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Original Article

Pathology Section

Expression of PD-L 1 in Urothelial Carcinoma and its Association with Clinicopathological Parameters

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ABSTRACT

Introduction: Urothelial cancer poses a substantial medical and public health challenge in most parts of the world. Programmed Death Ligand 1 (PD-L1) is a cell surface glycoprotein that plays an important role in the suppression of cellular immune responses to tumour and is now emerging as a new target for immunotherapy.

Aim: To examine PD-L1 expression in urothelial carcinoma and its relationship with various clinicopathological parameters.

Materials and Methods: A retrospective analysis study was conducted on 50 cases of Urothelial Carcinomas diagnosed in Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, Punjab, India from January 2016 to January 2019. Detailed clinical data of the patients' was collected and the analysis was undertaken in between February 2019 and April 2019. Tissues were formalin fixed, paraffin embedded and were studied for histopathological grading after staining with haematoxylin and eosin. All cases were subjected to immunohistochemistry for PD-L1 expression. Chi-square test was used to assess the relationship between PD-L1 positivity and various clinicopathological parameters. A p-value<0.05 was taken as statistically significant.

Results: Total of 50 cases were included and 54% (27 cases out of total 50) were low grade cases. The maximum incidence was seen in 5th-7th decade of life with male preponderance. While the rest 46% (23 cases) high grade. PD-L1 positivity was observed in 19 cases (38%). PD-L1 expression was significantly associated with high grade of tumour, increase in size of tumour and lamina propria invasion. Age, gender and muscle invasion however had no association with PD-L1 expression.

Conclusion: The present study concluded that expression of PD-L1 was significantly correlated with poorer clinicopathological variables including increasing size, higher grade and lamina propria invasion. PD-L1 positivity is therefore a bad prognostic marker.

Keywords: Immunohistochemistry, Immunotherapy, Oncology, Programmed death ligand 1

INTRODUCTION

Urothelial Cancer (UC) is a disease of older individuals with majority of patients older than 55 years of age and is four times less common in women than in men. It is the sixth most common cancer in men and the seventeenth most common cancer in women worldwide according to International Agency for Research on Cancer (IARC) [1]. A combination of both genetic and environmental factors play a role in its pathogenesis. Genetic factors like loss of p53 (Lynch syndrome) and germline mutation in MutL homolog (MLH) and MutS homolog (MSH) along with smoking, exposure to aromatic and aniline dyes, arsenic, schistosomiasis and pelvic irradiation can all cause bladder cancer [2].

A relatively newer strategy in oncotherapy is the use of agents to modulate the immune system to enhance its anti-tumour activity. The development of novel immune checkpoint inhibitors has drastically changed the landscape of cancer treatment in recent years. One such marker is PD-L1 [3].

PD-L1 is an inhibitory receptor which on interaction with its receptor leads to T-cell inactivation. It therefore plays an important role in the suppression of cellular immune responses and physiologically, it helps in the maintenance of T-cell tolerance [4].

A large array of solid tumours and haematologic malignancies have been found to over express PD-L1 correlating with adverse prognosis. In most solid tumours, its expression can be evaluated via immunohistochemistry. The various checkpoints inhibitors are now used as drug targets providing for an alternate means of therapy [5,6].

The present study aimed to evaluate the PD-L1 expression in urothelial carcinomas and its association with other clinical parameters.

MATERIALS AND METHODS

The retrospective analytical research was conducted on 50 cases (based on the availability of clinical data and cost of antibody and other reagents) of Urothelial Neoplasms received as biopsy specimens in the Department of Pathology, Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, Punjab, India, from January 2016 to January 2019. Detailed clinical data was recorded and the study was undertaken in between February 2019 and April 2019.

Inclusion criteria: Only transitional cell carcinoma cases were taken which included recently diagnosed untreated cases primarily.

Exclusion criteria: Patients on follow-up and those who had already received any form of therapy were excluded along with patients with incomplete clinical data.

