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PDL 1 IMMUNOHISTOCHEMICAL EXPRESSION IN INVASIVE HIGH GRADE UROTHELIAL CARCINOMA IN A SAMPLE OF IRAQI PATIENTS

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ABSTRACT

Background: Urothelial carcinoma (UC) is one of the important causes of cancer related morbidity and mortality. It comprises approximately 90% of all primary cancer of bladder. Recently, new treatment options for patients with UC with associated biomarkers have been approved. Specifically, programmed death-ligand 1/programmed cell death protein 1 (PD-L1/PD-1) immunotherapies have been approved in patients with locally advanced or metastatic UC with PD-L1 expression. Programmed cell death ligand 1 (PD-L1) is a cell surface glycoprotein that belongs to the B7/CD28 co-stimulatory factor superfamily. It functions as an inhibitor of the immune response through promoting T-cell apoptosis by either binding to programmed cell death-1 (PD-1) receptor, or a putative non-PD-1 receptor on the surface of T lymphocytes. Similar to self-antigen recognition, cancer cell can escape immune surveillance by upregulating PD-L1. Moreover, the PD-1/PD-L1 signaling axis may induce immune inhibitory/exhaustion signaling of activated T cells, and thus significantly impair the anti-tumor immune response, Therefore, it is hypothesized that blockade of the PD-1/PD-L1 pathway may restore the native anti-tumor function of T cells and facilitate tumor regression. **The aim of this study:** Investigating the PDL1 immunohistochemical expression in Iraqi patients with invasive urothelial carcinoma and its correlation with the age, gender and lymphovascular invasion. **Materials and Methods:** This cross sectional study includes 50 bladder samples, collected between January 2022 and June 2024. All the cases was histopathologically evaluated and immunohistochemically stained for PDL 1. The samples of bladder (invasive urothelial carcinoma) were collected from Safeer al imam hussein Hospital, Immam-Hussein Medical City and private pathology laboratories in karbala. **Results:** In this study, 50 sample of invasive urothelial carcinoma were enrolled and all of them were of high grade, so these result may indicate that invasiveness associated with high grade and tumor cells PDL1 expression may be valuable for evaluation of tumor aggressiveness. Our result show male predominance (41 patient, 82% of the samples) and an age range from 42 to 94 years. The PDL1 was expressed in 60% of the cases, and 33 of cases show positive LVI 18 cases of them show positive PDL1 expression (54.5%). **Conclusion:** The high prevalence positivity of PDL1 irrespective of age and gender can aid and points to the possible use of immunotherapeutic agents in all sex and age groups.

KEYWORDS: Programmed cell death ligand 1 (PD-L1) is a cell surface glycoprotein that belongs to the B7/CD28 co-stimulatory factor superfamily.

CHAPTER ONE INTRODUCTION AND LITRERATUREREVIEW

INTRODUCTION

Urothelial carcinoma (UC) is one of the important causes of cancer related morbidity and mortality.[1] It is comprising approximately 90% of all primary cancer of bladder.[2] Its development seems to depend on a combination of genetic and environmental factors. The incidence is increased with exposure to cigarette smoke and aryl amines. Other environmental factors include aniline dyes auramines, phenacetin, and cyclophosphamide. Schistosoma haematobium is pathogenically related to both urothelial and squamous cell carcinoma of the bladder. It is believed that there is a slight increased risk for the development of bladder carcinomas in patients who had been treated with radiation therapy for prostatic carcinoma.[3]

According to Iraqi Cancer Registry 2022, Bladder cancer is the seventh most common cancer in Iraq. There were 1776 cases of Bladder cancer accounting for 4.5% of all cancer cases diagnosed in Iraq in 2022.[4]

According to The American Cancer Society, bladder cancer is the 4th most common cancer accounting for 7% of all cancer cases in males. It's the 8th most common cause of cancer death.[5]

The UC ranges from papillary to flat, non-invasive to invasive and low grade to high grade. Low-grade carcinomas are always papillary and are rarely invasive, but they may recur after removal.[6]

Tumor progression with increasing degrees of cellular atypia and anaplasia are associated with an increase in the size of the lesion with increasing tendency of invasion into deeper structures of the bladder wall. High-grade cancers are also papillary but occasionally flat; consist of fused, branching and delicate papillae with loss of polarity. Enlarge nuclear size with marked pleomorphism and hyperchromatic, multiple prominent nucleoli and atypical mitosis are prominent. They may cover larger areas of the mucosal surface and can invade deeper.[6]

Pathological stage is the most important factor that determines the prognosis and the mode of therapy of the UC.[7,8] The carcinoma without basement membrane invasion staged as pTa (non-invasive papillary urothelial carcinoma) and pTis (carcinoma in situ). While that with lamina propria invasion by the tumor is staged as pT1, and muscularis propria (MP) invasion is staged as pT2. Stage pT3 is given for peri-vesical soft tissue extension.[9]

Apart from depth of invasion, the histological grade of the tumor is considered the second most important predictive parameter for the biological behavior of urothelial carcinoma.[10] Tumor grade has also been recognized as an important prognostic indicator with regard to the potential for recurrence and progression.[11]

Programmed cell death ligand 1 (PD-L1) is a cell surface glycoprotein that belongs to the B7/CD28 co-stimulatory factor superfamily.[12] It functions as an inhibitor of the immune response through promoting T-cell apoptosis by either binding to programmed cell death-1 (PD-1) receptor, or a putative non-PD-1 receptor on the surface of T lymphocytes.[12] Similar to self-antigen recognition, cancer cell can escape immune surveillance by upregulating PD-L1. Moreover, the PD-1/PD-L1 signaling axis may induce immune inhibitory/exhaustion signaling of activated T cells, and thus significantly impair the anti-tumor immune response.[13] Therefore, it is hypothesized that blockade of the PD-1/PD-L1 pathway may restore the native anti-tumor function of T cells and facilitate tumor regression. immune checkpoint inhibitors that can block PD-L1 expression and then enhance T cell function in cancers have been brought identified.[13] A previous meta-analysis suggested that patients with urothelial carcinoma with higher ratios of PD-L1- positive cells responded significantly better to anti-PD-1/PD-L1 therapy than those with lower ratios of PD-L1-positive cells.[14]

AIM OF THE STUDY

In this study we aimed to investigate the PDL1 in Iraqi patients with invasive urothelial carcinoma and its correlation with the age, gender and lymphovascular invasion.

LITERATURE REVIEW

1.1. The anatomy and histology of urinary bladder

-Anatomy

Urinary bladder, an extra peritoneal muscular organ acts as urine reservoir, holding up to 400 to 500 mL of urine, situated anteriorly in the pelvis. The empty bladder looks like a three-sided pyramid. Dome of bladder is most anterosuperior point of bladder. Base of bladder lies posteriorly and inferiorly. Trigone is located at base of bladder and extends to bladder neck. Ureteral orifices are located at proximal and lateral aspects of trigone. Bladder neck is the most distal portion of bladder and opens into urethra.[15]

-Histology

The wall of the urinary bladder is formed by four layers from inside to outside[16]

- Lining epithelium.
- Lamina propria.
- Muscularis propria.
- Serosa /Adventitia.

Lining epithelium: The urinary bladder lining is a specialized stratified epithelium, the urothelium. The urothelium is exclusively in urinary structures such as the ureter, urinary bladder, and proximal urethra. The urothelium is composed of three layers.[16]

Lamina Propria: This is the sub urothelial layer separating the urothelium and underlying muscularis propria (detrusor muscle). It is separated from the overlying urothelium by a basement membrane. Its composition is an extracellular matrix with elastic fibers, capillaries, lymphatics, immune cells, afferent and efferent nerve endings, fibroblasts, myofibroblasts, adipocytes, interstitial cells of Cajal or telocytes, an indistinct smooth muscle layer, and the muscularis mucosae.[16]

Muscularis propria: Also known as the detrusor muscle, it consists of three layers: inner longitudinal, middle circular, and outer longitudinal. These layers are well defined around the neck of the urinary bladder; however, in the rest of the bladder wall, they run randomly, without orientation. The bladder's body has a higher smooth muscle content compared with the trigone, reflecting a well- developed network of myofibroblasts of lamina propria and muscularis mucosae.[16]

Serosa: This thin connective tissue layer covers the bladder dome and is continuous with the peritoneal layer of the abdominal wall. It also contains blood vessels of various sizes.[3]

Adventitia: This loose connective tissue layer serves as the bladder's outer layer in areas of the bladder where there is no serosa.[3]

1.2 Urothelial carcinoma

1.2.1 Incidence of UC

Urothelial carcinoma compromise approximately 90% of all primary tumors of the bladder.[3]

According to Iraqi Cancer Registry 2022 Bladder cancer is the seventh most common cancer in Iraq. There were 1776 cases of Bladder cancer accounting for 4.5% of all cancer cases diagnosed. Bladder cancer affected 1376 males accounting for (77.5%) of cases and 400 of female 22.5%. The five governorates with the highest ASR were Karbala at 17.8 /100,000 followed by Wasit at 15.5 /100,000.[4]

The American Cancer Society's estimates for bladder cancer in the United States for 2020 are about 81,400 new cases, 76% being in males and 24% in females. And about 17,980 deaths from bladder cancer, 72% being in males and 28% in females.[5]

In males, bladder cancer is the 4th most common cancer accounting for 7% of all cancer cases. It is the 8th most common cause of cancer death accounting for 4% of all cancer deaths. Its incidence and death rate are less common in females.[5]

1.2.2 Risk factors for urothelial carcinoma: Non-modifiable risk factors

-Genetic factors: Multiple epidemiological studies have examined the role of genetic factors as risk factors for the development of carcinoma of urinary bladder. Most of these studies identified a small increase in the risk among relatives of individuals with bladder cancer.[17]

-Gender: Men are 3-4 times more likely to develop bladder cancer than women.

