Original Research Article

Association of PD-L1 immunoexpression with tumor grade in colorectal adenocarcinoma

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A R T I C L E   I N F O

A B S T R A C T

Background: Globally, colorectal cancer (CRC) is the primary cause of cancer-related deaths, and in emerging countries, its prevalence is continuously increasing. With all the advancements in surgery and treatment, the outlook for CRC patients is still not good. Even with the use of standard prognostic markers, there are presently no effective prognostic techniques for colorectal cancer. The long-term survival of many malignancies has been significantly enhanced by immune checkpoint blockades (ICB), suggesting that the immune checkpoint mechanism is crucial in inhibiting tumor-specific immune responses in the tumor microenvironment. By inhibiting T effector cell activity, the "PD-1 (programmed cell death-1)"/PD-L1 (programmed cell death-ligand 1) axis contributes significantly to immune suppression control and allows tumor cells to evade the host's anti-tumor immune surveillance. While early study suggested that immunotherapy was not relevant for patients with colorectal cancer, more recent studies have demonstrated that immunotherapy was beneficial for a certain subset of patients. This suggests that the prognosis prediction of colorectal cancer may benefit from a thorough evaluation of the local immune response. This study set out to assess the association between PD-L1 immunoexpression and tumor grade in CRC.
Materials and Methods: This was a cross-sectional observational study. Paraffin blocks of total 64 cases were selected from the patients who were diagnosed as adenocarcinoma from resected samples received in the department of pathology at BSMMU from July 2021 to June 2023. Immuno-histochemical staining for PD-L1 was performed using 28-8 clone along with appropriate positive control.
Results: In this study, PD-L1 immuno-expression was found in 14(21.9%) out of 64 cases. However, no expression was found in rest of the 50 (78.1%) cases. This study showed association of PD-L1 expression with high grade (Grade-3) tumors.
Conclusion: Evaluation of expression of PD-L1 may emerge as a new marker and target for the immunotherapy of colorectal cancer.

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1. Introduction

Colorectal cancer (CRC) is one of the main causes of cancer-related deaths globally, and its prevalence is continuously growing in developing countries, according to Wyss et al. (2019).[1] The principal means of treating

colon cancer requires extensive care, including surgery and chemotherapy after surgery; nevertheless, the major causes of treatment failure include metastasis and recurrence.[2] Despite improvements in surgery and therapy, the prognosis for CRC patients is still not good. Furthermore, even with the use of traditional prognostic variables including tumor location, tumor size, histological type, grading, and TNM staging, there are presently no valid predictive

techniques for colorectal cancer. Thus, finding more biomarkers might be helpful in developing precise CRC prognostic techniques.[3] Immune tolerance is present in many human cancer types, which is a major barrier to the disease. Recently, research has focused on the tumor microenvironment to examine the role that interactions between immune system components and cancer cells play in cancer surveillance.[4] To protect themselves from the body's immunological reactions, tumor cells employ a range of defensive mechanisms found in the tumor microenvironment.[5] Members of the B7 family of cell-surface glycoproteins, such as interferon-gamma, are among the inflammatory mediators and cytokines that trigger the expression of PD-L1 on the surfaces of different types of inflammatory cells.[6] On their cell membranes, malignant epithelial tumors, including colorectal cancer, produce the ligand Programmed Death Ligand-1 (PD-L1), which activated lymphocytes that express the PD-1 receptor may bind to selectively. The interaction results in down-regulation of antigen-stimulated lymphocyte proliferation and cytokine generation.[3] Studies show that malignancies that are PD-L1 "positive" have a higher chance of benefiting clinically from check-point inhibitor treatment, and that PD-L1 overexpression in the tumor microenvironment is associated with an increase in effector T-cell infiltration. Measuring PD-L1 expression in tumors or immune cells is necessary for more accurate patient classification for anti-PD-1 immunotherapy. Thus, this work was conducted to evaluate PD-L1 expression in colorectal cancer in light of the therapeutic use of PD-L1.

2. Materials and Methods

This study, which was approved by the institutional review board, was a cross-sectional observational study carried out at Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. Paraffin blocks were obtained from resected samples of 64 cases of colorectal cancer. Exclusion criteria included neoadjuvant therapy recipients, carcinoma in situ, biopsy specimens, and tumors with large necrosis regions. Information from the departmental records was gathered, including demographic and clinically significant details.

