FACULDADE DE MEDICINA
UNIVERSIDADE D
COIMBRA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

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PD-L1 expression and tumour-infiltrating lymphocytes as Biomarkers in Urothelial Carcinomas

ARTIGO CIENTÍFICO

ÁREA CIENTÍFICA DE ANATOMIA PATOLÓGICA

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04/2021

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Abbreviations

BCG	Bacillus Calmette-Guérin
Basal/SCC-like	Basal/Squamous Cell Carcinoma-like
CD	Cluster of Differentiation
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CDT	Companion Diagnostic Test
CHUC	Centro Hospitalar Universitário de Coimbra
CK20	Cytokeratin 20
CK5/6	Cytokeratin 5/6
CPS	Combined Positive Score
d-MMR	Mismatch Repair Deficiency
DSS	Disease Specific Survival
EMT	Epithelial-Mesenchymal Transition
FDA	Food and Drug Administration
Gata3	Gata 3 Protein
GU	Genomic Unstable
H&E	Haematoxylin-Eosin
HPF	High Power Field
IC	Immune Cell Score
ICI	Immune Checkpoint Inhibitors
IFNγ	Interferon Gamma
IHC	Immunohistochemistry

LDT	Laboratory Developed Test
LumNS	Luminal Nonspecified
LumP	Luminal Papillary
LumU	Luminal Unstable
Mes-like	Mesenchymal-Infiltrated-like
MIBC	Muscle Invasive Bladder Cancer
MSI-H	Microsatellite Instability-High
NMIBC	Non Muscle Invasive Bladder Cancer
OS	Overall Survival
PD-1	Programmed Cell Death 1
PD-L1	Programmed Death Ligand 1
PD-L2	Programmed Death Ligand 2
SAP	Serviço de Anatomia Patológica
Sc/NE-like	Small Cell/Neuroendocrine-like
TCGA	The Cancer Genome Atlas
TILs	Tumour Infiltrating Lymphocytes
TMA	Tissue Microarray
TMB	Tumour Mutation Burden
UC	Urothelial Carcinoma
Uro	Urothelial-like

Abstract

Introduction

In the last years, urothelial carcinomas (UC) have been tested to novel immune checkpoint inhibitors (ICI).

Programmed death ligand-1 (PD-L1) is a ICI, which has been associated with an increase of survival rate and can be evaluated by immunohistochemistry (IHC).

Three commercial Kits (Ventana SP142, Ventana SP263, DAKO 22C3), which use different technical platforms, protocols and evaluation scores, are available. However, several studies show different agreement between tests.

Currently, several Pathology laboratories use laboratory developed test (LDT) optimized internally, instead of the Companion Diagnostic Test (CDT).

The main goals of this study is to evaluate the IHC expression of PD-L1 in UC by comparing SP 142, SP263 and 22C3 clones using the CDT and LDT protocols, and also compare PD-L1, CD8 and p16 expressions.

Materials and Methods

Forty-three samples of high grade UC diagnosed in 2019 and 2020 were selected from the archive of the Serviço de Anatomia Patológica (SAP) of Centro Hospitalar Universitário de Coimbra (CHUC) and tissue microarray (TMA) were constructed. Immunostaining was performed with anti-human antibodies CD44, Gata3, Cytokeratin 20 (CK20) and Cytokeratin 5/6 (CK5/6) to categorize tumours as Luminal or Basal type; also PD-L1 clones (SP142, SP263 and 22C3 using CDT method and 22C3 LDT method). The information collected was organized in a database and subjected to statistical analysis using the SPSS.

Results

Female patients have higher PD-L1 22C3 ($p=0.051$) and SP263 ($p=0.06$) positives compared with male. Tumour infiltrating lymphocytes (TILs) are greater also in female patients when using 20 lymphocytes / High Power Field (HPF) cutoff (median expression). TILs positive cases are lower in basal IHC subtype carcinomas ($p=0.045, p<0.05$) when considering the cutoff of 50 lymphocytes / HPF and have a tendency to be higher in histologic grade 2 tumours ($p=0.0814$). Positive correlations between PD-L1 22C3 with CD8 expression (cutoff 50) ($p=0.024, p<0.05$) and PD-L1 SP263 with CD8 (cutoff 50) ($p=0.002, p<0.05$) were found. Almost perfect concordance between PD-L1 22C3 and PD-L1 SP263 (k=0.86) was observed.

Conclusion

High concordance between LDT and CDT enhances the use of PD-L1 LDT in daily routine diagnose. Our study suggests that TILs could be a potential predictive biomarker in combination with different PD-L1 clones, preferentially with SP142 and 22C3.

Keywords

Urothelial Cancer; PD-L1 assays; CD8; TIL; Biomarkers; Molecular Subtypes.

Resumo

Introdução

Nos últimos anos, os carcinomas uroteliais (UC) têm sido testados para novos inibidores de checkpoint imunológico (ICI).

O ligante de morte programada-1 (PD-L1) é um ICI, que tem sido associado a um aumento da taxa de sobrevivência podendo ser avaliado por imunohistoquímica (IHC).

Estão disponíveis três kits comerciais (Ventana SP142, Ventana SP263, DAKO 22C3), que usam diferentes plataformas técnicas, protocolos e métodos de avaliação. No entanto, vários estudos mostram diferente concordância entre testes.

