RESEARCH ARTICLE

Optimization of Programmed Death Ligand-1 73-10 clone Immunohistochemical Protocol on Urothelial Carcinomas

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Abstract

Background: In urothelial carcinomas, Programmed Death Ligand-1 (PD-L1) expression assessed by immunohistochemistry is essential in order to predict the response of patients to immunotherapy.

Objective: The aims of our study were (1) to optimize the automated immunohistochemistry staining technique for the PD-L1 antibody 73-10 clone (Leica Biosystems), using a control tissue, on the Leica BOND-MAX immunostaining platform at the Center for Advanced Medical and Pharmaceutical Research – Laboratory of Normal and Pathological Morphology (MORPHO) and (2) to validate the technique by applying it on a series of 40 urothelial carcinomas.

Methods: The cases included in our study corresponded to muscle-invasive urothelial carcinomas diagnosed on radical cystectomy specimens. The cases were retrieved from the Pathology Department of Targu-Mures Emergency County Hospital database (between 2011-2018). The immunohistochemical staining was optimized according to the producer's recommendation and adapted to our laboratory. Palatine tonsil specimen served as positive control tissue. PD-L1 positivity was assessed in all urothelial carcinoma cases either by evaluating the tumor and immune cells, or by applying the Combined Positive Score.

Results: Our technique enabled us to obtain a well-optimized, high quality immunohistochemical PD-L1 staining on the control tissue, which was further successfully applied on the urothelial carcinoma cases. By assessing the PD-L1-positive tumor cells and/or immune cells percentages, 15 cases were considered PD-L1 positive. Using the Combined Positive Score, 16 cases were evaluated as positive.

Conclusion: Through the optimization process we achieved an excellent staining reaction for the PD-L1 73-10 antibody, that can be used successfully when assessing urothelial carcinoma cases.

Keywords: PD-L1, urothelial carcinoma, immunohistochemistry.

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Introduction

Bladder cancer is the ninth most common cancer worldwide, the majority of the cases being urothelial carcinomas (UCs) [1] . UC is known as one of the deadliest types of cancer, with a five-year survival rate of less than 5% when diagnosed in an advanced or metastatic tumor stage [2,3]. The main risk factor for developing UC is smoking, which accounts for approximately half of the cases [4]. Exposure to ionizing radiation or aromatic amines are also important factors to be considered when discussing the risk of developing UC [4]. The management of UC patients involves a multidisciplinary approach, including chemotherapy, radiotherapy and radical surgery [1]. Nowadays, there is a high interest in immunotherapy as an innovative and less harmful treatment for UC.

Programmed Death Ligand 1 (PD-L1) is an immune response inhibitor that is expressed on activated T cells, macrophages, dendritic cells and specific tumor cells[5] . PD-1/PD-L1 axis has a beneficial effect in maintaining immune tolerance, but on the downside it can also allow neoplastic cells to proliferate [5]. Tumor cells produce PD-L1 as a way to evade anti-tumor responses. PD-1/PD-L1 inhibitors bind to PD-1/PD-L1 in order to avoid their interaction and to reactivate the normal functionality of T cells [1].

In recent years, immunotherapy has emerged as a revolutionary technique in the management of cancer, including UC. The use of this therapy in UCs relies on the PD-L1 positivity as assessed by immunohistochemical (IHC) testing. This highlights the importance of performing a well-validated PD-L1 IHC reaction that ensures reliable and highly reproducible results. Food and Drug Administration (FDA) and European Medicines Agency (EMA)

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recommend the use of PD-1/PD-L1 immune checkpoint inhibitors as therapy only for the cases that exhibit a strong positive IHC reaction to PD-L1.

As the PD-L1 clone 73-10 is a relatively novel antibody and has not been approved for diagnostic applications, the optimization of a robust immunohistochemical protocol is of particular importance. Such effort is necessary to assess its performance characteristics, support its validation, and facilitate its potential adoption in routine pathology practice.