After being collected from the Department, paraffin blocks of each chosen instance were examined. An adequate positive control was used for immunohistochemical staining for PD-L1 following diagnostic confirmation. Parts that were 3–4 mm thick were sliced, gently placed on a water bath surface at 45° C, and then wrinkle-free distributed onto slides coated with 0.1% poly L-lysine for 15 minutes at 37°C before being allowed to air dry. The slides were then left on a hot plate set at 60°C for a half-hour bake. Following xylene treatment for the slides, pure alcohol, 90% alcohol, and 70% alcohol were used to rehydrate them in order to dewax them. To extract antigens, slides were placed in a pressure cooker that had been prepared and filled with citrate buffer. The slides were then cooked and let to cool naturally.

To block the endogenous enzyme activity hydrogen peroxide was added in a moist chamber at room temperature.

Primary antibody used was pre-diluted, usable monoclonal rabbit PD-L1 Clone 28-8 (Abcam). After that, the secondary antibody DAKO REALTM EnVision TM (HRP RABBIT/MOUSE) (ENV) was applied. A positive control was the term placenta's syncytiotrophoblasts, which showed PD-L1 staining. (Abcam, 2022). The study's internal control consisted of immune cells expressing PD-L1, namely macrophages and lymphocytes seen in the lymphoid tissue of healthy colorectal tissue.[7]

After adding antibody enhancer (super enhancer), the primary antibody was enhanced and incubated for 20 minutes in a wet chamber. The secondary staining procedure used was the peroxidase anti-peroxidase technique. The antigen-antibody combination was colored using DAB. After that, hematoxylin counterstaining was done.

2.1. ***Scoring of 'PD-L1 expression' in colorectal cancer

IHC assay is the most common method for detection of 'PD-L1 expression' status. Detection of PD-L1 expression in tumor cells of colorectal cancer helps in patient stratification to guide anti- PD-1/PD-L1 therapy. Evaluation of PD-L1 expression in colorectal cancer is done by immunostaining of the cell membrane of the epithelial tumor cells and the stromal cells. A neoplastic cell is counted as PD-L1-positive if there is a membranous staining, irrespective of staining intensity and whether the membrane depicts complete or partial PD-L1 positivity.

Diffuse positivity (cytoplasmic and/ or membranous staining) is common in immune cells. For this reason, if there is cytoplasmic but no membranous staining; a tumor cell is considered PD-L1 negative.[7]

The percentage of positive cells in total tumor cells (TPS) is scored semi-quantitatively as 0 (<1% positive), 1 (1%-25% positive), 2 (25%-50% positive) and 3 (50%-75% positive) and 4 (>75% positive).[8]

Intensity score of PD-L1 expression is graded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong).[9]

The immune-reactivity score (IRS) is used to assess both the percentage of positive cells in total tumor cells (TPS) as well as the intensity score (IS). Immuno-reactivity score (IRS) is calculated by summing these values, culminating in final values ranging from 0 to 7. Positive PD-L1 expression is defined as an IRS value of ≥3.[9] Samples having a final score of ≤4 are considered to be low and those with score of > 4 are considered to be high.[10]

The results of the study were statistically analyzed using the Statistical Package for the Social Sciences (SPSS)

version 25.0 (IBM Corp. SPSS statistics, Chicago, Illinois, USA) for windows. Data were expressed as mean ± SD for the quantitative variables, numbers, and percentage. Comparison between multiple groups were made using Chi square test and Fisher's exact test for qualitative data. A value of $P < 0.05$ was taken as significant.

3. Results

This study employed a cross-sectional observational design. It was carried out at BSMMU's Department of Pathology. Patients with adenocarcinomas identified from resected specimens obtained in the pathology department at BSMMU throughout the research period comprised the study population. In this study, patients of various ages and genders were involved. Paraffin blocks of 64 cases were selected. Age, sex, tumor stage, grading, and other histopathological and demographic factors were evaluated, and PD-L1 immune-histochemical expression using 28-8 clone was noted.

A total of 64 cases showed age variations ranging in between 18 years to 75 years. Most of the cases (31.3%) were found in the 6th decade. Mean age of patients was 49.81 (± 13.96) years.

In this study out of total 64 cases, 40 (62.5%) cases were male and 24 (37.5%) cases were female with male to female ratio 1.7:1.

When the laterality of occurrence was considered, it was observed that in 28 (43.8%) cases the tumor existed in right colon while in 36 (56.3%) cases tumor occurred in left colon.