Atualmente, vários laboratórios de Anatomia Patológica utilizam o teste desenvolvido em laboratório (LDT) otimizado internamente, ao invés do teste de diagnóstico complementar (CDT).

Os principais objetivos deste estudo são avaliar a expressão IHC de PD-L1 em UC comparando os clones SP142 e SP263 usando os protocolos CDT e 22C3 usando os protocolos LDT, bem como comparar as expressões de PD-L1, CD8 e p16.

Materiais e Métodos

Foram selecionadas 43 amostras de UC de alto grau diagnosticados em 2019 e 2020 no arquivo do Serviço de Anatomia Patológica (SAP) do Centro Hospitalar Universitário de Coimbra (CHUC) e construídos *microarrays* de tecidos (TMA).

A imunomarcação foi realizada com anticorpos anti-humanos CD44, Gata3, Citoqueratina 20 (CK20) e Citoqueratina 5/6 (CK5/6) para classificar os tumores como do tipo Luminal ou Basal; também clones PD-L1 (SP142, SP263 e 22C3 utilizando o método CDT e o método 22C3 LDT). As informações obtidas foram organizadas num banco de dados e submetidas à análise estatística utilizando o SPSS.

Resultados

Doentes do sexo feminino apresentam maior número de casos PD-L1 22C3 ($p=0.051$) e SP263 ($p=0.06$) positivos em comparação com os homens. Os linfócitos intratumorais (TILs) são mais elevados em pacientes do sexo feminino, quando se usa o ponto de corte de 20 linfócitos / campo de grande ampliação (HPF) (expressão mediana). Os casos positivos para TILs são inferiores nos carcinomas do subtipo basal ($p=0.045, p<0.05$) quando considerado o ponto de corte de 50 linfócitos / HPF e tendem a ser superiores nos tumores de grau histológico 2 ($p=0.0814$). Foram encontradas correlações positivas entre PD-L1 22C3 com expressão de CD8 (ponto de corte 50) ($p=0.024, p<0.05$) e PD-L1 SP263 com CD8 (ponto de corte 50) ($p=0.002, p<0.05$). Foi observada uma concordância quase perfeita entre PD-L1 22C3 e PD-L1 SP263 ($k=0.86$).

Conclusão

A alta concordância entre os protocolos LDT e CDT permite usar o PD-L1 LDT no diagnóstico de rotina diária. O nosso estudo sugere que os TILs podem ser um potencial biomarcador preditivo em combinação com os diferentes clones PD-L1, preferencialmente com o SP142 e o 22C3.

Palavras-Chave

Carcinoma Urotelial; Ensaios PD-L1; CD8; TIL; Biomarcadores; Subtipos Moleculares.

Introduction

Urothelial Carcinoma (UC) correspond to more than 90% of malignant bladder cancer constituting the 6th most common cancer in men and the 9th leading cause of cancer related-death [1]. Bladder cancer represents the 10th most diagnosed cancer worldwide [1]. They are generally classified as papillary and infiltrative UC [2, 3], but they can be clinically categorized as Non-Muscle Invasive Bladder Carcinoma (NMIBC), Muscle Invasive Bladder Carcinoma (MIBC) and Metastatic Carcinoma [4].

Treatment options to enhance survival and quality of life include surgery, radiotherapy, chemotherapy and targeted therapy including immunotherapy, namely Immune Checkpoint Inhibitors (ICI), one of the most recent strategies to fight cancer in the context of personalized medicine [4-6].

The Programmed Death Ligand 1 (PD-L1) signalling pathway is an important target and recent cancer research developments lead to the discovery of several ICI. However, the history of immunotherapy in the field of UC started with the use of Bacillus Calmette-Guérin (BCG) intravesical immunotherapy for NMIBC, approved by the Food and Drug Administration (FDA) in 1990 [7]. Still today it is the *gold standard* in the treatment of NMIBC, with response rates of up to 70% [5].

In recent years, with the revolution in immunotherapy, monoclonal antibodies have emerged, especially targeting the interaction of PD1/PD-L1. There are currently 5 drugs approved by the FDA and available on the market (atezolizumab, nivolumab, durvalumab, pembrolizumab and avelumab) [4, 8].

By disrupting the tumour immunity cycle processes, tumours can avoid being signalled by the immune system and limit the extent of immune destruction. Many tumours do so by expressing the inhibitory ligand PD-L1. Under normal conditions, the PD-L1 signalling pathway can help maintain immune homeostasis. In cancer, the PD-L1 pathway can protect tumours from cytotoxic T cells [9].

Recent studies have been suggesting that the expression of PD-L1 may be one of the main immunosuppressive drivers in several types of cancer [10]. Blocking PD-L1 signalling can result in activity replacement and anti-cancer intensification of T cells [11].

The evaluation of PD-L1 by Immunohistochemistry (IHC) is mandatory for the selection of patients for first-line immunotherapy based on PD-L1 ICI [4, 12-15]. PD-L1 expression has been detected in tumour cells as well as in Tumor Infiltrating Lymphocytes (TILs) [9, 11].

Different clones of anti-PD-L1 antibodies are available, developed and optimized for different drugs, using different technical platforms, protocols and evaluation scores. The evaluation of agreement between different tests is essential [12, 16-19]. Several studies have identified varying levels of agreement between validated and approved tests [12, 16-19].