The tissue was formalin fixed, paraffin embedded and was then stained by haematoxylin and eosin for histopathological typing and grading. All the cases then were subjected to immunohistochemistry by using antibodies against PD-L1 (Rabbit monoclonal antibodies, pdl171aa - Biocare Medical). A 3-5 µm thick sections were cut and mounted onto slides precoated with Poly–L–lysine hydrobromide. Antigen retrieval was done using Diva Decloaker with citrate buffer at pH 6.0. Endogenous peroxidase inhibition was done using 3% hydrogen peroxide solution followed by protein blocking. After this, the slides were incubated overnight with anti-PD-L1 antibodies (primary antibody) and were conjugated with Horse Radish Peroxidase (HRP). Diamino-Benzidine (DAB) was used as a chromogen. In between different steps Tris buffer was employed as wash buffer. The slides were subsequently counterstained with haematoxylin and were examined by light microscopy.

Positivity Criteria: Tumour cells showing either partial or complete membrane or cytoplasmic staining (brown) in 5% or more of cells were considered as positive for PD-L1. Similarly, histological evidence of cell surface membrane or cytoplasmic staining in <5% of cells was taken as negative. The cut- off value of 5% was based on previous similar studies owing to the lack of standardised guidelines for the same [7-9].

Controls: Positive and negative controls were run with every batch of the IHC. Splenic tissue was used as positive control. Positive control tissue had coloured end product at the site of target antigen whereas negative control tissue section did not. So, the tissue having coloured end product had antibody specific antigen [8].

STATISTICAL ANALYSIS

The primary data was entered in Microsoft Excel and analysed using Statistical Package for Social Sciences (SPSS) version 20.0. The results were presented in the form of tables. The descriptive statistics frequency and percentages were calculated. The association between the categorical variables was analysed by Chi-square test with 5% level of significance.

RESULTS

Most of the patients belonged to the age group of 51-70 years. The oldest patient was 90-year-old whereas the youngest was 35-year-old. Majority of the patients were males constituting 84% of the total with M:F ratio of 5:1 as shown in [Table/Fig-1].

Age groups (Years)	Total Positive Cases	Males	Females
30-50	10	8	2
51-70	29	24	5
71-90	11	10	1
Total	50	42	8

[Table/Fig-1]: Showing age & sex distribution.

Painless haematuria was the general complaint but many reported increased frequency and burning micturition as well. On radiology/cystoscopy, the tumour size varied from 0.1 to 8.6 cm [Table/Fig-2]. All the lesions had papillary configuration.

Size (cm)	No. of cases (n)	Percentage (%)
0.1-3.0	27	54
3.1-6.0	16	32
≥6.1	7	14

[Table/Fig-2]: Showing size of the tumour radiologically.

Out of total 50 cases, 27 cases were of low grade and 23 cases were of high grade papillary urothelial carcinoma as shown in [Table/Fig-3]. Furthermore, high grade papillary urothelial carcinoma cases showed higher incidence of lamina propria invasion. Muscularis propria was included in only 33 biopsies. Out of these, only 6 cases showed muscular invasion. All were high grade tumours [Table/Fig-4].

Histological grade	Subtype	No. of Cases	Percentage (%)
Low Grade Papillary Urothelial Carcinoma	Non invasive type	25	50
	Invasive Type	2	4
High Grade Papillary Urothelial Carcinoma	Non invasive type	2	4
	Invasive type	21	42
Total		50	100