The aim of our study was to optimize the PD-L1 IHC staining protocol on Leica BOND-MAX platform using a positive control tissue. Further on, we aimed to test and validate our IHC protocol by applying it on a series of 40 UC cases.

The present study was performed using the infrastructure available in the MORPHO (Normal and Pathological Morphology) Laboratory of the Center for Advanced Medical and Pharmaceutical Research (CCAMF), at the George Emil Palade University of Medicine, Pharmacy, Science and Technology (GE Palade UMPhST) of Targu Mures.

Methods

Tissue Samples

The samples in our study were retrieved from the archival files of the Pathology Department of Târgu-Mureș Emergency County Hospital. The cases were muscle-invasive UCs diagnosed on radical cystectomy specimens from 01.01.2011 to 31.12.2018. The study was approved by the Ethics Committees of Targu Mures Emergency County Hospital (letter of approval no. 3568/ February 23, 2024) and GE Palade UMPhST of Targu Mures (letter of approval no. 2969/March 25, 2024). Informed consent was obtained from all patients included in the study.

IHC protocol

Our study included 40 UC cases. All cases were fixed in 10% neutral buffered formalin and underwent automated processing, according to standard procedures. For each case a representative formalin-fixed, paraffin-embedded (FFPE) tissue block, encompassing the tumor area (the invasion front) was selected by 3 pathologists with a special interest in urological pathology (AL, AB, ANB).

Four-µm sections from the representative FFPE tumor tissue blocks were cut using a microtome and mounted on charge adhesive slides. The slides were left to dry in an oven for one night at 56° C and two days at 37 °C . Until staining, they were stored at a temperature of 2-8° C in order to preserve antigenicity [6].

Palatine tonsil tissue was used as a positive control for optimizing the PD-L1 antibody IHC staining, according to the manufacturer's recommendation. For all UC cases a small fragment of palatine tonsil was attached to the slide, which served as an internal quality control of the IHC reaction and ensured an accurate interpretation (true positive or true negative PD-L1 immunostaining). Figure 1 shows the macroscopic appearance of the UC slides.

Figure 1: Immunohistochemistry slides of the urothelial carcinoma cases Palatine tonsil is present on each UC slide, serving as an internal positive control for the IHC reaction, to avoid false negative or false positive interpretation of the study cases (tumor area – blue arrow; control tissue – black arrow); Cases A and B express positivity in tumor (true positive cases), whereas case C is PD-L1 negative, with positive staining only in the control tissue (true negative case).

All IHC reactions were carried out using a Leica BOND-MAX automated immunostainer (Leica Biosystems, Melbourne, Australia) and antibody visualization was achieved using the Polymer Refine Detection Kit (Lei-

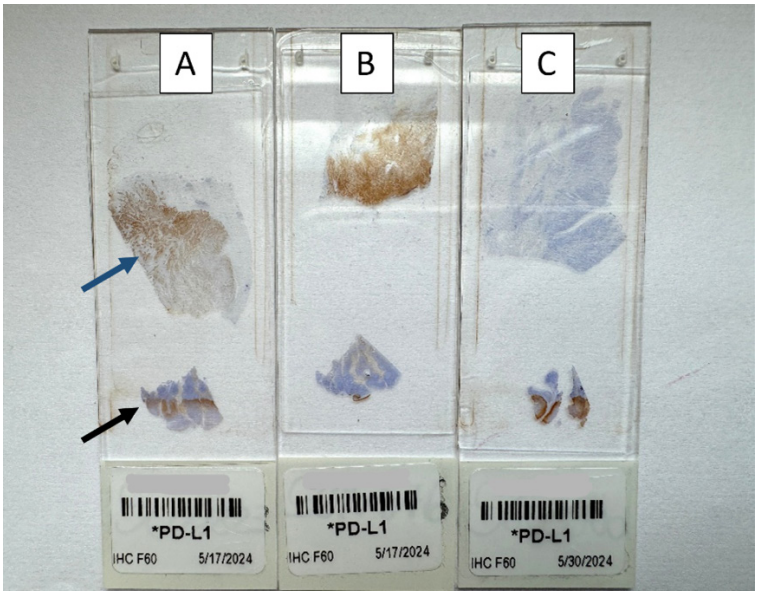

Fig. 1. Immunohistochemistry slides of the urothelial carcinoma cases.

ca Biosystems). Heat-Induced Epitope Retrieval (HIER) was performed automatically using BOND Epitope Retrieval solutions. The samples were stained with the PD-L1 73-10 clone (Leica Biosystems, BOND ready-to-use primary antibody).
A summarised IHC protocol for the PD-L1 antibody is listed in Table I.