Some Pathology Labs use tests developed and optimized internally, instead of the Companion Diagnostic Test (CDT) used in the therapeutic trials that led to the approval of the drug [18]. Furthermore, PD-L1 expression can be influenced by several factors [20], such as tumour microenvironment, especially immune infiltration [21]. Also, we know that the most frequent genetic alterations in UC are cyclin-dependent kinase inhibitor 2A (CDKN2A, 34%) that encodes p16, FGFR3 (21%), phosphatidyl-inositol 3-Kinase catalytic subunit alpha (PIK3CA, 20%) and HER2neu (17%) [22].

Herein, we selected 43 UC from 2019 and 2020 to evaluate the IHC expression of different PD-L1 clones: SP142 (CDT), SP263 (CDT) and 22C3 using the Laboratory Developed Test (LDT). Our main goal is to compare differences in intensity, type and percentage of PD-L1 expression in luminal and basal tumours subtypes. PD-L1 clones expression, TILs (cutoff of 20/HPF and 50/HPF) and p16 were measured to be correlated. Finally, we also evaluated the technical quality of each IHC assay.

Materials and Methods

Characteristics of the study cohort

Forty-three samples of different grades of UC diagnosed in 2019 and 2020 were selected from the archives of the Serviço de Anatomia Patológica (SAP) of Centro Hospitalar Universitário de Coimbra (CHUC).

The study was approved by the Ethics Committee of CHUC (number: UID.CEC.OBS.SF.08 / 2021).

Briefly, this study included 34 males and 9 females, with a male:female ratio of 4:1. Mean and median age at presentation was 73 and 74 years respectively (range, 47 to 95 y). UC cases correspond to high grade infiltrative carcinomas in different grades:

thirty-six (84%) tumours were at grade 2, five (12%) at grade 3, one (2%) ate grade 4. Only 1 UC case was an invasive squamous cell carcinoma. UC were at different stages: 5 in stage I (11.6%); 20 in stage II (46.5%), 9 in stage III (20.9%), 9 in stage IV (20.9%). The clinical and pathologic features of the study cohort are presented in **Table 1**.

Table 1. Clinicopathologic Features for cases of UC of the Bladder

	UC (N=43)
Age (Range)	47-95
Mean	73
Median	74
Gender	
Male	34 (79%)
Female	9 (21%)
Pathologic Diagnose	
Invasive UC High Grade (G2)	36 (84%)
Invasive UC High Grade (G3)	5 (12%)
Invasive UC High Grade (G4)	1 (2%)
Squamous Cell Carcinoma	1 (2%)
Molecular Subtype	
Luminal	21 (48.8%)
Basal	20 (46.5%)
Pathologic Stage	
1	5 (12%)
2	20 (47%)
3	9 (21%)
4	9 (21%)

Samples and tissue microarray

Tissue sections with 4 µm thick were cut and stained with haematoxylin-eosin (H&E) for morphologic and diagnostic evaluation. Representative samples of the tumours were selected by a pathologist using an optical microscope, and tissue microarray (TMA) was constructed with a core needle biopsy with a diameter of 3 mm from a donor paraffin block of 12 different tissues, obtained from the tumour samples using a dedicated tissue array instrument, Lab Vision™ TMA builder (Thermo Fisher Scientific, Massachusetts, USA). Tissue cores were arrayed into host paraffin wax blocks, creating arrays of 4 × 3 dots. To combine donor cores with the recipient block, the paraffin wax was reheated for five minutes at 70°C.

Luminal and basal subtype IHC

Immunostaining was performed to anti-human antibodies CD44, Gata3, Cytokeratin 20 (CK20) and Cytokeratin 5/6 (CK5/6) to categorize tumours as luminal (CK20 and or Gata3 positive) or as basal (CK5/6 and/or CD44 positive) (**Figure 1**).

