Standardizing immunohistochemistry methodology for evaluation of PD-1 and PDL-1 expression in upper tract urothelial carcinoma

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CAMPEDEL L, SEISEN T, VARINOT J, CANCEL-TASSIN G, RUFFION A, DE MAZANCOURT ES, DECAUSSIN-PETRUCCI M, ROBERT G, VUONG N-S, PHILIPP M, COMPERAT E, ROUPRET M, CUSSENOT O. Standardizing immunohistochemistry methodology for evaluation of PD-1 and PDL-1 expression in upper tract urothelial carcinoma. *Can J Urol* 2021;28(3):10719-10724.

Introduction: *Controversy regarding the prognostic and/or predictive role of PD-1 and PD-L1 expression for upper tract urothelial carcinoma (UTUC) could partly be explained by inconsistencies in the immunohistochemistry (IHC) methodology. Objective is to standardize the methodology for routine evaluation of PD-1 and PD-L1 expression in UTUC patients.*

Materials and methods: *Twenty-two cases treated with radical nephroureterectomy between 1996 and 2015 at 11 French hospitals were randomly selected to compare different methodologies for evaluation of PD-1 and PD-L1 expression. IHC was carried out on whole tissue sections and 0.6 mm- or 2 mm-core tissue micro-arrays (TMAs) using PD-1 NAT105 and PD-L1 28.8 or E1L3N on both tumor cells and tumor-infiltrating immune cells (TILs). Results obtained with whole tissue sections (WTS) were compared to those obtained with 0.6 mm- and 2 mm-core TMAs. Concordance was evaluated using Kappa coefficient.*

Results: *For evaluation of PD-1 and PD-L1 expression, the best concordance with WTS was observed using the PD-1 NAT105 and PD-L1 28.8 antibody on 2 mm-core TMAs, with 5% cut off for positivity on TILs and tumor cells, respectively (Kappa = 0.8).*

Conclusions: *The most accurate methodology for routine evaluation of PD-1 and PD-L1 expression in UTUC may be based on 2 mm-core TMAs using NAT105 and 28.8 antibodies with a 5% cut off for positivity on TILs and tumor cells, respectively.*

Key Words: tissue microarray, upper tract urothelial carcinoma, immune checkpoint inhibitors, PD-1, PD-L1 quantification, whole tissue sections

Accepted for publication March 2021

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Acknowledgements
We thank for their support and facilities:
INCa French National Cancer Institute (Grant 2019-181)
- IRCI The International Rare Cancer Initiative
- Alliance GETUG-AFU French cancer cooperative group in oncourology
CC-AFU (Cancerology Committee of French Association of Urology)
- Urothelial tumors group

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Introduction

Over the past few years, remarkable advances have been made in immunotherapy for various cancers including urothelial malignancies, particularly with the approval of novel immune checkpoint inhibitors, such as those involved in programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) blockade. Interestingly, a role for PD-1 and PD-L1 expressions as prognostic and predictive biomarkers has been reported for urothelial carcinoma of the bladder[1-4] but data is more controversial with regards to upper urinary tract urothelial carcinoma (UTUC),[5] accounting for only 5% of all urothelial malignancies.[6] Specifically, some reports showed that PD-1 and/or PDL-1 expressions had prognostic and/or predictive values,[7-9] while other suggested no significant association with survival and/or response to treatment.[10]

Several methodological aspects could explain these conflicting results for UTUC. First, different tissue sampling techniques, including whole tissue sections[8-10] and tissue microarrays (TMAs),[7] have been used without any direct comparison of their diagnostic performance. For example, although 0.6 mm-core TMA with two-fold redundancy is currently considered as the standard method for biomarker analysis in many tumor tissues,[11,12] three cores did not perform as well as whole tissue sections for evaluation of PD-L1 expression in clear cell renal cell carcinoma.[13] Second, different PD-1 antibodies, including NAT105[7] or AF1086[9] and PD-L1 antibodies, including E1L3N[7,8] or 5H1[10] have been used at multiple cut off for positivity without any evaluation of their concordance. For example, non-small cell lung cancer-specific data suggests that concordance could be low between PD-L1 E1L3N and SP142 antibodies.[14] Third, different cells, including tumor-infiltrating immune cells (TILs) or tumor cells have been analyzed, making it even more difficult to draw any definitive conclusion on the prognostic and predictive roles of PD-1 and PD-L1 for UTUC.

Against this drawback, we aimed to standardize the methodology for routine evaluation of PD-1 and PD-L1 expression in UTUC by analyzing diagnostic performances of different sampling techniques using several PD-1 and PD-L1 antibodies with various cut offs for positivity on TILs or tumor cells.

Materials and methods

Specimen and data collection

Using our national collaborative network, UTUC specimens from 456 patients treated with radical nephroureterectomy (RNU) between 1996 and 2015 at 11 French hospitals were retrieved to build a multi-institutional tumor bank. All clinical and pathological information were retrospectively collected at each participating center using a computerized database.