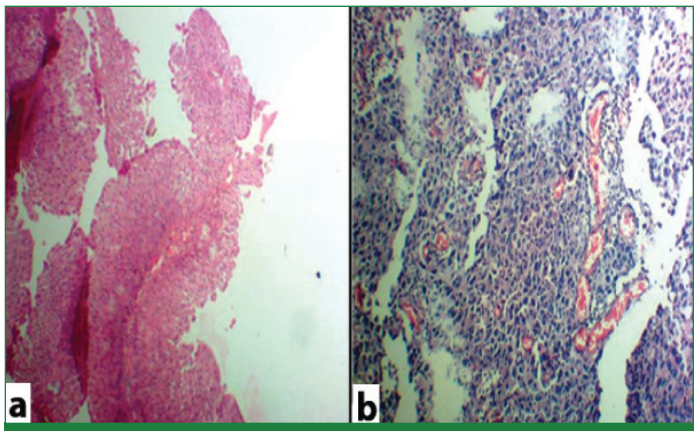
[Table/Fig-3]: Histological grade and lamina propria invasion in cases.

PD-L1 Expression

Nineteen cases (38%) out of total 50 cases showed immunopositivity for PD-L1 [Table/Fig-5].

Association of PD-L1 expression with age and sex

Maximum PD-L1 positive cases were seen within 51-70 years' age group. The 19 cases which showed PD-L1 expression consisted of 15 male patients and only 4 females. However, no statistically significant relation was found with either of these factors [Table/Fig-6].



[Table/Fig-4]: Papillary Urothelial Carcinoma: a) Low- Grade (H & E X100); b) High- Grade (H & E X100).

PD-L1 Expression	No. of cases (n)	Percentage (%)
Positive	19	38
Negative	31	62
Total	50	100

[Table/Fig-5]: Showing cases with PD-L1 positivity.

Age (Group)	No. of PD-L1 Positive Cases	No. of PD-L1 Positive Males	No. of PD-L1 Positive Females
30-50 Year	4	3	1
51-70 Year	12	9	3
71-90 Year	3	3	-
Total	19	15	4

[Table/Fig-6]: Showing association of PD-L1 positive cases with sex and age distribution. p-value = 0.69 for age and 0.44 for sex insignificant Chi-square.

Association of PD-L1 expression with tumour size

Most of the tumours ranged between 0.1- 3.0 cm in size (27 cases out of 50). It was noted that with increase in size of tumour the number of PD-L1 positive cases also increased [Table/Fig-7].

Size (cm)	Total no. of cases (n)	No. of PD-L1 positive cases	Percentage (%)
0.1-3.0	27	6	22.2
3.1-6.0	16	8	50
≥ 6.1	7	5	71.4

[Table/Fig-7]: Showing relation between size of tumour and PD-L1 positive cases. p-value= 0.028 significant chi-square

Association of PD-L1 expression with grade, lamina propria and muscularis propria invasion

High grade invasive carcinomas exhibited the greatest PD-L1 expression giving a statistically significant correlation between PD-L1 expression with grade and lamina propria invasion [Table/Fig-8]. However, the same was not true for PD-L1 positivity and muscle invasion [Table/Fig-9].

Histological grade	Subtype	Total no. of cases (n)	No. of PD-L1 positive cases	Percentage (%)
Low Grade Papillary Urothelial Carcinoma	Non invasive type	25	6	24
	Invasive type	2	0	0
High Grade Papillary Urothelial Carcinoma	Non invasive type	2	1	50
	Invasive type	21	12	57.4

[Table/Fig-8]: Showing relation of PD-L1 positive cases with lamina propria invasion. p-value= 0.013 significant chi-square

Histological Grade	Muscularis propria	Total no. of cases (n)	No. of PD-L1 positive cases	Percentage (%)
Low Grade Papillary Urothelial Carcinoma	Invaded	0	0	0
	Free	18	3	16.6
	Not included	9	3	33.3
High Grade Papillary Urothelial Carcinoma	Invaded	6	3	50
	Free	9	4	44.4
	Not included	8	6	75