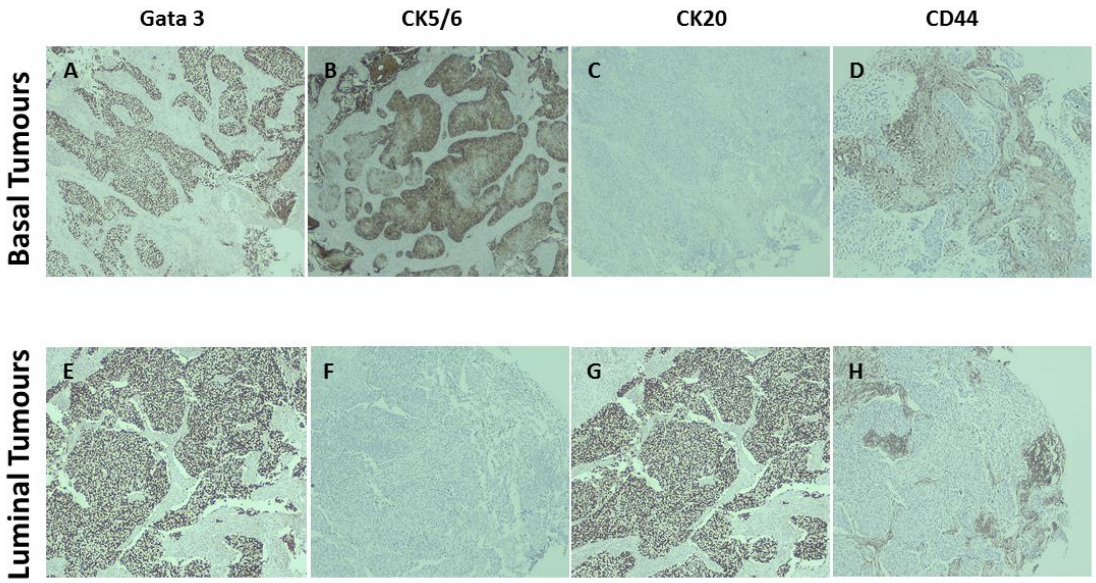

Figure 1. Basal and luminal urothelial invasive carcinoma cases defined by immunohistochemistry. Basal type UC characterized by nuclear intense Gata3 expression, Gata3 100x (A); intense membrane and cytoplasm CK5/6 expression, CK5/6 100x (B); without CK20 expression, CK20 100x (C); and with CD44 expression, CD44 100x (D). Luminal type UC with Gata3 expression, Gata3 100x (E); negativity for CK5/6, CK5/6 100x; intense and diffuse CK20 expression, CK20 100x (G); and without CD44 expression, CD44 100x (H).

CD8 and p16 IHC

Immunostaining was performed to anti-human antibodies CD8 (Leica, NewCastle, United Kingdom) and p16 (Ventana Medical Systems, Tucson, USA) (**Figure 2**).

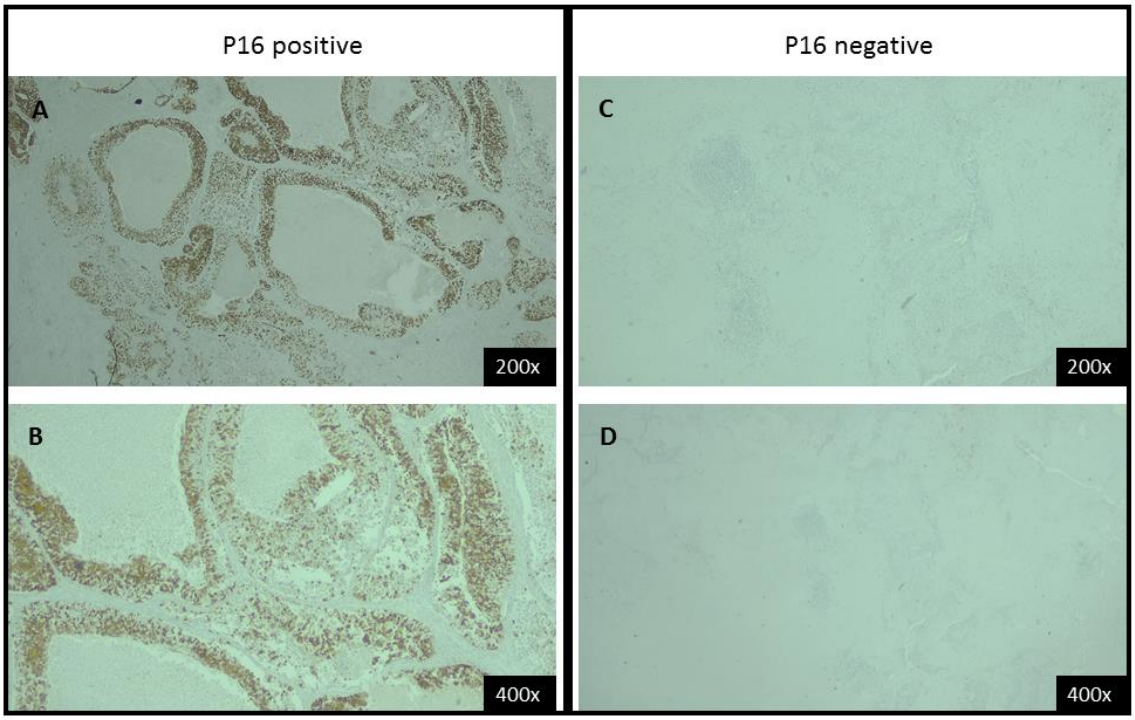

Figure 2. p16 intense nuclear positivity, p16 200x (A); and 400x (B); p16 negative case, p16 200x (C); and 400x (D).

PD-L1 IHC

IHC was performed also to PD-L1 clones: SP142, SP263 and 22C3 using CDT methods and LDT (for 22C3 clone) (**Figure 3**).

