I-IV) was significantly worse than that of negative patients (p=0.004 and p=0.044, respectively (Figures 2a and 2b). This was also observed for the second calculated parameter disease free survival (p=0.002 and p=0.01). Moreover,
overall survival of patients with tumors overexpressing both genes PD-L1 and PD-L2 were even worse than that of patients with tumors not expressing significantly both ligands (p=0.0005) (Figure 2c). The prognostic significance of PD-L gene expression was correlated with the protein expression profiles analyzed within the tumors. The overall survival of the 49 out the 116 patients (42.2%) with cancers positive for PD-L1 protein expression was significantly worse than that of the remaining 67 patients with negative expression of the ligand in their tumors (p=0.03). In addition, when compared with each other, PD-L1 and PD-L2, the 49 patients with tumors positive for PD-L1 protein expression had a worse overall survival than the 48 patients positive for PD-L2 (p=0.041).

Gene analysis showed significant worse overall survival for patients (completed follow-up of 60 months after surgery) with PD-L1 and PD-L2 overexpressing tumors when categorized by T category, N1/N2 category, M1 category as well as advanced UICC stages III/IV (Table 1). These results indicate that PD-L1 and PD-L2 expression was associated with a progressive course of the disease regarding nodal and metastatic status as well as UICC stages.

Cox regression analysis showed that PD-L overexpression in the tumor was an independent prognostic factor when considering overall survival (p=0.0002). Moreover, lymph node spreading (N1/2 status) was likewise an independent risk factor (p=0.001) while other factors were not significantly different.

**Correlation between PD-1 expression and tumor-infiltrating T lymphocytes**

We determined protein and gene expression levels of immune T cell-associated PD-1 expression and compared with the individual outcome of the patients. For this purpose, double immunostaining of PD-1 with markers for CD4+ and CD8+ immune cells were analyzed. Double positive CD4+/PD-1+ and/or CD8+/PD-1+ T cells were found to be present in 67 (57.8%) individual tumors. Among these cells the proportion of PD-1 positive tumor infiltrating CD4+ T cells profoundly increased in late stage cancers (UICC stages III/IV versus I/II: 57.8% vs. 24.4%, normal control 11.3%, p=0.001) (Figure 3a). In contrast, the proportion of PD-1 positive infiltrating CD8+ T cells decreased from early to late stages (UICC stages I/II vs. III/IV: 25.6% vs. 9.2%, p=0.0055 (Figure 3a).

To further analyze whether infiltration patterns of T cells with regulatory characteristics like the expression of Foxp3 was associated with the observed CD4+/CD8+ T cell infiltration profiles, co-expression studies of PD-1 with CD4+ and Foxp3 double positive T cells were performed using double and triple staining techniques. Expression of PD-1 on Foxp3+ CD8+ T cells was intensified in late stage cancers compared to early cancers and normal tissue (UICC stage III/IV vs. UICC I/II: 57.8% vs. 25.5%, normal tissue 8.6%) (Figure 3a) suggesting that the presence of such T cells with regulatory characteristics is associated with tumor progression. Real Time PCR analysis confirmed overexpression for PD-1, for CD4 as well as for Foxp3 gene expression in late stage cancers (UICC III and IV) as compared to early stage cancers (UICC I and II) p<0.005, p<0.001, and p<0.001 respectively (Figure 3b). In contrast, CD8 gene expression was decreased in advanced stage cancers (p<0.0001, UICC III and IV) (Figure 3). These latter results are in accordance with results obtained by immunohistochemical analysis and suggest that negative signaling via PD-1 expressed on infiltrating immune cells (PD-1 positive CD8+ T cells) might be one mechanism of downregulation of the tumor immune response that is involved in the progression of the tumor.

Multivariate analysis showed that patients with tumors infiltrated by immune cells expressing PD-1 on their surface were significantly more likely to die from CRC as compared to patients without tumor infiltrating immune cells positive for PD-1 (risk ratio, 2.21; 93% confidence interval, 1.21-3.72; p=0.003). This indicates, that PD-1

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Table 1: Correlation between PD-L1 and PD-L2 expression status and 5-year-survival rate in combination with clinicopathologic characteristics in patients with colorectal cancer (in %).
positive immune cells become negatively controlled through PD-L1 and/or PD-L2 signaling cancer cells within a specific tumor microenvironment and possibly by additional T cells of regulatory behavior.

