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# Comparison of CD3 and CD8 immunoscoring with histological grade of the tumor in urothelial carcinoma of bladder

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## ABSTRACT

**Background**: Inflammatory response within transitional cell carcinoma (TCC) bladder is known to be predictive of tumor prognosis. Lower scores are associated with worser outcome and *vice versa*. Simpler and cost-effective methods of immunoscoring using immunohistochemistry (IHC) have been described. This study uses two easily available T-cell markers CD3 and CD8 and compares their immunoscore with tumor grade and understands the association. **Aims and Objectives**: The aims of this study were as follows: (1) To perform immunoscoring for cytotoxic T lymphocytes -CD3+ and CD8+ lymphocytes in urothelial carcinoma and (2) to correlate the immunoscore with tumor grade and calculate its significance in predicting prognosis. **Materials and Methods:** A prospective cross-sectional study of 2 years duration from October 2018 to October 2020 was conducted at a tertiary level super specialty government institution. All the transurethral resection of bladder tissue biopsies reported as urothelial malignancies during the study period were included in the research. The tumors were graded histologically into high grade and low grade on histology based on the World Health Organization 2016 classification of Urothelial Carcinomas/International Society of Urological Pathology grading system. They were then subject to IHC with two T-cell markers CD3 and CD8 and immunoscoring was done using the method described by Galon. **Results**: A total of 42 cases of urothelial malignancies were diagnosed during the study period. Cases with higher tumor grade were 25 and lower tumor grade were 17. The association between the lower immunoscore and higher tumor grade was statistically significant. **Conclusion:** Immunoscoring is a useful adjunct to the routine histological evaluation of TCC. Easily available T-cell markers can be used as simple easy and cost-effective method for immunoscoring.

**Key words:** CD3; CD8; Immunoscore; Transitional cell carcinoma; Urinary bladder

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## INTRODUCTION

Bladder cancer was ranked 10th among all the cancers and contributed to 3.4% of cancer burden worldwide according to GLOBOCAN 2018.[1] In India, there are 18,921 new cases every year.[2] The bladder tumors have a heterogeneous clinical behavior. The death rate and morbidity increase from superficial to invasive disease. Tumor histology and clinical stage of the disease are the usual prognostic indicators. Configuration of the tumor, multiplicity size, and gene diagnosis may also have prognostic value.[3,4] Bladder cancer can be classified histologically as urothelial or non-urothelial. Urothelial cancer represents more than 90% of bladder cancers.[5] Many researchers have shown that tumor microenvironment plays an essential role in progression of malignant tumors and tumor-associated lymphocytes (TIL) can act as an independent prognostic factor. TIL response has been proven to be associated with favorable prognosis in transitional cell carcinoma (TCC) malignancies of the bladder.[6] Studies have tried to prove the association between TIL and urothelial cancer.[7-10] Spatial distribution of TILs in the epithelial and stromal

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compartments in the tumor margin and core has been studied. TNM staging can have different clinical outcomes following surgery. It is well known that clinical outcome can be different in patients with the same clinical stage. The currently used AJCC/UICC/TNM staging provides no information on response to treatment or prognosis in TCC.[7] Therefore, there is a need to supplement the histopathological evaluation of the tumors along with immunoscore as a part of diagnostic assessment as it will provide prognostic information.

A simple immunoscoring system was derived by Galon et al.,[7] who is one of the pioneers in the immunological research related to tumors. He used three parameters number, type, and distribution of immune cells. A score of I0 to I4 is given in this method which was originally applied to colonic tumors. We applied this immunoscoring method for CD3+ T-cells and cytotoxic T-cells (CD8+) using immunohistochemistry (IHC) in the central core and invasive margin (IM) of transitional cell tumors of urinary bladder. Higher immunoscore represents greater infiltration of CD3+ T-cells and cytotoxic CD8+ T-cells. We compared this score to the histological grade of the tumor based on the established World Health Organization (WHO) guidelines and statistically compared it to the immunoscore.

### Aims and objectives

1. To perform immunoscoring for cytotoxic T lymphocytes - CD3+, CD8+ lymphocytes in urothelial carcinoma.

2. To correlate the immunoscore with tumor grade and calculate its significance in predicting prognosis.

## MATERIALS AND METHODS

This study was conducted at a tertiary referral hospital in the capital city of Telangana, Hyderabad. The study has been approved by the Institutional Ethics Committee (No- ECR/300/AP/2013/RR-16).