-Age: The risk of bladder cancer increases with age.[18]

Modifiable risk factors

- smoking is the most common risk factor for bladder cancer, smoking is responsible for approximately two-thirds of bladder cancers in men and one-third in women. Smokers have a fourfold increased risk of bladder cancer compared with nonsmokers. The number of cigarettes smoked, length of years smoked, and age when smoking began all increase an individual's bladder cancer risk. Because the bladder's function is to store urine, there is ample time for carcinogens in the urine to affect the bladder. The carcinogens associated with smoking remain in constant contact within the genitourinary system until eliminated, thus the high rate for urothelial cancers.[19]
- Occupational exposure to aromatic amines and polycyclic aromatic hydrocarbons are other important risk factors.[20] Cyclophosphamide and ifosfamide (utilized to treat many solid tumors, hematological malignancies, and rheumatologic diseases) have been associated with an increased risk of UC of the bladder about fourfold to nine fold.[21] The impact of diet and environmental pollution is less evident.[20]
- Inflammation: Although the mechanism of action is unknown, patients with chronic urinary tracts infections (UTIs), chronic use of urinary catheters and bladder stones are at an increased risk for bladder cancer.[19] Schistosomiasis, a parasitic infection that can get into the bladder, is also a risk factor for bladder cancer. In countries where this parasite is common (mainly in Africa and the Middle East such as Egypt), squamous cell cancers of the bladder are seen much more often. The toxins of bacterial infections Escherichia Coli and pseudomonas is highly associated with bladder cancer.[22]
- Radiation: Patients treated with pelvic radiation for genitourinary and gynecologic cancers, such as prostate and cervical cancer, have a higher rate of bladder cancer; however, with advancements in radiation therapy, this number is expected to decrease.[19]

1.2.3. Diagnosis of urothelial carcinoma 1-Clinical features

Approximately 80-90% of patients with bladder cancer present with painless gross hematuria. Most patients with this classic presentation should be considered to have bladder cancer until proof to the contrary is found.[23] Hematuria is not the only manifestation of an underlying bladder cancer. Irritative bladder symptoms such as

dysuria, urgency, or frequency of urination occur in 2030% of patients with bladder cancer.[22]

2- Diagnostic tests

-Urine cytology: Voided urinary cytology is a useful non-invasive adjunct to cystoscopy because of its overall high specificity about (95 to 100 percent) but low sensitivity about (66 to 79 percent). It is also used to identify high grade tumors and monitor patients for persistent or recurrent disease following treatment.[24]

-Cystoscopy: Cystoscopy is currently considered the gold standard for the detection of primary tumors and for the follow-up of patients after transurethral resection of bladder tumors. There exists a variety of follow-up strategies; the most common has involved cystoscopy every 3 months for the first 2 years, followed by cystoscopy every 6 months for 2 to 3 years, and then cystoscopy annually.[25] Multiple biopsy specimens from various sites in the bladder can be obtained and papillary tumors can be totally resected during the same procedure.[26] Patients with abnormal findings during cystoscopy or bladder wash cytology should undergo transurethral resection of the bladder tumor (TURBT), which provides essential information for definitive diagnosis, staging, and grading and allows for removal of visible tumor and sampling of surrounding muscle to assess depth of tumor.[27]

-Imaging

1- **Ultrasonography:** Ultrasound is a commonly utilized imaging modality that is widely available and low-cost in relation to advanced imaging modalities such as MRI and PET/CT. Frequently, ultrasound may be the initial examination performed in patients with hematuria, as it is recommended as the initial diagnostic evaluation for microscopic hematuria by the American College of Radiology (ACR) Appropriateness Criteria.[28]

2- **Computed tomography (CT) scanning**: specifically CT urography (CTU), is the most commonly used imaging method worldwide to diagnose and stage urothelial malignancies, for the localization, locoregional staging, and detection of distant metastases.[28]

3- **MRI:** Bladder cancer is staged mainly with magnetic resonance imaging (MRI), because no radiation process is involved in MRI, it presents no danger to individuals who are required to avoid radiation, such as pregnant women or children, and it is easy to perform.[29]

-Immunohistochemical stains for UC

GATA3 can be used as a sensitive and specific marker for urothelial carcinoma can be effectively used to exclude other genitourinary malignancies, and renal cell carcinoma, at metastatic site.[30]

Uroplakin II demonstrates a significantly higher sensitivity than Uroplakin III in conventional and variants of UCs. Thus, UPII is more valuable marker than UPIII in immunohistochemical analysis for confirming the urothelial origin of carcinomas.[31]

1.2.4 Genetic alterations

Recent evidences strongly support the hypothesis that there is a difference between the molecular abnormalities in non-invasive or low grade UC and invasive/or high grade UC. Progression of bladder tumors is the result of accumulation of genetic alterations involving the clonal expansion of altered cells with growth advantages through sequential multi-step pathways. Although very similar at the morphological level, molecular studies showed that urothelial carcinomas present as a heterogeneous group of tumors that may evolve along either of two pathways with distinct biological behavior and clinical prognosis.[32]

The majority of tumors (70%) are papillary, low-grade and non-invasive. They are thought to arise from urothelial hyperplasia or papillomas and are often multifocal with high recurrence rate. These tumors infrequently progress to muscle invasion (10–15%). Fibroblast Growth Factor Receptor 3 (FGFR3) activation and phosphorylation activates HRAS, which induces the MAPK pathway through a kinase cascade, and the PI3K/AKT/mTOR pathway, leading to cell growth and proliferation.[32,33]

Pathways associated with high-grade and invasive urothelial carcinoma are involved with cell-cycle regulation. Normally, p53 is inactivated by being bound and sequestered by MDM2. The activation of p53 through DNA damage or cell stress activates expression of p21, halting cell-cycle progression. Activation of p53 can also lead to an apoptotic response. The Rb protein is normally bound to E2F, regulating the G1/S phase of the cell cycle. On phosphorylation by cyclin dependent kinases, Rb releases E2F, leading to cell-cycle progression. Upstream of both the Rb and p53 pathways is by the CDKN2A gene, which codes for the p16 and p14 tumor suppressor proteins. Mutations in any of these genes result in cell-cycle dysregulation and are associated with high-grade and invasive urothelial carcinoma.[33]

1.2.5. Microscopic morphology

Table 1: The WHO 2022 classification of tumors of the urothelial tract includes the following.[34]

Urothelial tumors
○ **Noninvasive urothelial tumors**
• Urothelial papilloma
• Inverted urothelial papilloma
○ Papillary urothelial neoplasm of low malignant potential
○ Noninvasive papillary urothelial carcinoma, low grade

○ Noninvasive papillary urothelial carcinoma, high grade ○ Urothelial carcinoma in situ • **Invasive urothelial neoplasms** ○ Invasive urothelial carcinoma
Squamous cell neoplasms of the urinary tract ○ Squamous papilloma of the urothelial tract • Squamous cell carcinomas of the urinary tract ○ Verrucous carcinoma of the bladder ○ Pure squamous carcinoma of the urothelial tract
Glandular neoplasms • Adenomas ○ Villous adenoma • Adenocarcinomas ○ Adenocarcinoma, NOS
Urachal and diverticular neoplasms ○ Urachal carcinoma ○ Diverticular carcinoma
Urethral neoplasms • Urethral accessory gland carcinomas ○ Littre gland adenocarcinoma of the urethra ○ Skene gland adenocarcinoma of the urethra ○ Cowper gland adenocarcinoma of the urethra
Tumors of the Müllerian type ○ Clear cell adenocarcinoma of the urinary tract ○ Endometrioid carcinoma of the urinary tract

The current 5th edition of WHO classification for bladder cancers is organized based on tumor lineage: urothelial, squamous and glandular tumors.[35]