Slide interpretation

Positive PD-L1 IHC staining on palatine tonsil was consistent with the following points: a weak to moderate membranous staining of the macrophages within the germinal center; a strong staining of the epithelial crypt cells; no staining in the majority of lymphocytes including mantle zone and germinal center B-cells; no staining in the superficial epithelial cells [7].

Table . Leica BOND-MAX PD-L1 immunostaining assay.

Step	Details
Dewax	Bond Dewax Solution - 100°C .
Epitope Retrieval	Bond Epitope Retrieval 1 (pH 6) or 2 (pH 9).
Peroxide blocking	5 minutes.
Washing	Bond Wash Solution.
Antibody incubation	15-60 minutes.
Washing	Bond Wash Solution.
Post-primary	8 minutes.
Washing	Bond Wash Solution, 3x2 minutes.
Polymer	8 minutes.
Washing	Bond Wash Solution, 2x2 minutes.
Distilled water	Rinse.
DAB	10 minutes.
Distilled water	Rinse.
Counterstain	Hematoxylin, 5 minutes.
Distilled water	Rinse.
Washing	Bond Wash Solution.
Distilled water	Rinse.

DAB – 3, 3' diaminobenzidine

Figure 2: illustrates the morphological features of PD-L1 expression in palatine tonsil, as demonstrated by analysis performed in our laboratory.

Strong membranous immunoreactivity of epithelial cells lining the palatine crypts (A, 100 ×; B, 200 ×); Moderate to strong membranous immunoreactivity of dendritic cells in a secondary lymphoid follicle (black arrow) (C, 100 ×; D, 200 ×) and no staining reaction in the superficial epithelium (blue arrow).

Positive PD-L1 staining in UC was defined as partial or complete membranous staining for tumor cells (TC) and cytoplasmic and/or membranous staining for immune cells (IC) [8]. The staining intensity of both TCs and ICs was quantified using the following system: 1+ weak, 2+ moderate, 3+ strong.

For the 73-10 clone used in our study there are no scoring recommendations in literature. We evaluated our cases using interpretation systems from other clinically approved PD-L1 clones[5] . Our interpretation was based on the percentage of positive TCs and/or ICs, as for the SP263 clone, where the positivity cut-off was set at ≥ 25% for positive TCs and/or ICs. In addition, we applied the Combined Positive Score (CPS), as used for the 22C3 clone. The CPS is defined by the number of PD-L1 positive cells (tumor and inflammatory cells) divided by the total number of viable tumor cells, multiplied by 100 [9]. A CPS ≥10 was considered positive.

A minimum of 100 viable tumor cells was required for each UC case in order to be considered as adequate for PD-L1 IHC assessment. Inflammatory cells that are not related to the tumor were not evaluated [9].

Results

1. Demographic and pathologic data

Clinico-pathological characteristics of the study cases are presented in Table II. The age ranged from 46 to 77 years, with a mean age of 63 years. More than a half of the cases were diagnosed in an advanced tumor stage, 3 and 4 respectively (n=29, 72.5%).