### Study design

It is a cross-sectional and a prospective study.

### Study period

This study was October 2018–2020.

### Source of data

Histologically, primary urothelial carcinoma cases of various histological types, Pathology Department, Osmania General Hospital, Hyderabad.

### Inclusion criteria

The following criteria were included in the study:

1 Informed consent to participate in this study
2 Patients with histologically diagnosed urothelial malignancies.

### Exclusion criteria

The following criteria were excluded from the study:

1. Benign bladder tumors
2. Non-urothelial malignancies
3. TCC cases associated with malignancy of prostate.

### Sample size

This study was 42.

### Sampling technique

Transurethral resection of bladder tissue (TURBT) biopsies.

### Procedure of data collection

TURBT biopsies of urothelial malignancies were fixed in 10% buffered formalin overnight grossly examined (Figure 1), whole embedded and paraffin embedded, sections were cut, stained with hematoxylin and eosin. Microscopically, sections were examined and tumors were histologically graded into high grade and low grade according to the histological grading system of World Health Organization 2016 classification of Urothelial Carcinomas/International Society of Urological Pathology.

In addition, the microscopic sections were assessed for tumor associated inflammatory response. A gathering of >20 lymphocytes was considered as presence of TIL in at least one high power field per section. Blocks showing the highest inflammatory cell response were selected for IHC. TILs admixed with polymorphs and plasma cells were also considered as inflammation. IHC with CD3 and CD8 was performed in 4μ sections.

### Immunoscoring

The immunoscore (0–4) was calculated based on the density of CD3+ and CD8+ TILs in the tumor on the IHC sections according to the method described by Galon et al.[7] The immunoscore (I0–I4) was calculated based on the density of CD3+ and CD8+ TILs in both the CT (central core) and IM of the tumor. For example, if both markers were elevated in both CT and IM, the highest score of I4 was given. If one marker was high in CT but was low in IM, while the other marker was high in both regions, then a score of I3 was given. Similar method was applied to I2 and I1. If both markers were low in both regions, then the lowest possible score of I0 was given. The areas of highest lymphocyte density were selected and the immunoscoring was performed on bright field microscopy and then results were analyzed. We did not perform digital imaging as it is not available at our institution. The scores I0 and I1 were


**Figure 1:** Gross image of TURBT specimen

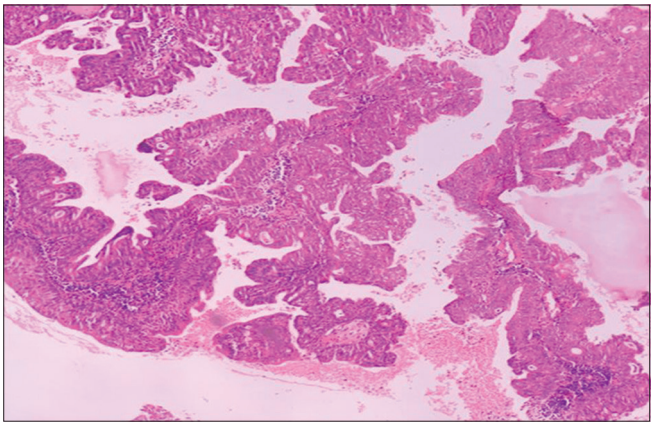

**Figure 2:** H and E ×10 image of HGPUC

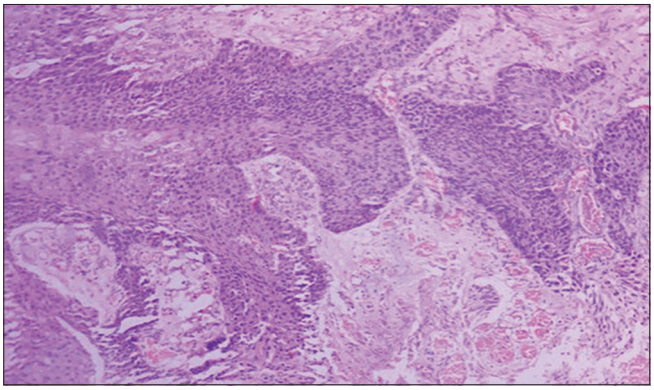

**Figure 3:** H and E ×10 image of LGPUC

grouped as lower immunoscoring and I2, I3, and I4 were grouped as higher scoring. These scores were compared to the histological grade of the tumor and significance was calculated.