- Exceptions for urachal, diverticular and urethral accessory gland tumors.
- Separate chapters for neuroendocrine neoplasms, mesenchymal tumors, hematolymphoid malignancies, melanocytic tumors and metastatic tumors.
- Genetic tumor syndromes of urinary and male genital tract are covered in dedicated chapter.
- Histologic subtypes are now preferred over variants.[35]
- Papillary tumors are deemed high grade if containing ≥ 5% high grade component; < 5% is noted as low grade with < 5% high grade component, prompted by poor interobserver reproducibility.[36]
- The descriptor inverted is reserved for papillary tumors with almost exclusively inverted architecture.
- Urothelial proliferation with undetermined malignant potential is no longer considered a distinct entity but rather an early low grade noninvasive papillary urothelial carcinoma or extension at the tumor edge / shoulder lesion.[36]
- Urothelial dysplasia no longer has a separate section; the term has been retained for preneoplastic lesions falling short of carcinoma in situ diagnosis.[36]
- Clear cell urothelial carcinoma was renamed clear cell urothelial carcinoma (glycogen rich) for clearer distinction from clear cell adenocarcinoma with Müllerian differentiation.[35]
- Signet ring / diffuse has been removed from plasmacytoid subtype terminology.[35]
- New edition advocates for pT1 tumor substaging via histoanatomical (tumor relative to muscularis mucosa and vascular plexus) or micrometric approach (measuring of invasive tumor component); however, it does not favor any specific methodology of subcategorization or tier system.[35]

Predictors of immune checkpoint inhibitor response: PDL1 expression in tumor and host immune cells, tumor mutation burden and microsatellite instability / mismatch repair defect status.[37]

1.2.5.1. Noninvasive urothelial lesions

1- Urothelial papilloma

- Rare urothelial neoplasm, comprises ~1% of all papillary bladder neoplasm
- Patients are generally younger than those with urothelial carcinoma.[38]

Microscopic (histologic) description

- Predominantly exophytic tumor
- Discrete papillary structures with central fibrovascular cores with hierarchical branching pattern but without fusion
- Papillary structures are lined by urothelium of normal thickness and cytology; often with prominent umbrella cells layer
- There should be no marked cytologic atypia, increased thickness of the urothelium or increased mitotic / apoptotic figures.[39]

2- Inverted urothelial papilloma

Inverted urothelial papilloma is a benign urothelial neoplasm with an endophytic growth of complex and interconnecting trabeculae**.** Endophytic proliferation of normal thickness urothelium forming anastomosing cords, islands and trabeculae.[40]

Histologically Sharply circumscribed, endophytic proliferation of thin and complex anastomosing cords, islands and trabeculae of cytologically bland urothelial cells with virtually no nuclear atypia.[40] invaginating trabeculae composed of 5 – 10 layers of urothelial cells with central streaming and peripheral palisading cells, embedded in lamina propria. Mild degenerative cytologic atypia may be seen, Mitotic figures are absent or extremely rare.

Surface is lined by the normal urothelium with no or minimal exophytic papillary component. Urothelial cells with vacuolation and foamy xanthomatous cytoplasmic changes may be seen.[41]

3- Papillary urothelial neoplasm of low malignant potential (PUNLMP)

- Neoplastic proliferation of the urothelium in a papillary configuration, with no invasion through the basement membrane. Thickened urothelium or increased cellularity, without marked cytological atypia.
- Noninvasive papillary urothelial neoplasm with exophytic or endophytic (inverted) configuration; a cut off of > 80% is proposed by the Genitourinary Pathology Society to designate a urothelial neoplasm of inverted type.
- Epithelial lining of fibrovascular cores is thicker than normal urothelium: urothelial cells show monotonous appearance and slight cytoplasmic and nuclear enlargement.
- No variation in nuclear size, shape or chromatin pattern.
- Preserved polarity of urothelial cells, Mitoses are rare and basally located.
- No hyperchromatic nuclei in urothelial cells in intermediate layers of neoplastic epithelium.[42]

4- Noninvasive papillary urothelial carcinoma, low grade

Neoplastic proliferation of the urothelium in a papillary configuration, with no invasion through the basement membrane.

Histologically Neoplastic urothelium lining fibrovascular cores long, slender papillae with minimal fusing or branching

- Orderly architecture at low magnification, some loss of polarity and mild pleomorphism at medium magnification
- Cells generally uniform in size, may have slight variation but no significant nuclear pleomorphism or nucleomegaly, occasional slight irregularities in nuclear contour
- Mitoses may be present but not atypical and usually confined to lower half of urothelium
- Inverted growth pattern (exophytic and endophytic components) may be present.[43]

5- Noninvasive papillary urothelial carcinoma, high grade

Neoplastic proliferation of the urothelium with a papillary configuration and no invasion beyond the basement membrane.

Histologically Fibrovascular cores lined by neoplastic urothelium, Complex, solid to fused papillae common. Architectural disorder; nuclear pleomorphism readily visible at low and intermediate power

- Crowded overlapping cells, dyscohesion common and partial denudation
- Nucleomegaly present, irregular and clumped chromatin
- Frequent prominent nucleoli and mitoses (brisk and maybe atypical)
- Concomitant low grade carcinoma may be present
- Inverted growth pattern may coexist (both exophytic and endophytic growth).[44]

6- Urothelial carcinoma in situ

Urothelial carcinoma in situ (CIS) is a high grade, flat, noninvasive urothelial neoplasm involving full or partial thickness of the urothelium.[45]

Microscopic (histologic) description

- Cytological features

- Nuclear enlargement (often 5 - 6 times the size of a lymphocyte), with or without pleomorphism.
- May have prominent nucleoli, irregular nuclear contour or uneven chromatin distribution.
- Frequent mitotic figures seen in the mid to upper layers of the urothelium; atypical figures may be present.[46]

- Architectural features.

- Loss of cellular polarity (urothelial cells lose their perpendicular orientation to the basement membrane).
- Nuclear crowding with or without nuclear overlapping.
- Irregular thickness characterized by hyperplasia, attenuation or denudation.

1.2.5.2. Invasive urothelial carcinoma

Urothelial carcinoma that has penetrated the basement membrane and invaded into the lamina propria or deeper.

- Histologic characterization and depth of invasion are the most important factors for determining prognosis.[47]
- Urothelial carcinoma is morphologically heterogenous with many variants and subtypes.[48]
- Invasive urothelial carcinoma involving the lamina

propria (T1) is often treated with conservative intravesical therapy and mucosal resection.
- Invasive urothelial carcinoma involving the muscularis propria (T2) is often treated with radical cystectomy.[49]

Microscopic (histologic) description
- Neoplastic cells arranged in irregular nests or single cells invading the lamina propria and muscularis propria.
- Retraction artifact is often seen and can mimic vascular invasion.
- High grade nuclear features: nuclear pleomorphism, hyperchromasia, high N/C ratio with frequent mitotic figures.[46]

1.2.5.3. Variants of infiltrating urothelial carcinoma

1. Infiltrating urothelial carcinoma with divergent differentiation

Tumors in which some percentage of ‘‘usual type’’ urothelial carcinoma is present along with other morphologies. Common morphologic manifestations of divergent differentiation appear along squamous, glandular, small cell, and trophoblastic lines. Squamous differentiation indicated by presence of keratinization and intercellular bridges occur in 40% of cases. Glandular neoplasms constitute the second most common form of divergent differentiation, seen in up to 18% of invasive tumors and defined by presence of small tubular or gland-like spaces. This variant most commonly associated with high-grade and locally advanced disease.[50]

2. Nested variant of urothelial carcinomas

This is very rare but aggressive variant. By definition these tumors cannot be high grade or have overlying surface carcinoma in situ. Irregular and confluent small nests and abortive tubules are composed of urothelial cells infiltrating the lamina propria or muscularis propria, usually without surface involvement. The tumour cells usually exhibit mild atypia (mild pleomorphism, slightly increased nuclear/cytoplasmic ratios, occasional prominent nucleoli, and rare mitotic figures) and resemble cystitis glandularis and cystitis cystica. Deep tumour-stroma interface is jagged and infiltrative. Often more atypia and focal anaplasia with increasing depth of invasion are one of the features. Typical urothelial is often present. Retraction artefact may be seen.[51]

3. Microcystic urothelial carcinomas

Microcystic variant has an incidence around 1% of bladder tumours and it is histologically composed of varying sizes cysts with intraluminal secretions, usually lined by transitional cells or low columnar cells with mucinous differentiation. It is deeply infiltrative, with early invasion of muscularis propria and usually associated with high-grade urothelial carcinoma.[52]

4. Micropapillary urothelial carcinomas

It is typically characterized by rather confluent “back-to-back” retraction spaces and multiple epithelial aggregates/nests in a single retraction space,[53] Most are invasive into the muscularis propria at diagnosis, and lymph node metastases are common.