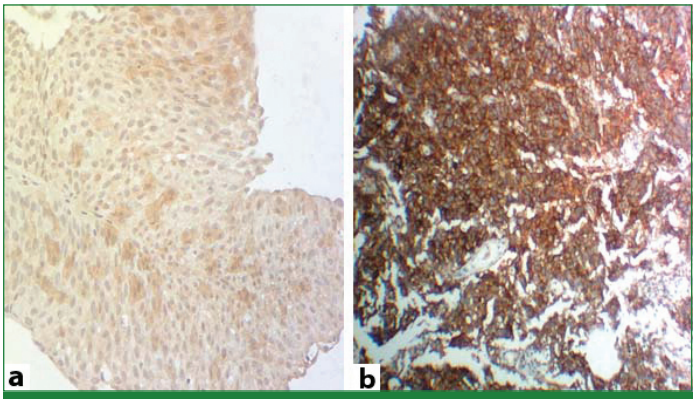
[Table/Fig-9]: Showing relation of PD-L1 positive cases with histological grade of tumour and muscle invasion.
p-value= 0.161 insignificant chi-square

Association of PD-L1 staining intensity and histology of tumour

Mild immunostaining was not seen in any case. All the 13 high grade papillary urothelial carcinoma cases showed strong PD-L1 immunostaining [Table/Fig-10,11].

PD-L1 Staining intensity	PD-L1 Positive cases in low grade type (>5% cells positive)	PD-L1 Positive cases in high grade type (>5% cells positive)	Total cases
Mild	0	0	0
Moderate	4	0	4
Strong	2	13	15
Total	6	13	19

[Table/Fig-10]: Showing association of staining intensity with histological grade of tumour.
p-value= <0.001 highly significant chi-square



[Table/Fig-11]: PD-L1 Immunostaining: a) Moderate Intensity (Low grade Papillary Urothelial Carcinoma) (Cytoplasmic)- (IHC; X400); b) Strong Intensity (High Grade Papillary Urothelial Carcinoma) (Membranous)- (IHC; X100).

Association of PD-L1 staining intensity and histology of tumour

Mild immunostaining was not seen in any case. All the 13 high grade papillary urothelial carcinoma cases showed strong PD-L1 immunostaining [Table/Fig-10,11].

DISCUSSION

Bladder tumours are the most common malignancies of the urinary tract accounting for nearly 90-95% of UCs. 15-25% of bladder tumours are invasive at diagnosis [2]. Globally, due to increase in the prevalence of smoking the health burden by UC is likely to rise in the future [1].

PD-L1 also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1) is a protein derived from the CD274 gene [10-11]. A naive T-cell requires two signals from Antigen presenting cells (APCs) for activation. The first signal involves interaction of major histocompatibility complex with the T-cell receptor for antigen recognition, and it confers specificity to the immune response. The second "costimulatory signal," is delivered by costimulatory molecules expressed on APCs to receptors expressed on T-cells. Binding of T-cells to a co-stimulatory molecule leads to T-cell activation whereas a co-inhibitory signal renders a T-cell anergic. In normal physiology, receptors that deliver co-inhibitory signals function as immune checkpoints. PD-L1 is one such co-inhibitory molecule which binds to PD-1 on T-cells and leads to their inactivation [4].

PD-L1 is constitutively expressed on bone marrow-derived dendritic cells, mast cells, macrophages and mesenchymal stem cells [12]. It helps in suppressing the immune system during events such as autoimmune diseases, pregnancy, tissue allografts, and other disease states such as hepatitis [4,10]. The constitutive expression of PD-L1 in the cornea and retinal pigmented epithelium and PD-1–PD-L1 interaction defends the eye from activated T-cells [13].

PD-L1 has been described as a double-edged sword in recent oncology literature. It has been discovered that tumours expressing PD-L1 might reduce the host immune responses for tumours by engaging the PD-1: PD-L1 pathway [14].

A meta-analysis study was performed by Wu P et al., to assess the relationship between PD-L1 expression and overall survival in solid tumours. It was found that PD-L1 overexpression showed significant correlation with worse overall survival at 3 years for gastric cancer, oesophageal cancer, urothelial cancer and hepatocellular carcinoma [6].