## RESULTS

A total of 50 TURBT biopsies were diagnosed as malignant during the study period. Out of which 42 (80%) were urothelial malignancies. Total of 42 cases of urothelial carcinoma were included in the study. The overall age

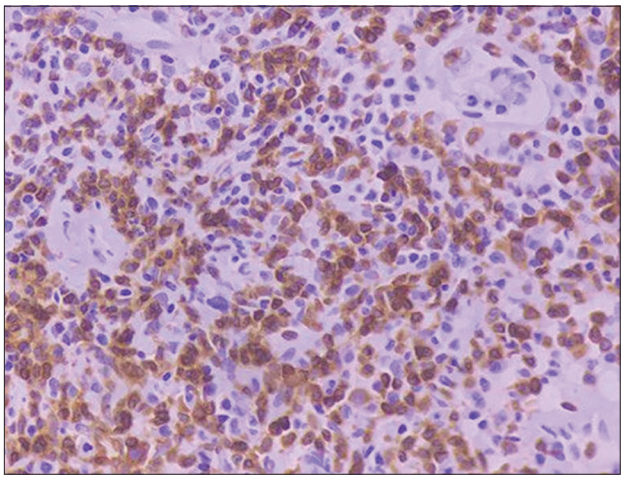

**Figure 4:** High expression of CD8+ in LGPUC

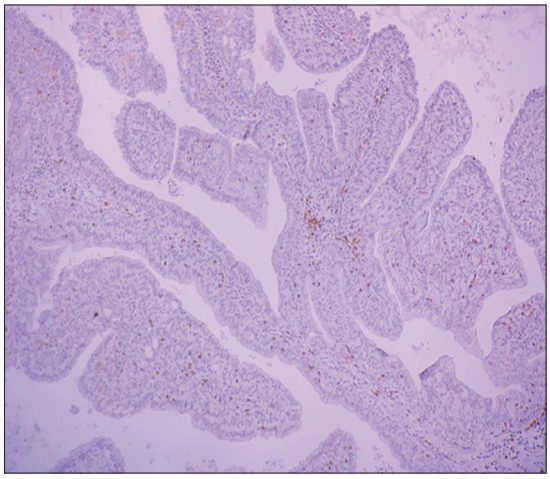

**Figure 5:** Focal positivity of CD3+ in HGPUC

incidence of bladder tumors was common and most common age group was 6$^{th}$ decade. The overall sex incidence of bladder malignancies was 1:4 with male preponderance. Among the urothelial tumors, 32 cases were male and ten cases were female and male-to-female ratio was 3:2.

On histological grading (Table 1), 17 cases (40.47%) were low grade and 25 cases (59.52%) were high grade. Immunoscoring of urothelial carcinoma showed 32 cases (76.18%) showing low score and 10 cases (23.80%) showing high score. Ten cases (23.8%) showed higher expression of CD3+ and CD8+ cytotoxic T lymphocytes and were diagnosed as low-grade tumors on HPE.

Twenty-five cases (59.52%) showed low expression of CD3+ and CD8+ cytotoxic T lymphocytes and diagnosed as high-grade tumors on HPE. Seven cases (16.6%) have showed low expression of CD3 positivity and CD8 positivity diagnosed as low-grade tumors on HPE. There

**Table 1 :**

| Serial No. | Histological Types | Histological Grades | Immunoscoring of CD3, CD8 | No. of cases | % |
|---|---|---|---|---|---|
| 1 | PUNLMP | Low Grade | High[I2,I3,I4] | 2 | 4 |
| 2 | LGPUC | Low Grade | High[I2,I3,I4] | 8 | 16 |
| | | | Low[I0,I1] | 7 | 14 |
| 3 | HGPUC | High Grade | Low[I0,I1] | 21 | 42 |
| 4 | PUC with Sq Diff | High Grade | Low[I0,I1] | 4 | 8 |

was no case with high tumor grade showing a higher immunoscore. It can be observed that majority of cases with low immunoscores exhibit high tumor grades and majority of cases with high immunoscores and exhibiting low tumor grades and this association between the immunoscore and tumor grade is significant.

### Statistical analysis

Data were entered in Microsoft Excel and analysis was done using SPSS version 20. Descriptive statistical analysis was done. Results on categorical measurements are presented as percentages. Significance is assessed at 5% level of significance. $P<0.05$ is considered statistically significant and $P<0.001$ is considered statistically highly significant. Fisher's exact test was used to find out the significance of study parameters on a categorical scale between two groups.