Also, non-invasive carcinomas with micropapillary excrescences should not be designated as “micropapillary.”[3]

5. Sarcomatoid urothelial carcinomas (also called carcinosarcomas)

Tumors with sarcomatous and carcinomatous components. Sarcomatous component has spindled, round and pleomorphic giant cells showing any sarcomatous differentiation. Carcinomatous component may be urothelial carcinoma, adenocarcinoma, squamous cell carcinoma or small cell carcinoma. This aggressive carcinoma has often present with advanced stage at diagnosis.[54]

6. Lymphoepithelioma-like urothelial carcinomas

Lymphoepithelioma-like carcinoma (LELC) is a rare high-grade carcinoma that resembles nasopharyngeal lymphoepithelioma. First reported in 1991, bladder LELC has an incidence of about 1% of all bladder carcinomas.

Unlike other sites of the body, LELC in the bladder has not been associated with the presence of Epstein - Barr virus to date. The LEL variant of urothelial carcinoma is diagnosed by the presence of high grade/poorly differentiated tumor cells admixed with a prominent inflammatory cell infiltrate. The tumor cells have high nuclear: cytoplasmic ratios and indistinct cytoplasmic borders imparting a syncytium-like appearance.[55]

7- Plasmacytoid carcinoma

Morphologically similar to lobular breast cancer and diffuse-type gastric cancer. With discohesive monomorphic round neoplastic cells. They often have homogeneous eosinophilic cytoplasm and an eccentrically placed nucleus, and intracytoplasmic vacuoles are also common, and this could make diagnosis challenge with metastatic signet ring adenocarcinoma of stomach.[56]

8. Giant cell urothelial carcinoma

is a rare aggressive variant of urothelial carcinoma, commonly occur in older males. It is characterized by the presence of highly pleomorphic bizarre tumour giant cells arranged in diffuse sheets and solid nests, hyperchromatic nuclei, frequent mitoses and abundant amphophilic or eosinophilic cytoplasm.[57]

9. Clear cell urothelial carcinoma

is a rare variant of urothelial carcinoma (UC), this variant is associated with rapid progression to muscle invasion and metastasis, with an aggressive clinical course.[58] Microscopically seen as Flat or cuboidal neoplastic cells having abundant clear or eosinophilic

cytoplasm with glycogen and frequent hob nailing and a large nucleus, forming papillary or tubulocystic structures.[59]

10. Lipid cell variant of urothelial carcinomas

is a rare variant of urinary bladder cancer, comprised of lipoblast-like cells, forming irregular solid nests and sheets. Lipoblast-like neoplastic cells that had eccentric nuclei and cytoplasmic vacuoles were observed, not only in the resected specimen, but also in urine samples. On mucin histochemistry, the tumor cell cytoplasm contained no neutral or acidic mucus. The lipoblast-like cells were positive for cytokeratins (AE1/AE3, CK7) and adipophilin, known as a protein associated with neutral lipid synthesis.[60]

1.2.6. Staging of urothelial neoplasms

Pathological stage is the gold standard for diagnostic and prognostic purposes. Initial bladder cancer stage is based on histological evaluation of endoscopic specimens, differentiating involvement of the lamina propria (T1) from non-invasive papillary carcinoma (Ta) and invasion of the muscularis propria (≥T2). It is well known that T1-BC is upstaged to muscle invasive in 27– 51% patients at radical cystectomy. Possible explanations for staging errors based on transurethral resection (TUR) specimens include thermal artifacts, tangential sectioning, and absence of muscularis propria in specimens with tumor invasion, desmoplastic reactions and the presence of nested cancer variants.[61]

Table 2: TNM staging system according to the AJCC 8th edition.[62]

Primary tumour (T)	
TX	Primary tumour cannot be assessed.
T0	No evidence of primary tumour.
Ta	Non-invasive papillary carcinoma.
Tis	Carcinoma in situ: 'flat tumour'.
T1	Tumour invades lamina propria.
T2	Tumour invades muscularis propria. pT2a Tumour invades superficial muscularis propria (inner half). pT2b Tumour invades deep muscularis propria (outer half).
T3	Tumour invades perivesical tissue. pT3a Microscopically. pT3b Macroscopically (extravesical mass).
T4	Tumour invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall. T4a Tumour invades prostatic stroma,seminal vesicles, uterus, vagina. T4b Tumour invades pelvic wall, abdominal wall.
Regional lymph nodes (N)	
NX	Lymph nodes cannot be assessed.
N0	No regional lymph node metastasis.
N1	Single regional lymph node metastasis in the true pelvis (hypogastric, obturator, external iliac or presacral lymph node).
N2	Multiple regional lymph node metastasis in the true pelvis (perivesical, obturator, internal and external iliac, or sacral lymph node).
N3	Lymph node metastasis to the common iliac lymph Nodes.
Distant metastasis (M)	
M0	No distant metastasis.
M1	Distant metastasis. M1a Distant metastasis limited to lymph nodes beyond common iliac. M1b Non–lymph node distant metastases.

Table 3: AJCC prognostic stage groups of urothelial carcinoma of urinary bladder.[62]

Stage	T	L.N	M
0is	Tis	N0	M0
I	T1	N0	M0
II	T2a, T2b	N0	M0
IIIA	T3a, T3b, T4a Or T1,T2,T3,T4	N0 N1	M0 M0
IIIB	T1,T2,T3,T4a	N2,N3	M0
IVA	**T4b Any N Any T**	**M0 Or Any N**	**M1a**
IVB	**Any T**	**Any N**	**M1b**

1.2.7 Management of urothelial carcinoma

According to stage, grade, location of the tumor and the general health of the patients, the treatment for bladder cancer includes the followings

- **Surgery**

- Transurethral resection TURBT: is used for diagnosing bladder cancer and for treatment of non-muscle-invasive bladder cancer, may allow the preservation of a functional bladder.[63]
- Partial, or segmental, cystectomy: In highly selected patients with invasive bladder cancer, partial cystectomy offers acceptable outcomes. Selection criteria should include solitary tumors without concomitant carcinoma in situ (CIS) and amenable to resection with 1–2 cm margins in a normally functioning bladder.[64]
- Radical cystectomy: radical cystectomy (RC) with pelvic lymph node dissection is the standard treatment for muscle-invasive urothelial carcinoma of the bladder and is recommended for patients with non-muscle-invasive urothelial carcinoma (NMIUC) with high risk of progression.[65]

- **Immunotherapy:** Bacillus Calmette-Guérin (BCG) is one of the most successful immunotherapies in cancer treatment and remains the gold standard of care for patients with high risk, non-muscle invasive bladder cancer, with initial response rates of approximately 70%.. Recently there has been significant interest in novel immunotherapeutic agents in the management of cases where bacillus Calmette-Guérin fails, as well as cases of more advanced cancer. These investigational therapies can generally be classified into several broad categories, including recombinant bacillus Calmette-Guérin and cell wall derived therapies, cytokines, gene therapy, cancer vaccines, immune checkpoint inhibitors, oncolytic viruses, adoptive immunotherapies and immune agonists, as well as several additional immunomodulatory agents.[66]

- **Chemotherapy**

Intravesical or systemic chemotherapy may be used to treat bladder cancer. Mitomycin is the drug most often used in intravesical chemotherapy.[67]

Radiation therapy: External beam radiation therapy is most often used to treat bladder cancer in combination with chemotherapy or as palliative therapy.[68]

1.2.8 Prognosis of urothelial carcinoma

Many parameters have been used to predict outcome in patients with urothelial carcinoma, including:

1. Stage: The most important prognostic factor. Muscle invasion associated with decrease in survival. The 5-year survival rate for people with stage 0, stage I, stage II, stage III, and stage IV bladder cancer are about 98%, 88%, 63%, 46% and 15%respectively. Tumor size generally determine depth of invasion, risk of metastasis and probability of survival. Lymph node involvement is an ominous prognostic sign. The number, the size and extracapsular extension have impact on the prognosis.[69]
2. Microscopic grade: The grade has independent prognostic value. High-grade bladder cancers are deeply invasive and have a greater risk of disease progression and recurrence and a less favorable prognosis.[70]
3. Recurrence rate and time to recurrence: Bladder cancer has the highest recurrence rate. About 70% of people with bladder cancer will have a recurrence. Time to recurrence is an important prognostic factor. Tumors that recur within the first 2 years after diagnosis and successful treatment are more aggressive and have a higher chance of progression.[71]
4. Patient`s age: Bladder tumors occurring in young age patients are usually well differentiated and non-invasive and have an excellent prognosis.[72]
5. Gender: severity of urothelial carcinoma has shown that women are more likely to present advanced tumors and have worse prognosis than men at almost every stage of the disease.[73]
6. Location and number of tumors: Bladder neck tumors are significantly associated with higher frequency of muscle invasion, lymphovascular invasion, local and distant metastasis at the time of diagnosis and hence with advanced tumor stage and poor prognosis.[74] Also multifocality of urothelial carcinoma associated with higher recurrence and progression rates.[75]
7. Lymphovascular invasion: Assessment and reporting of LVI (lymphovascular invasion) in specimens from transurethral resection of the bladder tumour (TURBT) or biopsy in patients with non muscle-invasive bladder cancer or muscle-invasive bladder cancer might enable improved staging, prognostication and clinical decision-making. In NMIUC, presence of LVI in TURBT and biopsy samples seems to be associated with under staging and increased risks of disease recurrence and progression. In MIUC, presence of LVI is associated with features of aggressive disease and predicts recurrence and survival.[76]
8. Tumor histeotype: Urothelial carcinoma with divergent (squamous and glandular) differentiation followed a more aggressive course biologically, significantly higher risk for disease recurrence and mortality compared to patients with pure urothelial carcinoma. Clear cell and lymphoepithelioma like variants probably have prognosis similar to usual urothelial carcinoma. While other variants (nested, micropapillary, micro cystic, sarcomatoid, plasmacytoid) were found to be associated with worse prognosis compared to pure urothelial carcinoma and with squamous differentiation.[77]
9. DNA ploidy: An independent prognostic factor in bladder carcinomas. A high degree of correlation between DNA ploidy, microscopic grade, and local

and distant spread has been observed. Aneuploidy status was correlated with higher tumor T stage and grade.[78]