For the purpose of the present study, 22 cases were randomly selected from this tumor bank with equal distribution between muscle-invasive and non-muscle invasive disease. Clinical and pathological variables of interest included age, gender, smoking history, primary tumor location, tumor stage, as well as status for nodal invasion and concomitant CIS.

Whole tissue sections and tissue microarrays construction

A 4-µm whole tissue section was obtained from each tissue block while tissue microarrays (TMAs) were constructed by fusing archived formalin-fixed paraffin-embedded UTUC specimens using techniques described previously.[15-17] Before arraying, a 4-µm section from each tissue block was stained with hematoxylin and eosin (H&E). A morphologically representative area of the tumor was further selected and labeled with colored ink by our senior uropathologist. Three cores with the most commonly used diameter of 0.6 mm and two with a more representative diameter of 2 mm were then punched from the targeted tumor area of each donor block and transferred into separate recipient blocks using a tissue arraying instrument (Tissue Arrayer Manuel MTA1, Excilone).

After construction of the array block, multiple consecutive 4-µm sections were cut until all the tissue samples were represented on a single section. Each section was stained with H&E for histological verification of adequacy of the arrayed tumor tissues. Consecutive sections were placed separately on charged polylysine-coated slides for immunohistochemical analysis.

Immunohistochemical staining and quantification

Whole tissue sections and TMAs were deparaffinised, rehydrated, treated with 3% hydrogen peroxide in phosphate-buffered saline, and incubated at 95°C for 10 min in 10 µmol/L citrate buffer (pH 6.0). For PD-1 detection, they were incubated at 37°C for 32 min with the mouse NAT105 monoclonal antibody (Cell marque; pre-diluted), which is the only PD-1 antibody available in Europe. For PD-L1 detection, they were incubated at 37°C for 16 min with the rabbit monoclonal antibody either mostly used in clinical research, namely E1L3N (Cell Signalling Technology; diluted 1/100) or mostly used in clinical practice 28.8 (Dako; diluted 1/100).

Immunohistochemical staining was performed using the EnVision+ dual rabbit/mouse link system HRP (DAB+) (Dako) and UltraView Universal DAB detection

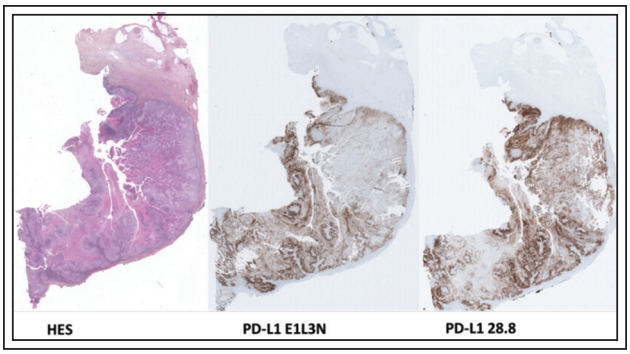

Figure 1. Whole slides (scan). HES staining, urothelial tumor invading the all ureter **(A)**. PD-L1 E1L3N antibody staining of 60% of the tumor cells **(B)**. PD-L1 28.8 antibody staining of 70% of tumor cells **(C)**.

kit (Ventana) in a Ventana Benchmark XT instrument according to the manufacturer's recommendations. Samples from tissues known to express each marker were taken as positive controls and a negative control was incubated with an irrelevant antibody. A minimum of ≥ 100 carcinoma cells was used to qualify a sample for quantification. The percentages of PD-1 and PD-L1 positive TILs and tumor cells were assessed using a semi-quantitative proportion score (0-100%), as described previously.[18,19] Briefly, a TIL was considered as positive if any part of its membrane or cytoplasm stained and the proportion of positive TILs was quantified using the ratio of area covered by positive TILs over tumor area, as described previously.[18] With regards to tumor cells, positivity was defined as any partial or complete staining of its membrane and the proportion of positive tumor cells was quantified using the ratio of positive over total tumor cells. It is noteworthy that PD-L1 positive necrotic areas as well as granular intra- or extra-cytoplasmic staining observed with PD-L1 28.8 antibody were excluded from the quantification. For further analyses, the cut offs of 1% and 5% were used to define PD-1 and PD-L1 positive UTUC on whole tissue sections and TMAs, Figures 1 and 2.

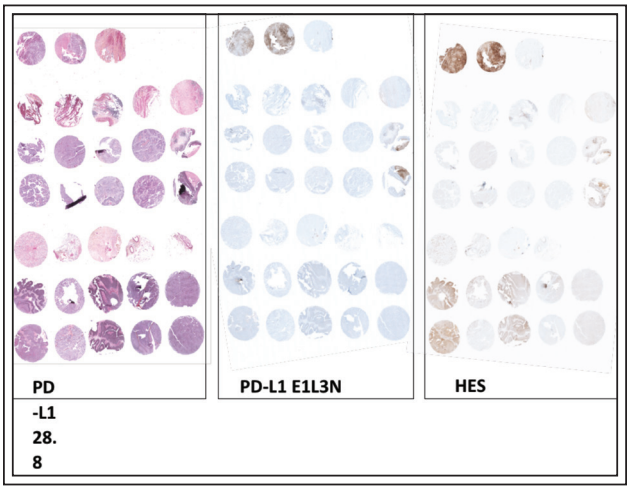

Figure 2. Each tissue microarray block is used to construct several slides for staining with HES **(A)**, PD-L1 E1L3N **(B)** or PD-L1 28.8 **(C)** antibody.