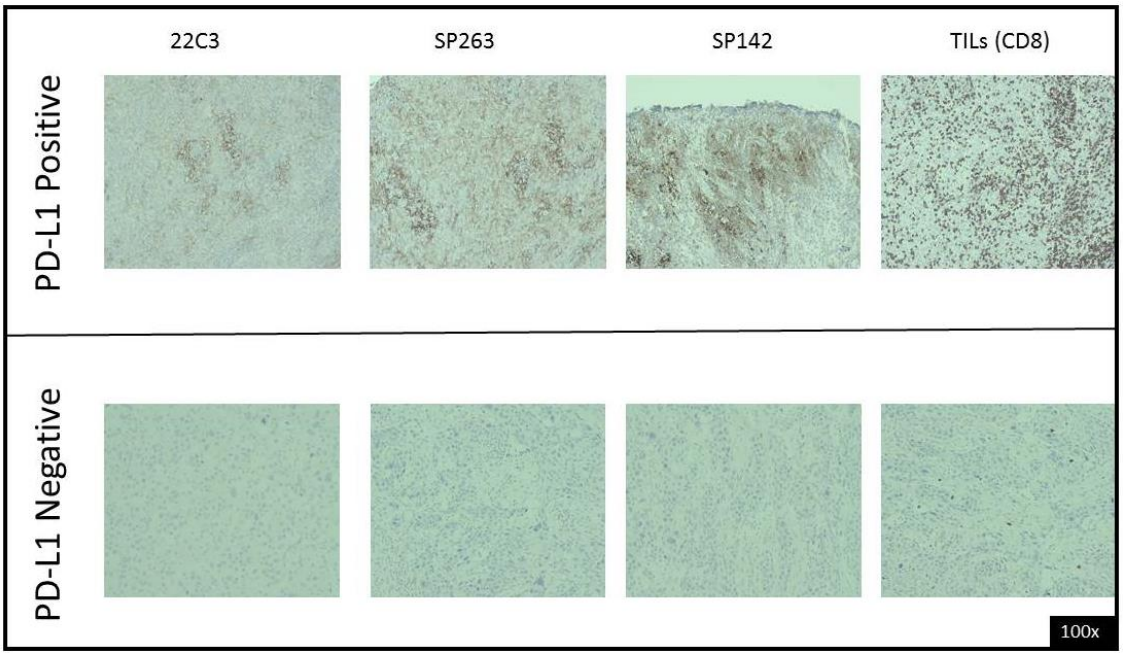

Figure 3. PD-L1 22C3, SP263, SP142 positive case with 150 CD8+ Lymphocytes by HPF (upper 4 images); PD-L1 22C3, SP263, SP142 negative case with 10 CD8+ Lymphocytes by HPF (lower 4 images).

For PD-L1 evaluation at least 6 tissue sections 4 µm thick were cut and mounted on to TOMO® adhesive coated slides (Matsunami; Bellingham, USA) each one with an on-slide tissue from tonsil as a positive and negative control, processed in the same manner as the tumour samples, and stored in a dry environment until use.

Six consecutive tissue serial sections were used to compare PD-L1 protein expression from clones SP142 and SP263 as CD test to PD-L1 clone 22C3 as LDT. All the slides were stained using the platform Benchmark Ultra automated stainer (Ventana Medical Systems, Tucson, Arizona) (**Table 2**).

Two slides, were immunostained to evaluate protein expression, from antibody anti-human PD-L1 for SP142 and SP263 clones (ready-to-use; Ventana Medical Systems, Tucson, Arizona, USA) (**Table 2**). One slide to evaluate the antigenic expression and the other as a negative control.

The two last slides were immunostained as a LDT with the antibody anti-human PD-L1, clone 22C3 (1:50; Dako, Glostrup, Denmark). One slide to evaluate the antigenic expression and the other as a negative control.

Antigen retrieval was performed with CC1, pH8 EDTA/Tris-based buffer (Ventana Medical Systems, Tucson, Arizona, USA) followed by primary antibody incubation according to manufacturer procedures.

Detection of immunostaining was performed with an indirect multimer based revelation system conjugated with Horseradish Peroxidase (HRP) (OptiView DAB IHC Detection Kit; Ventana Medical Systems, Tucson, Arizona, USA), revealed with a chromogenic precipitated by DAB.

All the immunostained sections were then counterstained with haematoxylin, dehydrated in a graded series of ethanol, cleared in xylene and mounted using a synthetic mounting medium.

Positive and negative controls were used, and human tonsil tissue was used as a positive control for the PD-L1 staining.

Table 2. Antibodies applied and immunohistochemistry protocol. General control builds with tissue sections from liver, kidney, prostate and placenta.

Primary Antibody	Clone	Manufacturer	On-slide control	Antigen Retrieval´ (EDTA/Tris, pH8)	Dilution and incubation time
PD-L1	SP142	VMS	Tonsil	95°C; 56´	RTU; 20´
PD-L1	SP263	VMS	Tonsil	95°C; 48’	RTU; 16’
PD-L1	22C3	Dako	Tonsil	100°C; 48´	1:50; 52´
CD8	4B11	Leica	Tonsil	100°C; 40	1:50; 40´
P16	E6H4	VMS	Tonsil	100°C; 24´	RTU; 16´
CK5/6	D5/16 B4	Dako	General control	100°C; 32’	1:200; 28´
CK20	SP33	Dako	General control	100°C; 16’	1:50; 40´
GATA3	L50-823	VMS	General control	100°C; 32’	RTU; 36´
CD44	SP37	VMS	General control	91°C; 16’	RTU; 37´

VMS: Ventana Medical System; RTU: Ready-to-use.

IHC scoring

The slides were evaluated and scored in light microscopy by two blinded experienced pathologists.

For CK5/6, CK20, CD44, Gata 3 and p16 samples were classified as positive or negative. For CD8, the number of CD8 positive lymphocytes per High Power Field (HPF) was registered and the data were categorized according to the cutoffs 50/HPF and 20/HPF (calculated median).