10. Proliferation index: The presence of a high mitotic count or a high S phase fraction and number of Ki-67 positive cells immunohistochemically, correlate with higher grade, aggressive clinical stage and larger tumor size.[79]
11. Chromosomal aberrations: Clonal karyotypic abnormalities in bladder cancer associated with shorter survival rates. Various karyotypic aberrations have been found to correlate with biological behavior (invasion or recurrence potential). Low-grade bladder tumors exhibit few chromosomal abnormalities, commonly involving deletions of chromosome 9q. While, high-grade bladder tumors tend to have variable chromosomal gains and losses. Aneuploidy of chromosomes 7, 9, and 17 is associated with aggressive tumors.[80]
12. P53 and Rb genes: Alterations in the p53 gene and its pathways are usually implicated in muscle invasive bladder cancer (T2-T4) and advanced stages, and are associated with augmented angiogenesis, invasiveness, metastasis, recurrence, and consequent poor prognosis.[81]
13. Loss of P27 and cyclin E: The loss of these two cell cycle regulators correlates with increased histologic aggressiveness and decreased patient survival. Also low expression (P21), an inhibitor of cyclin dependent kinase CDK, predicated tumor recurrence and poor prognosis in bladder cancer.[82]
14. Loss of E-cadherin: If E-cadherin expression is found to be negative or weak, close clinical follow-up of patients is necessary, even if the initial diagnosis noninvasive urothelial carcinoma of the bladder. This is because the rate of invasion in these cases is higher in recurrent cases.[83]
15. CD44 and MUC1: Both CD44 (a cell surface adhesion molecule) and MUC1 (which is restricted to the apical membranes of umbrella cells in a normal urothelium), are independent prognostic factors. Their overexpression was associated with tumor progression and the clinically aggressive behavior.[84]
16. Nonspecific lab values like C-reactive protein (CRP), and anemia was independently associated with a higher risk of cancer specific mortality.[85] Preoperative high albumin is coordinated with a high overall survival in patients with bladder cancer.[86]

1.3 Programmed death ligand 1 (PD-L1)

Programmed death ligand 1 (PD-L1), also known as B7-H1 or CD274, is the first functionally characterized ligand of the coinhibitory programmed death receptor 1 (PD-1). Together with its cognate ligand PD-L2, PD-L1 plays a key role in maintaining peripheral and central immune cell tolerance through binding to the PD-1 receptor.[87]

1.3.1 Structure

PD-L1 is a 290 amino acids type I transmembrane protein encoded by the CD274 gene on chromosome 9[12] at position p24.1.[88] First described in 1999 by Dong et al as the 3rd member of the B7 protein family, showing a 15%–20% homology with B7.1 & B7.2 proteins.[89] The full length of PD-L1 is encoded within seven exons, corresponding to a 40 kDa protein and consists of IgV-like and IgC-like extracellular domains, a hydrophobic transmembrane domain and a short cytoplasmic tail formed by 30 amino acids, with unclear signal transduction properties.[89,90]

1.3.2 Expression of PD-L1

PD-L1 expression is either constitutive or inducible. Constitutive, low level PD-L1 expression can be found, on resting lymphocytes, antigen presenting cells (APCs) and in corneal, syncytiotrophoblastic and Langerhans' islet cells of pncreas where it involved in tissue homeostasis in pro-inflammatory responses.[87] PD-L1 confers an 'immune privileged' status to certain tissues like testis, the anterior chamber of the eye and placenta, where inoculation of exogenous antigens is tolerated without induction of an inflammatory/immune response.[91] In the Context of inflammation and/or infection, PD-L1 is induced as a suppressive signal on hematopoietic, epithelial & endothelial cells.[12] PD-L1 expression is primarily regulated by toll-like receptor (TLRs), a subtype of non-catalytic receptors, which is highly expressed in APCs and activated by pathogen-associated molecular patterns. TLR-mediated regulation relies on the activation of the MEK/ERK kinases, which enhance PD-L1 messenger RNA (mRNA) transcription via nuclear factor kappa B. Interferon-γ (IFN-γ) receptors 1 and 2 are also implicated in regulating PD-L1 expression, largely through Jak/STAT-mediated activation of IRF-1. Interferon-mediated activation of Jak/STAT can also up-regulate PD-L1 expression through the MEK/ERK and the phosphatidyl-inositol 3 kinase PI3K/AKT pathway, which through phosphorylation of mammalian target of rapamycin, offers a permissive role on PD-L1 transcription [(92)]. Other regulators include: TNF-α, IL-17, IL-10, IL-4, TNF-β and others.[93] In carcinogenesis, other regulatory mechanisms take place including MYC, HIF1α, EGFR, RAS and others.[93] In lung cancer for instance, epidermal growth factor receptor (EGFR) mutations, correlate positively with PD-L1 expression, with EGFR inhibitors acting as repressors of PD L1 transcription.[94] In T cell lymphoma, the nucleophosmin (NPM)/anaplastic lymphoma kinase (ALK) fusion gene up- regulates PD L1 expression via constitutive STAT3 activation.[95]

1.3.3 PD-L1/PD-1 activation and signal transduction

The biological functions of PD-L1 depend on binding with PD-1 (CD279), a 288 amino acid long type 1 transmembrane receptor encoded by the PDCD1 gene which is physiologically expressed on lymphocytes and

myeloid cells. PD-1 is composed of an extracellular IgV-like domain & a transmembrane region. Its intracellular tail is composed of tyrosine based switch motif (ITSM) and immune receptor tyrosine based inhibitory motif sequences.[96] On ligation with PD-L1, recruitment of Src homology 2 domain which contain phosphatases 1 and 2 (SHP 1/SHP-2) to the ITSM causes dephosphorylation of signalling kinases such as CD3ζ, PKCθ and ZAP70 resulting in a global inhibitory action of T cell expansion.[12,97] Such inhibitory response is due to inactivation of the PI3K-Akt and Ras- MEK-ERK cascades.[95] Dephosphorylation of Casein kinase 2 (CK-2) which is 2 is a target of SHP-2 leads to unrestrained activation of PTEN, a physiological PI3K-Akt signalling antagonist.[98] Mostly the inhibitory effect of PD-1 on the Ras-MEK ERK cascade depends on direct inhibition of Ras and dephosphorylation of phospholipase Cγ.[99,100]

1.3.4 Functions of PD-L1

A) Central and peripheral tolerance

The PD-1/PD-L1 pathway is crucial for the development of immune tolerance, a process of negative selection of auto-reactive lymphocytes taking place in primary (central tolerance) and secondary lymphoid organs (peripheral tolerance).[101] the fact that is supported by high PD-L1 expression within the thymus[102] and on dendritic cells, where the PD L1/PD-1 interaction prevents the proliferation and differentiation of naïve T cells.[103] Knock-out PD-1/PD-L1 leads to autoimmunity in animal models with dilated cardiomyopathies, lupus-like diseases, and type 1 diabetes mellitus.[104] In humans, immune-related toxicity is a recognized class effect of anti-PD-1/PD-L1 antibodies, where Type 1 diabetes, encephalomyelitis, inflammatory bowel diseases, Rheumatoid Arthritis, autoimmune hepatitis and others are common complications.[105]

B) Immune exhaustion

Immune exhaustion, that is, the progressive impairment of effector T cell function following persistent antigen presentation, is a physiological mechanism that prevents tissue destruction in chronic infection.[106,107] A cardinal feature of T cell exhaustion includes the induction of various co inhibitory pathways including PD-1/PD-L1.[113] HIV-specific CD4/CD8 cells co-express PD-1(108) and a similar role for PD-1/PD-L1 has been found in viral hepatitis.[109] and tuberculosis,[110] where impairment of effector T cell function is induced through apoptosis, inhibition of T cell replication and maturation[111] as well as parallel induction of regulatory T cells.[112]