2. Qualitative data regarding PD-L1 staining and evaluation

The first assay on palatine tonsil was conducted according the following protocol: 30 minutes antibody incubation and 20 minutes epitope retrieval using the pH 6 solution. The results were not adequate. Therefore, three more tests were carried out with different protocols, that helped us to determine the optimal parameters for our IHC

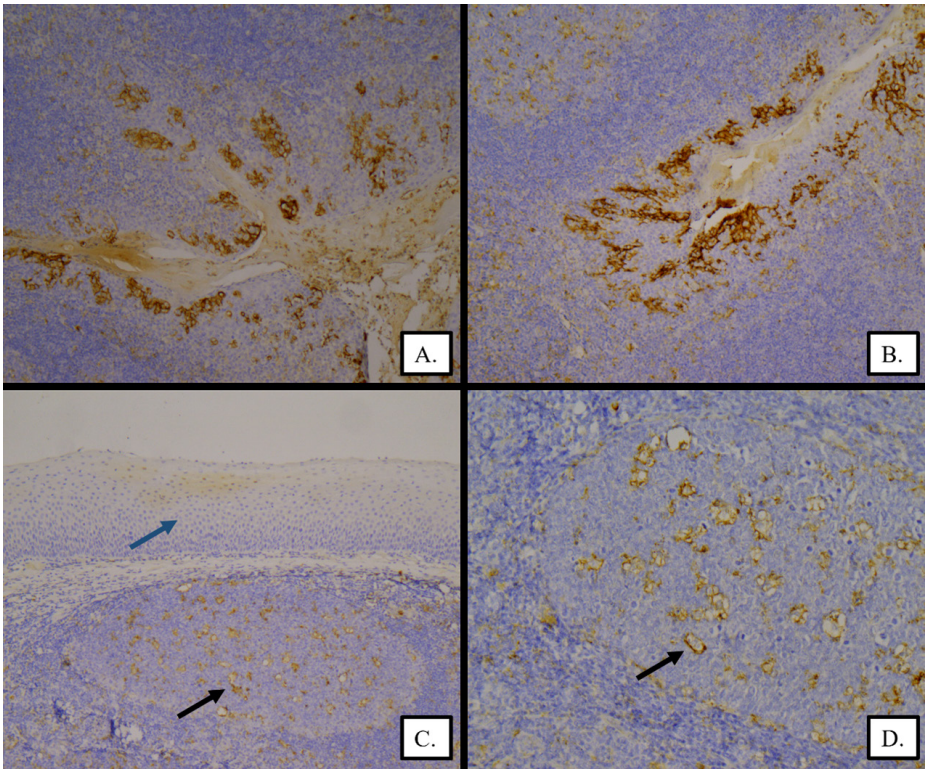

Fig. 2: Staining characteristics of the 73-10 PD-L1 clone in the control tissue – palatine tonsil.

Table II . Characteristics of the study population.

Characteristic	Total (n=40)	PD-L1 + (n=16)	PD-L1 – (n=24)
Demographic data			
Age (n, %)			
40-50 years	1 (2.5)	0	1 (4.1)
51-60 years	15 (37.5)	7 (43.7)	8 (33.3)
61-70 years	17 (42.5)	5 (31.2)	12 (50)
71-80 years	7 (17.5)	4 (25)	3 (12.5)
Gender			
Male	33 (82.5)	16 (100)	17 (70.8)
Female	7 (17.5)	0	7 (29.2)
Primary tumor, pT (n, %)			
pT2	11 (27.5)	5 (31.2)	6 (25)
pT3	20 (50)	7 (43.7)	13 (54.2)
pT4	9 (22.5)	4 (25)	5 (20.8)
Distant metastasis (n, %)	7 (17)	3 (18.7)	4 (16.6)

reaction. The parameters that were modified for each run are presented in Table III.

The fourth and final protocol – 1 hour antibody incubation, 20 min ER (pH 9) – demonstrated positive membranous staining of the epithelial crypt cells and macrophages and no staining of the superficial epithelium. Using this protocol, we carried out the IHC reaction on the UC cases.

The PD-L1 IHC staining in TCs showed partial or complete linear membranous staining of different intensities. For ICs, the staining was cytoplasmic and/or membranous, in general of strong intensity.

Figure 3: shows the characteristics of PD-L1 expression in TCs and ICs within the study cohort.

Figure 3: Staining characteristics of the 73-10 PD-L1 clone in UC cases.