## DISCUSSION

Bladder cancer is ranked 10$^{th}$ in incidence in the world. In India, it is ranked 17$^{th}$ in incidence and 19$^{th}$ in mortality, with a varying incidence across Indian population. It is hypothesized that bladder cancer is likely to show an increase in number of cases due to several factors, including aging, population growth, and socioeconomic development, in emerging economies, with a westernization of lifestyle, a shift is observed from cancers related to infections and poverty, to cancers related to lifestyle.[7,8]

Although bladder cancer can occur at any age, and many demographic studies have shown that individuals' aged ≥65 years have 11 times higher incidence than those younger than 65 years.[11] Most common age at diagnosis was 6$^{th}$ decade in our study in both urothelial and non-urothelial category. Bladder cancer is more common in men and is also labeled as the disease of older men, and the possible reason in gender-based disparity in incidence and mortality is the difference in hormonal pathways.

Genitourinary malignancies are the commonest tumors in males. Occupational and smoking habits may only partly explain the difference in the incidence of bladder cancer between men and women. Hormonal influence was suggested by some, considering the lower incidence of bladder cancer in parous compared to nulliparous women.[12] Moreover, mucosal changes during the menstrual cycle have been reported[13] suggesting an influence on cell proliferation and differentiation. Bladder cancer incidence rates were more common in men, four-fold, than in women worldwide.[1] We found a ratio of 3:2 in our study among urothelial; however, the overall sex incidence was comparable at 1:4.

On histological examination, four different urothelial tumors were diagnosed on histology of the TURBT specimens. They were graded histologically into high grade and low grade using the WHO histological criteria for grading of bladder tumors. Low-grade tumors were less and formed 17/42 cases. They included PUNLUMP and LGPUC (Figure 3). All the cases of PUNLUMP had high scores. Among cases of LGPUC, 10/17 had higher score and 7/17 had lower score. High-grade tumors included HGPUC (Figure 2) and PUC with squamous differentiation. All the three categories showed a low immunoscoring. To conclude, all the 25 cases of high-grade tumors showed low immunoscore and ten cases of low-grade tumors showed a high immunoscore and interestingly seven cases showed low immunoscore. These results suggest that immunoscoring is independent of tumor grading on histology particularly in tumors of lower histological grading and therefore needs to be assessed in every case. Given the power of a proper immune evaluation of cancer patients, the immunoscore is likely to be important for the field of cancer, beyond the field of tumor-immunology. In an effort to promote the immunoscore in routine clinical settings, an international task force was initiated. The results of this international validation may result in the implementation of the immunoscore as a new component for the classification of cancer, designated TNM-I (TNM-Immune).

We, in our study, performed IHC using two T-cell markers CD3 for T-cells in general and CD8 for cytotoxic T lymphocytes. The tumor grading on histopathology was calculated with the immunological profile using IHC for CD3 and CD8.

This approach has been advocated by[7] in many malignant tumors suggesting that they could play a role in prognosis and also provide a tool to assess the utility of using immunotherapy in patients.

The methodology similar to that established by Galon et al.[6,7] Galon established the scoring system using a worldwide task force and advocated its use in the prognostication of malignant tumors. This involved identifying and grading tumor immune infiltrates using IHC in the center and the IM of the tumor. They suggested the use of two easily available membrane stains CD3 and CD8 for this. A method of quantification and grading was also provided, in which we have used in our study. Their understanding was that this method provided a pathology-based, feasible in routine settings, simple, inexpensive, rapid, robust, reproducible, quantitative, standardized, and powerful method of immunoscoring.

We found correlation between lower tumor grade and high immunoscore expression of CD3+ (Figure 5) and CD8+ (Figure 4) lymphocytes. P-value suggested that indeed immunoscore might have an additional value in tumor histopathology reporting. In this study, we used two easily available IHC markers for T-cells CD3 and CD8 on bladder malignancies. Although we found statistical correlation between higher tumor grade and lower immunoscore and *vice versa*, there were 20% of low-grade tumors showing a higher immunoscore. These findings are of significance as they are in contradiction to the grade of the tumor. This requires that it should be made imperative that IHC for TIL's and immunoscoring should also be included in the histopathology report of bladder tumors.