According to location of tumor, most 16 (25%) cases were in rectum. Another 14 (21.9%) cases were in sigmoid colon and 3 (4.7%) cases in descending colon. In right colon, 11 (17.2) cases were in caecum, 12 (18.8%) in ascending colon and 8 (12.55) in hepatic flexure of transverse colon.

In this study only adenocarcinomas were included. Out of 64 cases, the vast majority of the cases were conventional adenocarcinoma (47 cases, 73.4%), followed by 15 (23.4%) cases of mucinous adenocarcinoma. The rest 2 (3.1%) cases were signet ring cell carcinoma.

Among the 64 cases, the majority (53/64, 82.8%) of the tumors were grade 2 tumors followed by 9 (14.1%) were grade 3 tumors. The rest 2 (3.1%) cases were grade 1 tumor (Figure 1).

Among the total 64 cases, PD-L1 expression was positive in 21.9% (14/64) of the cases in total. PD-L1 positive 14 cases were categorized into low expression (IRS<4) and high expression group (IRS>4). Out of 14 positive cases, 11 (17.2%) cases showed low expression and only 3 (4.7%) cases showed high expression of PD-L1. No expression was seen in 78.1% (50/64) of cases.

In this study, percentage of PD-L1 positive cells in total tumor cells was assessed. Out of 64 cases, 47 (73.4%)
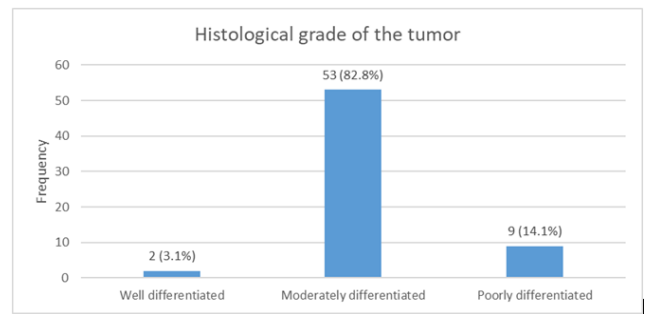

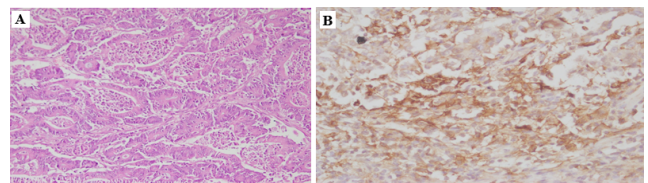**Figure 1:** Distribution of the cases according to tumor grade (n=64)

Figure 2: (**A**) Photomicrographs show adenocarcinoma of colon under light microscope (case no:62, H&E, 100x) and (**B**) Strong positive membranous staining for PD-L1 antibody in tumor cells (case no:62, IHC, 400x).
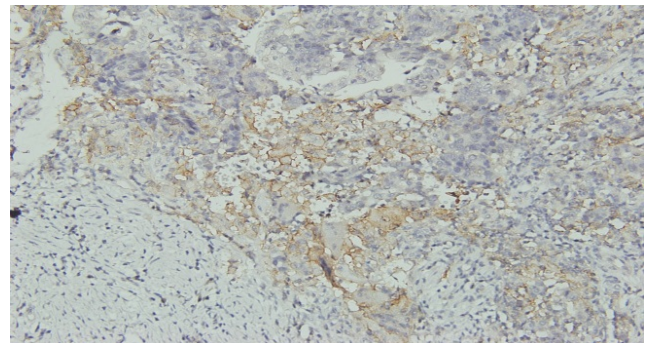

Figure 3: Photomicrograph shows moderate positive membranous staining for PD-L1 antibody in tumor cells (case no. 44, IHC, 100x).
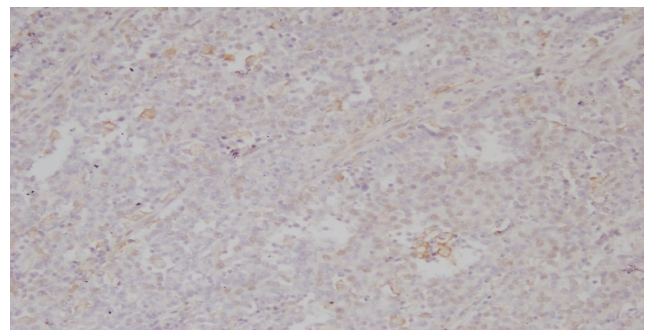

Figure 4: Photomicrograph shows weakpositive membranous staining for PD-L1 antibody in tumor cells (Case no. 45, IHC, 200X).