PD-L1 22C3 and PD-L1 SP263 expression status was determined by Combined Positive Score (CPS), which is the number of PD-L1 staining cells (tumour cells, lymphocytes,

macrophages) divided by the total number of viable tumour cells, multiplied by 100, according to the manufacturers recommendations and literature [23, 24]. A cutoff of 10 was applied to define negative (<10) or positive (≥10) cases. For PD-L1 SP263 we used also the 25 cutoff, accordingly with the protocols [24].

PD-L1 SP142 expression status was determined by Immune Cell Score (IC), using the cutoff 5 to define a case as negative (<5) or as positive (≥5), according to the manufacturer indications and with the literature [25]. IC score is defined by the area of the tumour occupied by stained TILs present in the tumour area or contiguous peritumoral stroma.

Different technical quality features were assessed: uneven staining, cold zones / blank spots, hot zones, DAB spots, background staining and decreased lateral staining.

Statistical analysis

Statistical analysis was performed using IMB SPSS software version 25.0 (IMB corporation, Armonk, NY, USA). Initially, a descriptive analysis of the results was done. Secondly, relational statistics was conducted. Metric variables were described by mean with standard deviation (SD) whenever there was a normal distribution of the values and by median with range, if not. Medians and ranges of continuous variables were compared using an unpaired two-sample Student t test. Categorical variables were compared using Pearson's chi-squared test and Fisher's exact test. For the comparison of categorical and numerical variants, the T-Test and ANOVA tests were used. The Pearson correlation coefficient, which is used in assessing the strength of the linear relation between two variables, was used to compare biomarkers. P-values <0.05 were considered as statistically significant.

Results

Technique quality assessment

Features related to technical quality were previously assessed. We did not register any technical issue.

Gender analysis

TILs (cutoff of 20 lymphocytes per HPF) was significantly higher in the female gender ($p=0.017$, $p<0.05$). When used a cutoff of 50 lymphocytes per HPF no differences were found ($p>0.05$).

Also, PD-L1 22C3 (*p=0.051*) and SP263 (*p=0.06*) has a tendency to be positive in female patients. No statistically significant differences were identified in the expression of PD-L1 SP142 (*p>0.05*).

Differences were not found between female and male taking into account the histological grade and stages at the time of diagnosis (*p>0.05*).

No statistically significant differences were found in the expression of p16 according to gender (*p>0.05*).

Age analysis

Patients were classified into two categories: under 65 and with or over 65 years. No statistically significant differences were found when compared age categories with p16, CD8, PD-L1 22C3, PD-L1 SP263 and PD-L1 SP142 expressions (*p>0.05*).

Characterization according to molecular subtypes

In 41 UC, IHC characterization was performed, allowing the classification of each UC as luminal subtype (n= 21, 48.8% of the cases) and as basal subtype (n=20, 46.5%) (**Figure 1**). Two cases (4.7%) were not classified according to the molecular subtype.

The phenotype features are present in **Table 3**. TILs (CD8 expression) is lower in basal subtype carcinomas (*p = 0.045*, *p <0.05*) when considering the cutoff of 50 lymphocytes / HPF.

Regarding the categories luminal and basal molecular subtypes, no statistically significant differences were identified regarding gender, age, stage, histological grade, as well as p16, PD-L1 22C3, PD-L1 SP263 and PD- L1 SP142 expression(*p>0,05*).

Evaluation of expression of p16 and CD8

There was a tendency for higher levels of CD8 expression (TILs) in histologic grade 2 tumours (*p=0.0814, p<0.05*) when compared with other grades. We did not found any statistically significant differences in CD8 expression according to age and stage (*p>0,05*).

On the other hand, a not quite significant association of p16 expression and luminal subtype was found (*p=0.059*). p16 expression showed no significant differences according to age, gender, stage, and histological grade. Also, no correlation was found between p16 expression and PD-L1 SP142, 22C3 or SP263 expressions.

No correlation was found between p16 and CD8 expression (*p>0,05*).

Evaluation of the expression of PD-L1 clones

We found statistic significant positive correlation between PD-L1 22C3 with CD8 expression (cutoff 50) (*p=0.024, p <0.05*). Similar results were seen for PD-L1 SP263 and CD8 expression (cutoff 50) (*p=0.002, p <0.05*). However, no correlation was found neither CD8 (cutoff 50) and PD-L1 SP142 (*p>0.05*), nor between p16 and PD-L1 (*p>0.05*).

PD-L1 expression was not correlated with histological grade and histological stage (*p>0.05*).

Correlation between different PD-L1 clones

A very significant correlation was found between PD-L1 22C3 (cutoff 10) with PD-L1 SP263 (cutoff 10 or 25) expression (*p<0.0001*). Substantial agreement (*K test = 0.624*) was found between PD-L1 22C3 and SP263 expression, and an almost perfect agreement (*K test = 0.86*) was found between PD-L1 22C3 and SP263 (cutoff 10) expression.

Comparing PD-L1 22C3 (cutoff 10) with PD-L1 SP263 (cutoff 25), we found 8 cases with no concordance. All these cases were PD-L1 22C3 positive and PD-L1 SP263 negative. When considering a lower cutoff (cutoff 10) for PD-L1 SP263 (cutoff 10) the number of no agreement cases reduced to 3 cases.