C) Downregulation of anticancer immune response

Persistent up-regulation of PD-1 is commonly found in tumor infiltrating lymphocytes, where PD-L1 expression is exploited by malignant cells to avoid immune destruction.[113] Interestingly, PD-1 activation by PD-L1 up-regulates Slug, Snail and Twist through the MAPK/ERK pathway suggesting the presence of a link between tumor invasiveness and anti-tumor immune control.[114] Regulation of programmed cell death ligands by hypoxia-inducible factor-1α implying an interplay with neo-angiogenesis; representing an independent hallmark of cancer progression.[115,116]

1.3.5 The Role of Immune System in Bladder Carcinoma

In solid tumors, including bladder cancer, the process of oncogenesis generally leads to genetic instability which results in the production of tumor-specific neoantigens that allow the immune response to target malignant cells.[117] Bladder carcinoma is characterized by a higher mutational burden compared to other tumors, and this heterogeneity could be due to the presence of different cancer stem cells; consequently, their clones lead to a mixture of signature and discordance between global mRNA profiling and immunohistochemical features that is crucial not only for tumor cells, but also for immune responses.[118] In fact, the immune microenvironment has a central role in neoplastic processes, and the ability of the immune system to recognize and eliminate transformed cells early in the tumorigenic process is called “tumor immunosurveillance”. Immunogenicity in bladder carcinoma differs among different histological subtypes and affects both innate and adaptive immunity.[118] The immune cells involved are represented by both B and T lymphocytes (CD4, CD8, and Th1), and dendritic cells (DCs) which are resident in the bladder, and by neutrophils, macrophages, mast cells, and NK cells which are recruited from the bloodstream. An effective anti-tumor immune response will be the result of a concerted effort of antigen-presenting cells (APCs) (DCs, macrophages), lymphocytes, NK cells, and the other abovementioned immune effectors.[119,120]

1.3.6 Mechanisms of Immune System Evasion in Bladder Carcinoma

To survive and escape from the normal immune response, tumor cells secrete various immunosuppressive and anti-apoptotic factors, such as TGF-beta, PGE2, IL-10, and IL-6, creating a highly tolerogenic microenvironment.[121,122] In addition, the tumor microenvironment is tightly linked to the accumulation of several types of immune cells with immunosuppressive phenotypes, such as myeloid-derived suppressor cells (MDSCs), tolerogenic DCs (tDCs), tumor- associated macrophages (TAMs), and regulatory T cells (T-regs).[121,122] A highly immunosuppressive microenvironment has been described in bladder carcinoma, in which the PD-L1/PD-1 axis may play a crucial role in neoplastic immune escape.[123,124] Firstly, PD-1 was identified in 1992 as a protein involved in apoptosis, and subsequently, its role in modulating the hyper-stimulated immune system was highlighted.[125] Particularly in inflammatory conditions, PD-L1 is expressed in immune response cells, including activated T and B lymphocytes, macrophages, DCs, APCs and some epithelial cells.[125,126] Furthermore, tumor cells produce PD- L1 as an “adaptive immune mechanism” to evade anti-tumor responses; this event

involves a suppressive signal to T lymphocytes, which in turn causes the immune response to diminish after interacting with either the PD-1 or B7.1 (CD80) receptors.[126] However, another important player in the regulation of T-cell activity is represented by CTLA-4, a crucial immune checkpoint receptor. In detail, CTLA-4 has regulatory effects on T-lymphocyte activation and it is expressed on regulatory T cells and also upregulated on conventional T cells after activation.[127]

Additionally, cytokines and other immunosuppressive factors secreted by tumor- recruited myeloid cells play multifaceted roles in the mechanisms of regulation of PD-L1 expression.[128]

Therefore, to efficiently escape the normal immune response, bladder cancer produces and secretes different cytokines with both pro- and anti-inflammatory roles. The tumor microenvironment is characterized by the presence of several immune cells with an immunosuppressive function, such as TAMs, MDSCs, and T-regs. Moreover, tumors secrete PD-L1, which interacts with the PD-1 receptor expressed by CD8+ T cells and APCs, resulting in an effective immune escape.[128]

1.3.7 PD-L1 Expression in Bladder Carcinoma

The assessment of PD-L1 expression in bladder carcinoma has been considered as an important part of understanding the tumor immune milieu and predicting therapeutic response.[129] In fact, PD-L1 expression in bladder carcinoma has shown a prognostic value[130] and an association with a higher pathologic stage has been demonstrated.[131] Specifically, it has been shown that high-grade bladder carcinomas express higher PD-L1 and PD-1 levels compared to low-grade ones, and PD-L1 might function as a mediator of stage progression.[132] However, the best application of PD-L1 evaluation in bladder carcinoma is in relation to its possible therapeutic implications. In fact, recent advances in anticancer treatments have led to the development of therapies that effectively restore the immune response against cancer cells, the so-called “immune checkpoint inhibitors” (ICIs).[132] One of the best examples is represented by PD-1/PD-L1 inhibitors, that bind to PD-1 or PD-L1 to avoid their interaction, thus reactivating the functionality of T-lymphocytes and preventing the immunological escape of tumor cells.[133] In this regard, the evaluation of PD-L1 expression in bladder carcinoma is relevant for determining the eligibility of patients for treatment with PD-1/PD-L1 inhibitors.[134] In addition, such drugs represent an emerging treatment option for bladder carcinoma, particularly in advanced stages, and the evaluation of PD-L1 expression is performed using immunohistochemical assays.[117]

1.3.8. Scoring system of PDL1 in urothelial carcinoma

Specifically, there are several PD-L1 assays and immunohistochemical scoring systems (Table 4) performed to obtain appropriate treatment decisions for PD- 1/PD-L1 targeted immunotherapy. In particular, the most commonly used scoring system in bladder carcinoma is called the combined positive score (CPS) and it is calculated by dividing the number of PD-L1 stained cells (tumor cells, lymphocytes, and macrophages) by the total number of viable tumor cells, multiplied by 100.[117] Other scoring systems, although less utilized in bladder carcinoma, are the tumor proportion score (TPS) and immune cell (IC) score, which are individually able to measure the percentage of tumor cells showing PD- L1 expression and the area occupied by PD-L1-positive immune cells (lymphocytes, dendritic cells, macrophages, and granulocytes) as a percentage of the whole tumor area.[135] Of note, different cut-off values for PD-L1 status determination have been adopted (Table 4).[122]

Table 4: Different PD-L1 scoring systems commonly used in bladder carcinoma.[122]

Scoring System	Description	Platform Used	Positivity Criteria
CPS	Ratio of PD-L1 stained cells (tumor cells, lymphocytes, macrophages) to total viable tumor cells, multiplied by 100	Dako Agilent 22C3 platform	CPS > 10
TPS	Percentage of tumor cells showing PD-L1 expression	Various platforms	TPS cut-off varies
IC Score	Area occupied by PD-L1-positive immune cells (lymphocytes, dendritic	Ventana PD- L1 SP142 platform	IC score of 2/3
Scoring System	**Description**	**Platform Used**	**Positivity Criteria**
cells, macrophages, granulocytes) as a percentage of whole tumor area			

CHAPTER TWO. PATIENTS AND METHODS

2.1 Sampling method

This cross sectional retrospective study included 50 bladder samples, collected between January 2022 and June 2024.

All the cases were histopathologically evaluated and immunohistochemically stained for PDL 1.

The samples of bladder (urothelial carcinoma) were collected from Safeer al imam hussein Hospital, Immam-Hussain Medical City and other private pathology laboratories in karbala.

- **Inclusion criteria**

• Paraffin embedded samples from Iraqi Patients with invasive high grade urothelial carcinoma that have

already been diagnosed in the period between January 2022 and June 2024.
- Only membranous positivity was included.

- **Exclusion criteria**
- All biopsies with technical errors in processing, staining & storage
- Low grade urothelial carcinoma
- Ureter and pelvic urothelial carcinoma
- Cases with no clinical data

2.2 Selection of the cases
2.2.1 Study groups
Cross sectional study with fifty cases of invasive papillary urothelial carcinoma of the urinary bladder (T2), all cases were high grade. The cases included 50 patients with invasive urothelial carcinoma of the bladder by TURBT (transurethral resection of bladder tumor).

All samples were fixed with formalin, then paraffin embedded and histopathologically re-assessed by a 2 histopathology consultants to establish the diagnosis, grade and the presence or absence of muscle invasion.

2.2.2 Control group
- Positive control: placental tissue or lymphoma cases were dependent as Immunohistochemistry control group as recommended by marker manufacturer.

-Negative control: negative control was earned by omitting the primary antibody.