Complete and strong membranous TC immunoreactivity in a case of UC (A, 100 ×; B, 200 ×); positive PD-L1 expression on ICs (blue arrow) and TCs (black arrow) (C, 100 ×; D, 200 ×)

UC – urothelial carcinoma, TC – tumor cell, IC – immune cell

Table III. Different assays applied to establish the final PD-L1 73-10 IHC protocol (no. 4).

Assay	Primary antibody incubation time (minutes)	ER time (minutes)	pH of the ER solution
1st	30	20	6
2nd	60	20	6
3rd	60	30	6
4th	60	20	9

ER – Epitope retrieval

3. PD-L1 IHC scoring assessment for the study UC cases

PD-L1 scoring was performed in consensus by three pathologists with experience in urological pathology, using a multi-headed microscope. By applying the TC/IC% score, 15 cases were considered PD-L1 positive. Using the CPS score, 16 cases were evaluated as positive. The staining intensity of TCs and ICs in the positive cases was mostly moderate or strong. All data regarding the PD-L1 immunohistochemical evaluation is presented in Table IV.

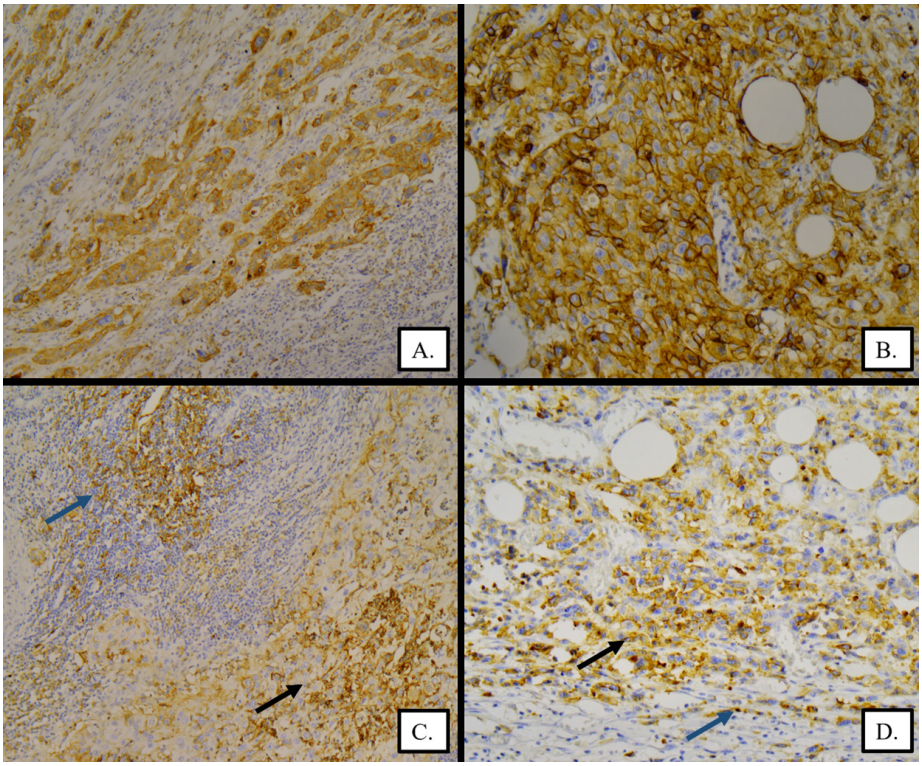

Fig. 3: Staining characteristics of the 73-10 PD-L1 clone in UC cases.

Discussion

The assessment of PD-L1 in UC is of utmost importance in establishing the patient's eligibility for treatment with PD-1/PD-L1 inhibitors [10]. This medication belongs to a class of therapy called immune checkpoint inhibitors – ICIs – which have the role to modulate the immune response and to mantain the balance between neutralizing threats and preserving self-tolerance [11].

Most FDA-approved PD-L1 clones target the extracellular domain of the PD-L1 protein. The 73-10 clone used in our study, binds to the intracellular domain and is considered to be more sensitive as demonstrated in major studies such as the Blueprint Phase 2 study. Because the 73-10 clone is incredibly novel and sensitive, it requires very specific protocol optimization.