Comparing 22C3 with SP142 PD-L1 expression, a significant correlation was found (*p=0.002, p<0.05*), with a moderate concordance (*K=0.486*). A discrepancy was observed in eleven cases: 4 cases were positive for PD-L1 SP142 and negative for 22C3 and 7 cases were negative for SP142 and positive for 22C3.

A significant correlation also was found between PD-L1 SP142 and SP263 (*p=0.002, p<0.05*) with moderate concordance (*K=0.453*). In 11 cases there was no concordance, 3 cases positive for SP263 and negative for SP142, and 8 cases negative for SP263 and positive for SP142. When considering the cutoff of 10 for PD-L1 SP263 the discordance cases was reduced to 8: 3 positive for SP263 and negative for SP142 and 5 negative for SP263 and positive for SP142.

Table 3. Distribution of urothelial carcinomas molecular subtype according to gender, age, PD-L1 expression and lymphocytes CD8 positive.

			Luminal Phenotype	Basal Phenotype
Gender	Male		15	17
	Female		6	3
Age	<65 years old		2	5
	≥ 65 years old		19	15
PD-L1	**22C3**	<10%	10	11
		≥10%	11	9
	SP263	<25%	14	15
		≥25%	7	5
		<10%	11	13
		≥10%	10	7
	SP142	<5%	12	12
		≥5%	9	8
Lymphocytes CD8+	<50 / HPF		14	19
	≥50 / HPF		7	1
	<20 / HPF		8	12
	≥20 / HPF		13	8

Discussion

In this study, no relevant technical issues were found between different PD-L1 clones. Therefore, our protocols LDT are optimized and ready-to-use, since we achieved the same IHC quality when compared with CDT. It might be explained by improved skills and technical formation of the professionals, ensured by continuous training, leading to a high scientific knowledge that allows resolving issues related to the technical protocol. To implement a LDT it is necessary to develop a validation program that must be followed by internal quality programs. Another key aspect is the active and regular participation in

international quality control programs. Our results are in accordance with others described in the literature reinforcing the use of LDT assuring quality control [26-29].

In our current study, we found a predominance of males and older individuals, supported by the literature [1, 3].

UC at stage 2 represents 47% of the cases studied; stage 3 and 4 correspond to 21% each. Usually, patients are selected for ICI when UC is classified as stage 4. However, we selected patients in different stages, to compare them with different PD-L1 antibody clones. Nevertheless, early assessment of PD-L1 expression at the time of invasive UC diagnosis might be an important variable to take into treatment decision made by clinicians with information related to targeted therapy. This is a clinical practice recommended in the recent NCCN guidelines [4].

According to the literature, high TILs density is associated with better overall survival (OS) and disease specific survival (DSS) in invasive UC of bladder. We found that female patients have higher expression of TILs. Our results are accordingly to the literature, where several studies showed that the female gender has a higher proportion of lymphocytes [30]. However, it is still unclear whether it is related to hormonal status, immune control / modulation mechanisms or microbiome. It is known that TILs (CD8 positive lymphocytes) tend to decrease during lifetime and higher levels are more frequent in the male gender. The effects related to age seems to be a greater defining factor than gender for immunological heterogeneity [31, 32].

Our results also demonstrated a lower CD8 expression in the UC basal type compared to luminal. Other authors identified a higher CD8 expression in basal type, indicating an “hot” phenotype [33]. In recently published consensus for molecular characterization of MIBC, basal cell type and luminal non specified (Lum NS) are more associated with immune infiltration. Luminal Papillary (Lum P) has papillary architecture and is associated with FGFR3, KDM6A and CDKN2A genetic alterations while Lum NS is mainly characterized by mutations in ELF3. A third luminal category - luminal unstable (Lum U) – is characterized by genomic instability and by higher rates of somatic mutations. Our study includes 21 luminal tumours labelled by IHC, a technique that is not capable to differentiate the three types of luminal UC (MIBC classification) and might have limitations in stratifying UC according to the molecular consensus. We did not find papillary architecture in all luminal cases, that may correspond to Lum NS and Lum U. We hypothesize that some of our luminal cases with higher TILs expression are Lum NS. Both, Lum NS and Lum U, can be immunogenic due to P53 mutations and a higher rate

of somatic mutations. Besides, a higher response to Atezolizumab was reported in Lum NS and Lum U [34].

In our study TILs (CD8 expression) is correlated with higher PD-L1 expression. It is known, that some UC subtypes, such as Lum P, with lower TILs has also lower PD-L1 expression. Lum U, a luminal subtype with higher TILs levels and PD-L1 expression, demonstrates a high response rate (68,7%) to ICI (Pembrolizumab) [35]. Robertson et al also identified a subgroup of Luminal cases (Luminal-infiltrated) with Epithelial-Mesenchyme (EMT) markers, which expresses PD-L1 and myofibroblast markers [36]. TILs and CD8 expression have been associated with PD-L1 expression and ICI responses [37-39]. Also in the PURE-01 and ABACUS, trials TILs and CD8 expression were associated with response to Pembrolizumab and Atezolizumab [38, 39]. In the ABACUS trial, Powles et al demonstrated that CD8 expression was associated with complete response. Also, tumours with lower CD8 expression were associated with resistance to ICI [38]. The association between CD8 and PD-L1 expressions has been identified by other authors corroborating our results [40-42]. IFNγ has been associated the induction of PD-L1 expression by activating the JAK/STAT signalling pathway, explaining PD-L1 higher expression in immune infiltrated tumours [43].