Role of immunosurveillance in the natural history of malignancy has been known for decades. This complex interaction has led to the development of immunotherapy as one of the treatment options in clinical practice. Immunoediting has been described in three scenarios by Schreiber et al.[14] Immunoediting has been described in 3 scenarios by Schreiber,[14] which include elimination of malignant cells, maintainence of equilibrium through which the immune cells contain and control the malignant cells and the process of escape, by which the tumor cells escape the immune cells promoting tumor growth and metastasis.[15]

Risk prediction or evaluation of patient prognosis forms an important entity in management of malignancies. In this, histopathological features play a major role which includes size of the tumor, integrity of the tissue, cell morphology, histological grade, expression of aberrant proteins, genetic markers, tumor margins, IM, depth of invasion, and vascularization. In addition, involvement of the draining lymph nodes is also taken into account. Distant metastasis is also considered. In this system, the cancer progression evaluation is longitudinally performed and applied to understand patient prognosis.[6] The traditional system fails to provide adequate estimate of the patient outcome. Partial or complete regression of metastatic tumors has also been noted.[16]

Lower TNM staging of I/II might be associated with residual tumor or recurrence of metastasis.[16] Tumors with the same clinical staging vary in prognosis. Immunomodulation plays a role in development of anti-tumor resistance.[6] TILs are known play an important role in many malignancies including bladder cancer, where immunotherapy with GCG is a known modality of management for risk non-muscle invasive cancer. The introduction of the role of host immune response in tumorigenesis and progression lead to the understanding that a histological analysis should also include the inflammatory and lymphocytic cells. These tumor infiltrating immune cells (TILs) are localized into dense cell infiltrates in the center of the tumor cell (CT) and at the infiltrating margins (IM) of the tumoral nests and adjacent lymphoid structures. The immune cells that maybe found in a tumor include macrophages, dendritic cells, mast cells, natural killer cells, naïve and memory lymphocytes, and all subsets of T lymphocytes. These observations and understanding that the tumor molecular features and immune reactions are inter-related have made assessment of these factors crucial.[17] This immunity evaluation by the pathologist will help in identifying patients who will benefit from immunotherapy.

Data collection from large cohorts of human cancers suggests that the number type and location of tumor immune infiltrates are of prognostic value. The potential clinical translation of this data helps in the establishment of immunoscore based on any two lymphocyte subset population. Nature cancer review meta-analysis[18] summarizes the effect of immune cells B-cells, NK cells, myeloid derived suppressor cells, macrophages, and subsets of T-cells on clinical outcome of more than 120 published articles. They reported that high densities of T-cells (CD3+) cytotoxic T-cells (CD8+) and memory T-cells on clinical outcome of many tumors. Higher densities of T-cells were associated with prolonged disease-free survival or overall survival. The prognostic value of lymphocytes in stromal, peritumor, and intratumor locations remains unclear.

Shankaran et al.,[19] studied immunodeficient mice and documented the fact that immune system might play a role in prevention of tumorigenesis. Van Der Meer et al.,[20] and Birkeland et al.,[21] proved that incidence of cancer is higher immunocompetent hosts in comparison to immunodeficient ones. These observations provide insight into the occurrence of tumors recently the role of competent immune system in prognostication or potential

role for immunotherapy in management of established tumors has been extensively researched. Studies done previously on bladder cancer and TILs gave conflicting results. Numerous studies have found that bladder urothelial malignancies with higher immunoscoring had better long-term out come and patient survival. Flamminger in their study on intratumoral T lymphocytes in prostate malignancies found that these cases with high immunoscore had longer disease-free survival post-treatment and also slower progression.[22] This study is limited by the small number of cases and lack of patient follow-up as our institution does not provide oncology services and patients are referred to another institute. We suggest the use of easily available IHC markers in analyzing immunoscore and also adding this to the detailed histopathology report.

### Limitations of the study

Sample size is small.

## CONCLUSION

Understanding the immune signature of tumors provides insights for the risk stratification, prognostic assessment, and all helps in deciding therapeutic approach and treatment options. We found the immunoscoring methodology used in our study to be simple, cost effective, and easy to perform on TURBT biopsies of the urothelial malignancies. It is essential to incorporate the "Immunoscore" into traditional classification, thus providing an essential prognostic and potentially predictive tool.

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**Authors' Contributions:**
**SM**- Acquisition of data, analysis and interpretation of data, literary review, preparation of the manuscript, revision, and final approval; **RKOH**- Concept, and design of the study, analysis, and interpretation of data, drafting of the article, literary review, revision, and preparation of manuscript; **SLG** - Concept and design of the study, intellectual content, final approval. **VPGJ**- Concept, and design of the study, intellectual content.

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