2.3 Equipments and Materials
2.3.1 Equipments
- Oven Memmert.
- Water bath Memmert, type WNE 22.
- Microtome Leica RM 2235.
- Positively charged slides bio SB.
- Slide glass cover slip
- Timer
- Microtome
- Glass Jars.
- Pipettes Appendorf.
- Pipettes' tips.
- Microwave Samsung.
- Slide box

2.3.2 Materials
Table 5: Chemicals.

Material	Type
Xylene	Analar (England)
Ethanol (absolute)	Merck (Germany)
Distilled water	
Rinse buffer	TBS (dako cytomation)
Target retrieval solution	Citrate (bio SB,USA)
Primary antibody	PDL1 (Clone:CD274) Rabbit Monoclonal Antibody (bio SB, USA)
Hematoxylin	Counter stain (Dako Cytomation)
Mounting media	Dako Cytomation Faramount
Secondary detection system	Bio SB Detection system

2.4 Immunohistochemical Method
1. We cutting and mount 2-5-micron formalin-fixed paraffin-embedded tissues on positively charged slides such as Bio SB Hydrophilic Plus Slides (BSB 7028).
2. drying in air for 2 hours at 58° C.
3. Deparaffinize, dehydrate and rehydrate the tissues.
4. Subjecting the tissues to heat induced epitope retrieval (HIER) using a suitable retrieval solution such as ImmunoDNA Retriever with a Citrate (BSB 0020-BSB 0023) or EDTA (BSB 0030- BSB 0033).
5. the heating method we used was: TintoRetriever PT Module Place tissues/slides in a pre-warmed staining coplin jar containing the ImmunoDNA Retriever with Citrate or EDTA at 95°-99° C. Incubate for 30-60 minutes.
6. After the heating treatment, transfer the slides in ImmunoDNA Retriever with Citrate or EDTA to room temperature and let it stand for 15-20 minutes.
7. For the manual staining, perform antibody incubation at ambient temperature, and for the automated staining methods, perform antibody incubation according to instrument manufacturer's instructions.
8. Washing the slides with ImmunoDNA washeror DI water.
9. We continue IHC staining protocol and wash the slides between each step with ImmunoDNA washer solution.

2.5 Scoring system
The criteria for positive immunohistochemical reaction of PDL1 is of membranous as indicated by the manufacturer literature. Anti-PD-L1 immunostaining was only evaluated in field ×20 on tumor cells.

A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

PD-L1 expression in urothelial carcinoma is determined by using Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. Although the result of the calculation can exceed 100, the maximum score is defined as CPS 100. CPS is defined accordingly:

$$CPS = \frac{\text{\# PD-L1 staining cells (tumor cells, lymphocytes, macrophages)}}{\text{Total \# viable tumor cells}} \times 100$$

Urothelial carcinoma tissue specimens that are tested for PDL1 expression are scored and divided into two groups based on their Combined Positive Score (CPS):
– CPS < 10 negative
–CPS ≥ 10 positive

2.6 Statistical Analysis

Statistical Analysis was performed by using the (SPSS) program of version 26.

The significant relation was done by using two sided Chi square test and Fisher's Exact test, which have been used to calculate the relation between different clinicopathological parameters and PDL1 reactivity.

Statistically, when a P value ≤ 0.05 it was considered to be statistically significant.

CHAPTERTHREE. RESULTS

3.1 Distribution of invasive Urothelial Carcinoma according to clinicopathological parameters

The cases included 50 patients with invasive urothelial carcinoma of the bladder by TURBT (transurethral resection of bladder tumor) all of them were high grade tumor with muscularis properia invasion [T2], with male predominance as they formed 82% of the sample (41 patients) and an age range from 42 to 94 years.

Table 6: Distribution of invasive urothelial carcinoma according to clinicopathological parameters.

Clinicopathological parameters		Invasive urothelial carcinoma	
		number	Percentage %
Age	≤ 65 year	16	32
	>65 year	34	68
Gender	male	41	82
	female	9	18
Lymphovascular invasion	present	33	66
	absent	17	34
Grade	high	50	100

3.2 Immunohistochemical results of PDL1 staining

According to the using of Combined Positive Score (CPS) to evaluate the membranous expression of PDL1 in invasive urothelial carcinoma, The programmed death-ligand 1(PDL1) testing showed that the majority of patients (60%) were positive (figure 1).

Table 7: Immuno-histochemical expression of PDL1 in invasive urothelial carcinoma.

PDL 1	Frequency	Percentage
Negative	20	40%
Positive	30	60%
Total	50	100%

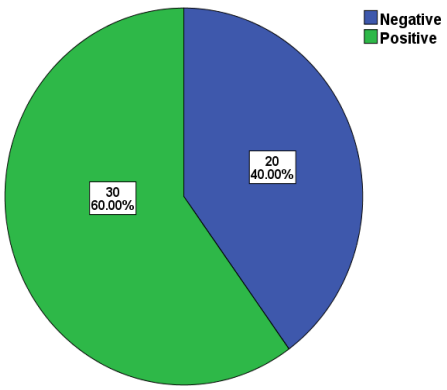

Figure 1: PDL1 distribution in invasive urothelial carcinoma of the urinary bladder patients.

3.3 Association of PDL1 expression with age group

The mean age of the patients was 68.36±10.93 year.

When the patients were classified into two age groups, the age distribution showed that PDL1 expression is more staining in age group >65year (18 cases, 52.94%).

However, no significant association was discovered for age distribution with expression of PDL1 (P value =0.137, table 8).

Table 8: Association of PDL1 immunohistochemical stain with age group in invasive urothelial carcinoma.

Age group	PDL1_expression		Total	P value
	negative	positive		
≤ 65 year	4 (25%)	12 (75%)	16	**P=0.137** (> 0.05)
>65	16 (47.05%)	18 (52.94%)	34	
Total	20 (40%)	30 (60%)	50	

3.4 Association of PDL1 expression with patient gender

PDL1 expressed in 24 out of 41 cases of invasive urothelial carcinoma in male gender, while it expressed in 6 out of 9 cases in female gender. No significant association was discovered for gender distribution of PDL 1 in this sample of patients (p value =0.724, table 9).

Table 9: Association of PDL1 immunohistochemical stain with gender group in invasive urothelial carcinoma.

Gender	PDL1_expression		Total	P value
	negative	positive		
F	3 (33.3%)	6 (66.7%)	9	**P=0.724** (> 0.05)
M	17 (41.4%)	24 (58.6%)	41	
Total	20 (40.0%)	30 (60.0%)	50	

3.5 Association of PDL1 expression with lymphovascular invasion

Thirty three of cases show positive lymphovascular invasion.

PDL1 expressed in 18 cases (54.5%) out of 33, and it was not expressed in 15 cases (45.5%) out of 33 in cases whose show presence of lymphovascular invasion.

No significant association was discovered for lymphovascular invasion distribution of PDL 1 in this sample of patients (p value =0.273, table 10).

Table 10: The lymphovacular invasion distribution of PDL1 in invasive urothelial carcinoma of the urinary bladder patients.

Lymphovascular invasion	Frequency & percentage	PDL1 expression		Total	P value
		negative	positive		
Absent	Count	5	12	17	
	% within	29.4%	70.6%	100.0%	
	Lymphovascular invasion				**p=0.273**
Present	Count	15	18	33	(>0.05)
	% within	45.5%	54.5%	100.0%	
	Lymphovascular				
	invasion				
Total	Count	20	30	50	
	% within	40.0%	60.0%	100.0%	
	Lymphovascular				
	invasion				

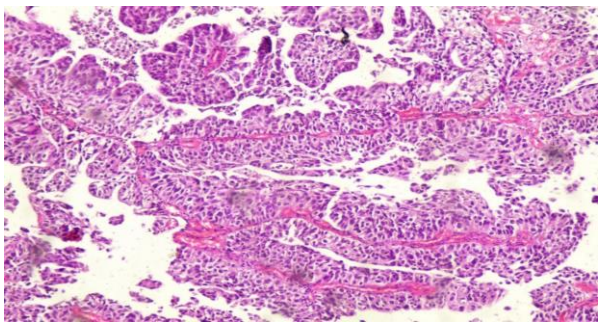

Figure 2. High grade invasive urothelial carcinoma H&E(x10).

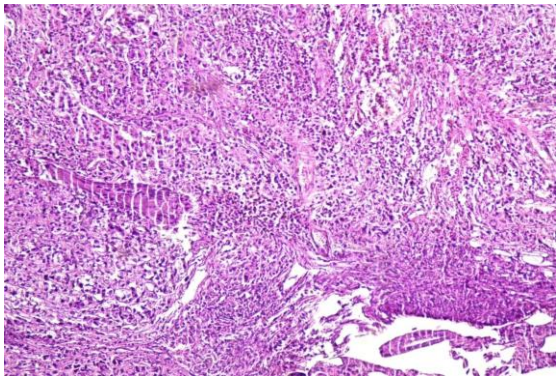

Figure 3. Muscularis properia invasion by tumor cells in invasive urothelial carcinoma, high grade, H&E(x40).