Other authors suggest another UC classification. Sjödahl et al defined five tumour- cell phenotypes: Uro (Urothelial-like), GU (Genomic Unstable), basal/SCC- like (basal/squamous cell carcinoma-like), Mes- like (Mesenchymal-infiltrated-like), and Sc/NE- like (Small cel/Neuroendocrine-like). Uro could be divided into Uro B (with features related to Basal type) and Uro C (with features related to GU) [37]. The authors suggest that Uro B and C are more immunogenic, like GU and basal types. Curiously, Uro C could have a high p16 expression [37]. We found higher expression of p16 in luminal type carcinomas, which accordingly with Sjödahl et al classification, can correspond to UC cases with phenotype Uro C [34, 37].

A tendency of higher expression of TILs in histologic grade 2 tumours was found. Published data demonstrate a higher TILs or CD8 expression in high grade UC. We must take into account that our sample included exclusively high grade tumours (grade 2, 3 and 4). The lower number of G3 and G4 UC is a limitation that may impact our results. Higher grades (G3 and G4) tends to accumulate molecular events related to immune escape and immune regulation, probably related with the immune exhaustion phenomenon. CD8 lymphocytes exhaustion has been demonstrated to be mediated by PD-L1, associated the reduction of IFN-γ and IL-12 cytokine and production of immunosuppressive cytokines.

Also, one study demonstrated that PPARγ, FGFR3 and β-catenin pathways activation in tumours is associated with reduced immune cells ("immune desert"). To further investigate this question a larger sample with a more proportional distribution is necessary [40, 41, 44-47].

We have demonstrated an almost perfect concordance between PD-L1 22C3 (LDT) and PD-L1 SP263 (CDT); moderate concordance between PD-L1 22C3 (LDT) and PD-L1 SP142 (CDT) and between PD-L1 SP263 (CDT) and PD-L1 SP142 (CDT). Concordance between different PD-L1 clones is well documented, being PD-L1 SP142 clone the one with less concordance [16, 17, 29].

Regarding the monoclonal antibodies PD-L1, the better combination of PD-L1 clones was SP142 and PD-L1 22C3. Together detected the most higher positive cases when compared with the other combination pair of PD-L1 clones. Each monoclonal PD-L1 antibody, PD-L1 SP142 and PD-L1 22C3 is associated with a different drug, and its combination allows to identify of a greater number of potential candidates for immunotherapy.

The immunohistochemical quantification of PD-L1 as a biomarker is still not very consistent [48].

New promising biomarkers have been developed and are starting to emerge. Tumour mutation load (TMB), mismatch repair status, TILs, TCGA (The Cancer Genome Atlas) profiling, and PD-L2 are some of the new candidates [48].

TMB is the number of acquired mutations in the tumour genome and its level is related to immunotherapy response [49]. Surprisingly TMB is a better biomarker predictor, with higher response rates compared with those obtained by PD-L1. However, it is necessary genome sequencing to calculate the TMB and not all laboratories have access to this technology [49].

Higher levels of TILs in the tumour microenvironment appear to be related to an increase in overall survival, but the results as a biomarker are inconsistent [50]. More studies will be required to confirm the role of TILs as a significant predictor of prognosis in bladder cancer [50].

Although PD-L1 does not be a perfect biomarker, it has been used as an important tool to identify potential candidates for PD-L1 drugs. The laboratory protocol is already

optimized, with external quality control, and the pathologists are well trained. Importantly, it is a low-cost IHC, with LDT validated.

This work has some limitations related to sample size and distribution of histologic grade and stage. Another important technical limitation, is the use of TMAs, reducing the area of tumour heterogeneity.

We also recognize that a more robust panel of IHC antibodies is needed to classify UC molecular subtypes. In the future it will be interesting to explore the tumour microenvironment, especially the relation between TILs, tumour cells and PD-L1, as well as other emergent biomarkers.

Conclusion

Our study demonstrated a significant correlation between PD-L1 22C3 (LDT), PD-L1 SP263 (CDT) and PD-L1 SP142 (CDT) with an almost perfect concordance between PD-L1 clones 22C3 (LDT) and SP263 (CDT). The PD-L1 combination of PD-L1 SP142 and PD-L1 22C3 identify more positive cases.

No relevant technical issues differences were found between PD-L1 clones comparing LDT and CDT reinforcing the importance of ongoing quality control, laboratory personnel training and competency assessment.

Higher TILs (CD8 expression) levels are correlated with higher PD-L1 expression, highlighting their use as biomarkers.

Acknowledgements

In order to carry out a study of this dimension, a lot of support is needed to reach the results.

I am grateful for all the support of the Serviço de Anatomia Patológica do CHUC, which with its experience and professionalism made the development of this work possible.

I would like to give a special thanks to:

- Professor Vitor Sousa for the support, sympathy and teachings, and the huge impulse in the conception, work planning and monitoring;
- Dr. Rui Gonçalinho for his assistance throughout the work;
- Dr. Fátima Ramalhosa for her sympathy, availability, agility and constant support in the development and production of the work;
- Dr. Paulo Teixeira for his support in the technical component;
- To my parents and friends, who, despite not having a direct influence on this work, are the pillar of everything I build.

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