In order to obtain unquestionable results, the IHC reaction must be optimized in each laboratory. There are various factors described in literature that could influence the quality of an IHC technique. Tissue fixation, antigen

Table IV. PD-L1 immunohistochemical evaluation of UC

Factors	Total cases(n=40)
TCs (n, %)	
TC ≥25%	15 (37.5)
TC<25%	25 (62.5)
Staining intensity for TCs (n, %)	
absence of staining	16 (40)
1+ (weak)	4 (10)
2+ (moderate)	5 (12.5)
3+ (strong)	15 (37.5)
ICs (n, %)	
IC≥25%	7 (17.5)
IC<25%	33 (82.5)
Staining intensity for ICs (n, %)	
absence of staining	8 (20)
1+ (weak)	2 (5)
2+ (moderate)	8 (20)
3+ (strong)	22 55)
CPS	
Positive ≥10	16 (40)
Negative <10	24 (60)

TC – tumor cells, IC – immune cells, CPS – Combined Positive Score

retrieval, detection system and antibody selection are all crucial steps to be considered when performing IHC.

• Tissue fixation

A single substance used for tissue fixation is not suitable for all antigens. For most tissues, 10% neutral buffered formalin is effective in preserving cellular morphology and preventing autolysis [12]. However, formalin can alter or destroy some epitopes and it is not recommended in techniques involving nucleic acids analysis [13].

As for the duration of fixation, in the past it used to be up to 72 hours, but nowadays is generally under 24 hours [14].

• Antigen retrieval

This step is crucial because formalin fixation can sometimes mask the epitopes. Generally, antigen retrieval is performed using heat – Heat Induced Epitope Retrieval (HIER) – or enzymes. In order to determine the optimal retrieval condition, each laboratory should test at least two antigen retrieval methods and compare the staining results [12]. The choice of antigen retrieval method should be made individually for each antigen-antibody pair, as some antibodies respond better to citrate (pH 6) whereas others perform better with EDTA (pH 9).

• Detection system

There are many detection systems available, the most commonly used being the avidin-biotin method, the phosphatase – anti-phosphatase method, the polymer-based detection system [12]. Detection systems have to be selected according to the tissue type, level of the marker of interest, localization of the antigen, the affinity of the antibody [15]. Each method has its own advantages and disadvantages. However, the polymer-based detection system is frequently used nowadays because it reduces or eliminates the background signal that is generated by the presence of endogenous avidin or biotin [16].

• Antibody selection

For antibodies that are not ready-to-use, serial dilutions have to be made to settle the optimal concentration. Before starting any assay, a full literature review is necessary to understand the antibody and what to expect from it [17]. To have a clear picture, the validation of an antibody should consist of at least a comparison of antigen retrieval at pH 6 and 9 plus incubation with two antibody dilutions [17]. Positive and negative controls should be included in each run to prevent potential errors of the staining platform [18,19].

The IHC technique can be performed either manually or automatically, but nowadays the manual approach is increasingly being abandoned in favor of automated methods, which offer several important advantages, including automatic antigen retrieval, reduced exposure of personnel to harmful or potentially carcinogenic reagents such as xylene and 3,3'-diaminobenzidine, shorter processing time (typically around three hours compared to the manual method, which often requires overnight antibody incubation), and improved reproducibility [20,21].

Even though the automated IHC technique is more standardized than the manual one, there are still important issues to be considered, that were challenges for our PD-L1 73-10 antibody's protocol optimization process.

The first issue we encountered was the detachment of tissue section from the slide during the staining protocol. This may be due to poor fixation, inadequate processing or overheating the tissue during antigen retrieval procedures [22]. Moreover, the time allowed for the slides to dry in the oven could influence this process. To overcome this limitation we left the slides to dry overnight at 56°C followed by an additional two days at 37°C. The results showed a clear improvement.