Discussion

The inability of T cells to recognize tumor cells due to an impaired antigen presentation on the tumor cell surface as well as an inhibition of T cell activation by immunosuppressive proteins at the tumor site are mechanisms which preclude an effective anti-tumor immune response. This permits further tumor growth and metastasis. Tumor-infiltrating T lymphocytes are considered as a manifestation of the host immune response [27]. Particularly CD8-positive effector T cells with help of specific CD4-positive helper T cells infiltrating the tumor are contributing to the elimination of tumor cells. However, infiltrating T lymphocytes could also have negative regulatory functions particularly on effector immune cells, thus, contributing to tumor growth and metastasis. Several clinical studies have suggested that tumor-infiltrating T lymphocytes play a critical role and may have prognostic significance in certain human tumors including colorectal cancer [28].

The negative regulatory programmed death-1/programmed death ligand (PD-1/PD-L) pathway in T cell activation has been suggested to play an important role in tumor evasion from host immunity by engaging PD-1 receptors on activated T and B cells [14,29]. In this study both ligands, PD-L1 and PD-L2, were found to be expressed in CRC. Interestingly, patients with PD-L negative tumors showed a significantly better survival than those expressing either PD-L1 or
PD-L2. Moreover, the impact of overexpressing one of the ligands (either PD-L1 or PD-L2) as determined on the gene level was different between patients at advanced cancer stages with nodal involvement (N1/2) and distant metastasis (M1). Multivariate analysis showed that PD-L1/PD-L2 mRNA overexpression was an independent prognostic factor negatively influencing the prognosis of patients with CRC. Thus, our results demonstrate PD-L1 gene overexpression as an independent prognostic factor in CRC and confirm clinical data in solid tumors [30-33]. Consequently, PD-L expression is suggestive to be involved in downregulating anti-tumor immunity and thus promoting further tumor growth and metastasis in patients with CRC.

The herein presented results from clinical CRCs support the hypothesis that expression of PD-L1, as recently supposed to be predominantly tumor cell-mediated, promotes cancer progression through downregulation of tumor-reactive T cells [18]. Previous experimental findings strengthen this theory. However, PD-L1 expression in CRC has not been fully addressed so far. Nevertheless, a strong correlation between PD-L1 expression on tumor cells and discrepant clinical outcomes has been observed [30,33]. Thus, the predictive value of PD-L1 expression on tumor cells is still controversial in CRC patients. However, we cannot ignore the value of PD-L1 expression in selecting patients who will benefit from immunotherapy. Some clinical trials have demonstrated that immunotherapy significantly improves progression-free survival in patients.

We have shown that patients with invasion of PD-1 positive immune cells in their colorectal cancers developed significantly more often concomitant PD-L expression in their tumors. In fact, the expression of PD-1 on infiltrating immune cells in tumors of patients with CRC was significantly upregulated at late stages of the disease. Regulatory T cells are capable of inhibiting the activity of other cells, including CD4- and CD8-positive T cells [23,34-36]. Interestingly, the number of infiltrating T cells with regulatory characteristics (CD4- and Foxp3 positivity) significantly increased in tumor tissues of our analyzed cancer patients from early to advanced tumor stages. This data are in accordance with the observation that a blockade of PD-L1 led to a decreased number of regulatory T cells [37]. Regulatory T cells can also suppress the function of cytotoxic CD8-negative T cells, thus inhibiting anti-tumor immune responses, largely through a mechanism that requires cell-cell contact. CD8-positive T cells are generally thought to play a central function of cytotoxic CD8-positive T cells, thus inhibiting anti-tumor immunity. It will be necessary to design prospective trials to validate predictive factors to select patients with colorectal cancer with the highest chance to benefit from immune agents.

Conflict of Interest
All authors confirm that they have no conflicts of interest concerning this study.

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References