Table 1: Association between PD-L1 expression and tumor grade (n=64)

		PD-L1 expression		p-value
		Positive	**Negative**	
Histological grade	Low grade (grade-1 and grade-2)	9(16.4%)	46(83.6%)	0.019*
	High grade (grade-3)	5(55.6%)	4(44.4%)	

*p value was determined by Fisher's exact test. Variables were expressed as frequency.

cases showed PD-L1 staining in <1% of tumor cells. It was observed that PD-L1 staining was positive in 1-25% of tumor cells in 9 (12.5%) cases. Six cases (10.9%) showed positive staining in 25-50% of tumor cells and only two (3.1%) cases showed positive staining in 50-75% of tumor cells.

Among 64 cases, only 2 (3.1%) cases showed strong intensity of PD-L1 (Figure 2A,B). Moderate staining of PD-L1 was observed in 10 (15.6%) cases (Figure 3) followed by weak intensity in 5 (7.8%) cases (Figure 4). No staining for PD-L1 was observed in 47 (73.4%) cases.

Out of 64 cases, score: 0 was observed in 47 (73.4%) cases. Only 3(4,7%) cases showed score:2. Among 14 PD-L1 positive cases, score: 3 was observed in 7 (10.9%) of cases followed by score: 4 in 4(6.3%) cases and score:5 in 3(4.7%) cases.

Among nine high grade (Grade-3) tumors, five (55.6%) cases showed PD-L1 expression. However, out of fifty-five low grade (Grade-1 and Grade-2) tumors, only nine (16.4%) cases showed PD-L1 positivity. There was a statistically significant association (p = 0.019) between tumor grade and PD-L1 status in tumor cells as seen with the Fisher's exact test (Table 1).

4. Discussion

Colorectal carcinoma (CRC) is one of the most frequently occurring cancers and one of the leading causes of cancer related death worldwide. Sometimes prognosis, treatment response and recurrence rate varies from patient to patient even within the same stage. Lately studies have been carried out focusing on biomarkers reflecting immune-regulation, which plays an important role in initiation, progression and metastasis. Adding the programmed death-1 (PD-1)-targeting agents Nivolumab or pembrolizumab to chemotherapy was recently shown to improve outcomes in both early-stage and metastatic colorectal cancer.

In this study 64 cases of CRC were included. The mean age of patients was 49.81 ± 13.96 years ranging from 18 to 75 years. The majority (38.5%) of the cases belonged to age group 51-60 years. In another study done in Bangladesh by Raza et al. (2016), the average age of the patient was found 47+14.8 years and the peak age group was in between 50-59 years which is very much similar to current study.[11] One study from USA showed that the median age at the time of diagnosis was 55±15 years.[12] Therefore, this study is similar in respect to age at the time of diagnosis, compared with other studies in the region. However, the study conducted in India found that CRC patients ranged in age from 19 to 82, with the majority of cases (62.5%) occurring in the sixth and seventh decades of life.[13] In the study of Enkhbat et al., conducted in Japan in 2018, the mean age of CRC patients was 69.7 years (range:41-93 years).[14] One study from China, conducted by Lie et al., (2021) showed that mean age of patients with CRC was 66 years.[10] Another study of in Korea, showed that mean age of CRC patients was 63.1 ± 12.5 years.

This study showed that, there were male predominance in colorectal cancer. Out of total 64 cases, 40 (62.5%) cases were male and 24 (37.5%) cases were female with male to female ratio 1.7:1. Similar finding was obtained by Raza et al. (2016) having male to female ratio 1.4:1.[11] A study conducted by Gupta et al., in India also showed that, male to female ratio in CRC patients was 1.4:1.[13]

In this study, it was observed that in 34 (53.1%) cases, tumor existed in the left colon while in 30 (46.9%) cases, tumor occurred in the right colon. Most of the malignancies in this investigation were found in the rectum (16 cases, 25%) and sigmoid colon (14 cases, 21.9%) respectively. In their study Raza et al. (2010) observed the similar findings: 74% tumors were located in left colon, while 26% were in right colon. Similar results were discovered in Korea by Lee et al., who found that the left colon was where the majority of cancers (71.4%) were found.[15] One study from Jordan, conducted by Al-Jussani et al., showed that most of the tumors in CRC were located in left colon.[16] Thus, the finding of this study that majority of CRCs are located in left colon is similar with previous studies.