PD-L1 positivity was seen in 19 (38%) out of the total 50 cases included. This percentage positivity is higher than that reported by Faraj SF et al., Boorjian SA et al., and Inman BA et al., who reported 18%, 12.4%, and 28% positivity, respectively [8,9,15]. Bellmunt J et al., reported a positivity of 20% [7]. The results were closest to study by Zhang J et al., who declared PD-L1 positivity in 45% of all examined tumour specimens [16]. Most of these studies used a ≥ 5% cut- off limit like the one used in the present study except Inman BA et al., who used a ≥ 1% cut-off [15]. Given the considerable difficulty in developing reagents and methods for detection of PD-L1 in archival tissue, the lack of specificity amongst the previously used commercial antibodies comes as no surprise. This fact also might, to some extent, be responsible for the discrepant results among some of the studies mentioned earlier. Another cause for the variable positivity could be lack of standard guidelines and the difference in scoring strategies used to evaluate PD-L1 expression among the various studies [8].

In the current study, owing to the male preponderance, more PD-L1 expression was seen in males as compared to females. However, despite this no statistical significance was found between PD-L1 expression and sex of the patient. The same was true for age of patient and PD-L1 immunostaining. This resembles the observations reported by Inman BA et al., [15]. On the other hand Faraj SF et al., indicated in their research that tumours from younger patients show higher PD-L1 positivity [8].

Our study also determines that PD-L1 positivity increases with increase in size of tumour. To the best of our knowledge no other study has attempted to correlate between PD-L1 expression and tumour size as an independent variable.

High grade tumours, especially the ones with lamina propria invasion are more likely to show PD-L1 overexpression as compared to low grade tumours. However, the muscle- invasive tumours show no predilection towards PD-L1 immunopositivity. Similar data was recorded by Bellmunt J et al., Inman BA et al., and Nakanishi J et al., [8,15,17].

Staining intensity and histological grade of tumour also showed a significant relationship. High grade papillary urothelial carcinomas exhibited strong staining intensity implying that the intensity of staining increases with increasing grade of tumour. These findings have been corroborated by Zhang J et al., [16].

Keeping the above findings in mind and also the work done by Boorjian SA et al., Xylinas E et al., and Pichler R et al., it can be safely assumed that PD-L1 has got effect on the prognosis, and overall survival [9,18,19].

The real role of PD-L1 comes with the introduction of the checkpoint inhibitors. Drugs like Pembrolizumab and Atezolizumab that target

the PD-L1/PD-1 pathway offer real hope for patients who are ineligible for cisplatin-based regimens on the basis of age, co-morbidities, or patient acceptance and whose tumours are PD-L1 positive. These drugs are relatively well-tolerated, without the propensity for renal damage; and therefore, may be a used as an alternative to cisplatin in many such patients [20-22].

Limitation(s)

There is an absence of specificity amongst the commercially available antibodies and reagents along with a lack of standardisation in the reporting guidelines. These factors along with a variation in the scoring system are considered to be responsible for the discrepant results among some of the studies mentioned earlier. Owing to the short duration and nature of the study follow-up of these patients could not be done.

CONCLUSION(S)

It is concluded that as PD-L1 expression increases with adverse prognostic factors such as increasing size of tumour, grade of tumour and lamina propria invasion; it is therefore a bad prognostic marker. Bladder carcinomas are considered to be immunogenic tumours and several immunotherapeutic drugs targeting PD-1 and PD-L1 have been tested and shown to be curative. These drugs also provide a useful alternative for cisplatin-based regimens. However, more studies are needed regarding the standardisation of scoring algorithm and the use of anti-PD-L1 drugs in combined regimes. Thus, all the cases papillary urothelial carcinomas especially the high grade invasive ones should be subjected to PD-L1 immunohistochemistry.

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