Antigen retrieval and the pH of the solutions influence the quality of the staining reaction [23]. During antibody optimization we tested two antigen retrieval solutions (pH 6 and 9) and the differences were visible even to the naked eye. EDTA proved to be more suitable for the PD-L1 antibody, despite the manufacturer's recommendation to use citrate.

Another important aspect is to have a positive control on the same slide as the test tissue. This positive control fragment (palatine tonsil tissue) acts as a quality indicator for the IHC reaction and represents a distinctive feature of our study. Only in this way can one reliably determine whether the reaction is positive or negative, while also eliminating uncertainties related to potential impairment of the staining system.

At the end of the IHC staining protocol, the slides must be washed in distilled water, dehydrated in alcohol, cleared in xylene and mounted; it is essential that the alcohol and xylene baths to be clean and free of impurities that could interfere with slide interpretation.

The optimization of the PD-L1 clone 73-10 IHC protocol is of significant clinical and logistical relevance for the management of UC. By adapting this assay, we established a highly efficient workflow that easily fits within standard laboratory shifts. This automated protocol prevents time-consuming overnight runs, minimizes manual handling errors and ensures accurate identification of PD-L1 expression in patients with UC.

While the exceptional sensitivity of the 73-10 clone offers a powerful tool for detecting PD-L1 expression, its widespread clinical use depends on assay harmonization. Prioritizing large-scale cross-validation studies is a vital step to ensure this clone's high sensitivity translates into more accurate treatment decisions for UC patients.

Limitations

A primary limitation of this study is the relatively small sample size (n=40), the cases being all retrieved from a single institution which may reduce the general applicability of the results. However, the study focuses on providing critical insights on optimizing an IHC protocol, that can be applied on future, larger-scale investigations.

Furthermore, this study did not include a direct com-

parison between the evaluated clone and other clinically-approved PD-L1 clones, such as 22C3 or SP263. Nevertheless, the main objective in the present study was to optimize the PD-L1 73-10 clone IHC staining protocol on Leica BOND-MAX platform using a positive control tissue and then to test and validate the IHC protocol by applying it on a series of 40 UC cases. Our data has demonstrated the staining efficacy of the PD-L1 73-10 clone in our cohort. These results are important and relevant, as the present study is part of a larger-multicenter study in which comparison of PD-L1 73-10 clone will be performed with other clinically-approved PD-L1 clones.

While this study successfully optimizes the technical workflow for the PD-L1 clone 73-10, it is limited by the lack of formal statistical analysis. Given that the focus was strictly on technical feasibility and protocol optimization, data evaluation was primarily descriptive and qualitative. Consequently, these findings should be viewed as a foundational technical framework. Further analytical validation with a larger cohort and dedicated statistical evaluation is necessary to confirm clinical utility.

Conclusion

Immunohistochemistry is a modern technique that plays an important role in the diagnosis and differentiation of various tumors, as well as in the evaluation of prognostic and therapeutic markers in cancer. For routine laboratory application, all antibodies should be optimized prior to use, in order to ensure reliable and highly reproducible results.

In this paper, we aimed to outline the technical steps required to establish an optimal protocol for the PD-L1 antibody 73-10 clone using the Leica BOND-MAX automated immunostainer (incubation time 60 minutes, ER 20 minutes – pH 9), as well as to highlight the pitfalls encountered in setting up and interpreting the IHC staining, and the strategies we used to overcome them. Important considerations in antibody optimization include tissue fixation, antigen retrieval, the detection system, and antibody selection. Furthermore, when working with antibodies that are difficult to interpret and score (such as PD-L1), the presence of an internal positive control on the same slide as the tumor is essential, as it helps minimize or significantly reduce false-negative and false-positive IHC results.

Author's contributions

LS (formal analysis, methodology, validation, writing-original draft)
IANB (investigation, project administration, funding acquisition)
AB (data curation, methodology, investigation, validation)
AL (data curation, methodology, investigation, validation)
ANB (formal analysis, conceptualization, methodology, supervision, writing-review and editing)

Conflict of interest

The authors declare no conflict of interest.

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