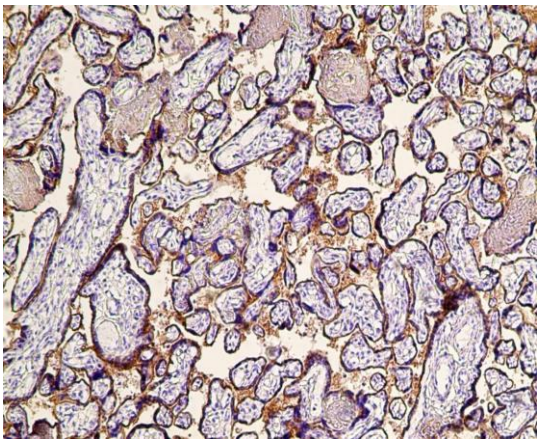

Figure 4: The membranous staining of PDL1 in placental tissue (control)(x10).

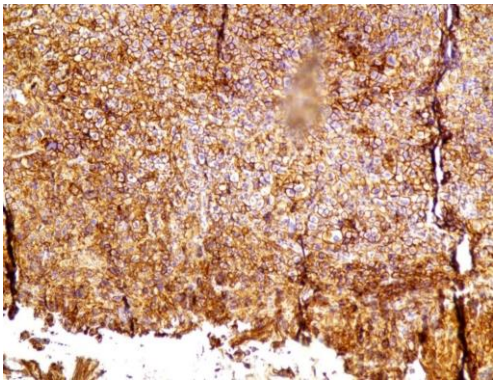

Figure 5. complete membranous staining of PDL1(CPS>10%, intensity 3) in tumor cells of invasive urothelial carcinoma (x10).

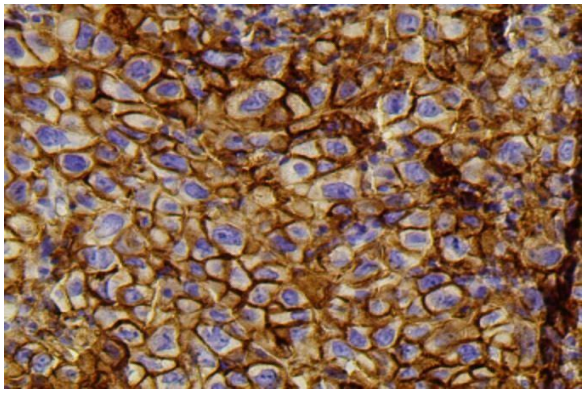

Figure 6. complete membranous staining of PDL1(CPS>10%, intensity 3) in tumor cells of invasive urothelial carcinoma (x40).

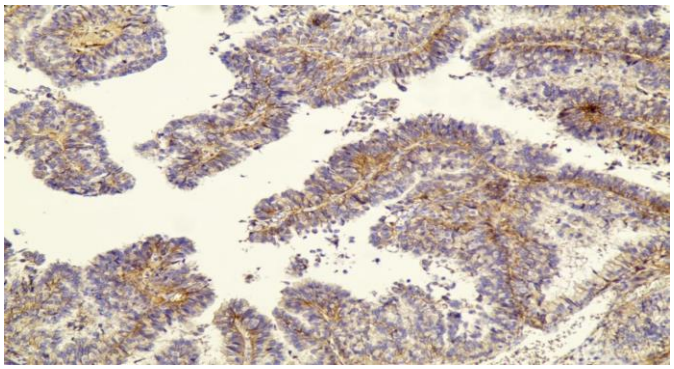

Figure 7. Papillary urothelial carcinoma, high grade showing the membranous staining of PDL1(CPS>10%, intensity 3) in tumor cells (x10).

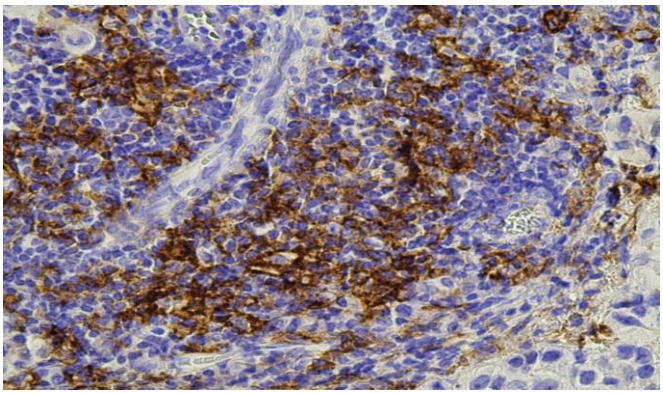

Figure 8. invasive urothelial carcinoma, high grade showing PDL1 staining of immune cells inside the tumor cell (x40).

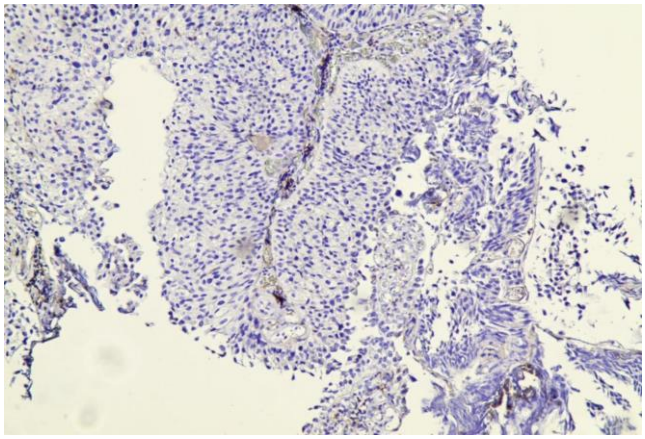

Figure 9: Invasive urothelial carcinoma, high grade showing negative PDL1 staining (x10).

CHAPTERFOUR. DISCUSSION

The PD-L1, a member of the B7 family, has the ability to regulate T cell functions through engagement with PD-1, and is expressed on dendritic cells (immature, mature, and follicular) and on many types of cancer cells.[136]

PD-L1 inhibitors are currently approved for treatment of locally advanced or metastatic urothelial carcinoma of the bladder and the upper urinary tract. Since anti-PD-L1 IHC is only indicated in patients with locally advanced or metastatic UC, it is common sense to select a tissue sample with at least invasive UC.[137]

Our study of PDL1 IHC included 50 samples of invasive urothelial carcinoma (T2) in Iraqi patients, the expression showed that the majority of patients (60%) were positive (30 cases out of 50) and negative in (40%) (20 cases out of 50) as shown in table 6. These results are similar to **Utpal Kumar (2021)**[138] study that found PDL1 expression in tumor cells was seen in 62% of cases, and it showed a high expression in high grade, muscle invasive and also lamina invasive tumors. **Nehal A. Heabah (2021)**[139] study results show positive PDL1 expression was detected in 51.7% of all cases, PDL1 expression was significantly associated with histological types, high tumor grade and muscle invasion. A research done in Egypt by **Ahmed Abd El-Moeze, Ismail Moustafa (2021)**[140] pointed that 30% of studied cases were positive for PDL1 expression, and it was detected among advanced pathological stage (pT).

These variations may be attributed to the sample size and demographics of the population in each study, and to the difference in the method of staining.

All of the cases were high grade urothelial carcinoma so these results may indicate that invasiveness associated with high grade and tumor cells PDL1 expression may be valuable for evaluation of tumor aggressiveness, and that PD- L1 status may be used to select patients more likely to respond to anti-PD-1/PD-L1 treatment. As **Yide Huang (2015)**[132] **Takashi Kawahara (2018)**[141] found that PDL1 was associated with more aggressive clinicopathological parameters of bladder cancer and more highly expressed in high grade bladder cancer than in low grade bladder cancer. also **Xiangli Ding (2019)**[142] found that PDL1 expression was associated with muscle invasive disease in patients with bladder cancer (with more aggressive clinical features) but a significant better response to PD-1\ PD-L1 targeted treatment.

The results of our study did not show any significant association between the expression of PDL1 in invasive urothelial carcinoma and the age of the patients, a ($p > 0.05$), The results also did not show any significant association between the expression of PDL1 in invasive urothelial carcinoma and the gender of the patients, a p value= 0.724 with a male predominance as they formed 82% of the cases, The disparity between genders is proposed to be the result of a differences in exposure to carcinogens (i.e., tobacco and chemicals) as well as due to difference in genetic, anatomic, hormonal, societal, and environmental factors.[73] As similar to **Bradley C Holland (2019)**[143] and **Utpal Kumar (2021)**[138] studies they also found that differences in age or gender did not show any significant association and difference in the expression of PDL1.

Thirty three of cases that show positive lymphovascular invasion, PDL1 was expressed in (54.5%), as to **Luca Campedel (2023)**[144] found that Patients who expressed PD-L1 or PD-1 were more likely to have lymphovascular invasion (52.4%) as compared to those who did not.

CONCLUSION

1. The high prevalence positivity of PDL1 irrespective of age and gender can aid and points to the possible use of immunotherapeutic agents in all sex and age groups.

RECOMMENDATIONS

1. Further studies of cases with a larger sample size, comparison between different stages and grades of tumor.
2. More detailed history about the Iraqi patients like smoking, occupation, previous radiation or treated with BCG immunotherapy.
3. Studying the PDL 1 expression in the invasive

urothelial carcinoma by other genetic techniques and making comparative studies with the immunohistochemical protocol.

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