The current study found that the vast majority of the cases were conventional adenocarcinoma, not otherwise specified (n = 47, 73.4%), followed by mucinous adenocarcinoma (n =15, 23.2%) and signet ring cell carcinoma (n = 2, 3.1%). Lee et al.(2018) also showed similar findings, as their study showed majority of the patients (90.5%) had adenocarcinoma, not otherwise specified.[15] 90% of cases were classified as adenocarcinoma, not otherwise specified; in the study of Eriksen et al.,[4] One study from Li et al., showed that 88.8% of cases were classified as adenocarcinoma, not otherwise specified.[10] Thus, the finding of this study, that majority of CRCs are of adenocarcinoma (NOS) type are similar with previous studies.

The majority (53/64, 82.8%) of the tumors were moderately differentiated (grade 2) followed by poorly differentiated (9/64, 14.1%) and well differentiated (2/64, 3.1%) in this study. In the study of Al-Jussani et al., that majority of CRCs were grade 2 (89.8%) as compared to other grades.[16] Lee et al. (2012) also found higher frequency (83%) of grade 2 tumors in their studies.[12] The study by Shan et al. (2019) also found that out of the 80 patients, 46 patients (57.5%) had grade 2 cancers.[2] Similarly in an Indian study by Gupta et al.,(2020) observed that majority of CRCs were in grade 2 as compared to other grades.[13] Hence, the finding of this study is similar to other studies.

In this study, PD-L1 staining was done with 28-8 clone. Positive expression was seen in 21.9% (14/64) of the cases. On the basis of imuuno-reactivity score of PD-L1 positivity, 17.2% (11/64) cases showed low expression and only 4.7% (3/64) showed high expression. No expression was seen in 78.1% (50/64) of cases. Similar result was found in the study of Noh et al., which showed more cases having low expression of PD-L1 than high expression.[9] Among the 58 CRCs studied by Jung et al., PD- L1 (28-8 clone) expression was detected in 18 (31%) cases.[17] The study of Kim et al., conducted on patients with CRC, showed that 20.9% of cases of CRC were PD-L1 (sp142 clone) positive similar to current study.[18] In the study of Gupta al. (2020), PD- L1 (Biocare kit) positivity of tumor cells was seen in 35% CRC cases.[13] In the study of Lang-Schwarge et al., PD-L1 (22c3 clone) positivity was seen in 30.5% CRC cases.[19] The variation in PD-L1 expression is due to use of different clones of PD- L1 and cut off values in different studies as shown by the studies.[15] Biocare kit in the study of Gupta et al., showed higher PD-L1 positivity in tumor cells than 28-8 clone. But it is not approved by FDA yet. Currently, 22C3 clone and 28-8 clone are the two FDA-approved companion diagnostic tests.[20] FDA has approved the anti- PD1 antibodies pembrolizumab (22c3 clone) and nivolumab (28-8) for the treatment of CRC.[21] Different studies showed more PD-L1 positivity using 22c3 clone rather than 28-8 clone. However, Nivolumab is one of the most extensively studied immune checkpoint inhibitor (ICI) in metastatic CRC.[22]

High grade (grade 3) tumors had higher number (55.6%) of positive PD-L1 cases than low grade (grade I and II) tumors (16.4%). There was a statistically significant association between tumor grade and tumor PD-L1 status. These results are comparable with the study of Kim et al.(2016) where grade 3 tumors had the highest number (65%) of positive PD-L1 cases.[23] However, in the study of Calik et al., most of the PD-L1 positive tumors were grade II. There was no significant association between tumor grade and PD-L1 expression in the study.[24] Similar result was also seen in the study of Al-Jussani et al.[16] However, In the study of Helmy et al., no significant association was found between PD-L1 expression and tumor grade.[25]

5. Conclusion

On the basis of immuno-reactivity score (IRS), 21.9% of the tumors showed PD-L1 positivity in the present study. It also demonstrated association between PD-L1 expression and high grade (Grade-3) adenocarcinoma. Extensive research employing PDL1 antibody may facilitate the determination of the prognosis and potential therapy options for individuals suffering from colon cancer.

6. Source of Funding

None.

7. Conflict of Interest

None.

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