Statistical analysis

Median and interquartile ranges (IQRs) were reported for continuous variables, while categorical variables were presented as frequencies and proportions. Kappa coefficient was calculated to assess concordance between whole tissue section, considered as the gold standard method, and 0.6 mm- or 2 mm-core TMA for evaluation of PD-1 and PD-L1 expression on TILs and tumor cells using PD-1 NAT105 antibody and PD-L1 E1L3N or 28.8 antibodies. Statistical analyses were performed using Graph Pad Prism and IBM SPSS software.

TABLE 1. **Baseline clinical and pathological characteristics of the study population (n = 22)**

Characteristic	**No. (%) of cases**
Age (yrs), median (interquartile range)	73 (47-84)
Gender	
Male	12 (54.5)
Female	10 (45.5)
History of smoking	
Current smoker	5 (22.7)
Former smoker	7 (31.8)
Never smoker	6 (27.3)
Not available	4 (18.2)
Localization	
Renal pelvis or calyces	12 (54.6)
Ureter	4 (18.2)
Multifocal	3 (13.6)
Not available	3 (13.6)
Pathological tumor stage,	
≤ pT1	9 (40.9)
≥ pT2	13 (59.1)
Pathological nodal stage	
pN0	5 (22.8)
≥ pN1	3 (13.6)
pNx	14 (63.6)
Concomitant CIS	
Present	3 (13.6)
Absent	16 (72.8)
Not available	3 (13.6)

Results

Study population and immunochemistry

Among the 22 patients included in the present study, 9 (41%) and 13 (59%) had non-muscle and muscle invasive UTUC, respectively. Median age at diagnosis was 73 years (IQR: 47-84). The male to female ratio was 1.1 and the vast majority of patients was either current (22%) or former (32%) smokers. Most of the tumors were located in the renal pelvis (63%). Baseline characteristics of the study population are shown in Table 1.

Although all immunohistochemistry results were available for whole tissue sections from the 22 included patients, several cores were lost during immunohistochemical staining for both 0.6 mm- and 2 mm-core TMA. Only those from 19 cases were analyzed for 0.6 mm-core TMAs, except for PD-1 NAT105 antibody (n = 20). With regards to 2 mm-core TMAs, 21 cases were available for analysis, except for PD-L1 antibody 28.8 (n = 20).

Evaluation of PD-1 expression

Whole tissue sections as well as 0.6 mm- and 2 mm-core TMAs showed no PD-1 positive staining on tumor cells using the NAT105 antibody. For evaluation of PD-1 expression on TILs, the best concordance with whole tissue sections was observed using the NAT105 antibody on 2 mm-core TMAs, without any difference

TABLE 2a. **Comparison of PD-1 and PD-L1 immunohistochemistry results obtained from whole tissue sections and 0.6 mm-core TMAs and 0.2 mm-core TMA**

Antibody	Cells analyzed	Positive cut off	Positive cases on WT n (%)	Positive cases on 0.6 mm-core TMA n (%)	Positive cases on 2 mm-core TMA n (%)
PD-1	TILs	1%	6/22 (27)	3/19 (16)	8/21 (38)
NAT105		5%	4/22 (18)	0/20 (0)	2/21 (10)
PD-L1	TCs	1%	4/22 (18)	2/19 (11)	2/20 (10)
28.8		5%	4/22 (18)	2/19 (11)	2/20 (10)
	TILs	1%	10/22 (45)	7/19 (37)	13/20 (65)
		5%	4/22 (18)	6/ 19 (32)	10/20 (50)
PD-L1	TCs	1%	2/22 (9)	1/19 (5)	2/21 (10)
E1L3N		5%	2/22 (9)	1/19 (5)	2/21 (10)
	TILs	1%	4/22 (18)	7/19 (37)	8/21 (38)
		5%	1/22 (5)	4/19 (21)	4/21 (19)

TABLE 2b.

Antibody	Cells analyzed	Positive cut off	Concordance WT/0.6 mm-core TMA		Concordance WT/2 mm-core TMA	
			%	K	%	K
PD-1	TILs	1%	95	0.7	90	0.8
NAT105	5%	84	n/a	95	0.8	
PD-L1	TCs	1%	84	0.2	95	0.6
28.8		5%	84	0.3	95	0.8
TILs		1%	74	0.5	60	0.2
		5%	89	0.7	70	0.4
PD-L1	TCs	1%	95	0.6	95	0.6
E1L3N		5%	95	0.6	90	0.5
	TILs	1%	84	0.6	76	0.4
		5%	84	0.3	86	0.4

WT = whole tissue; TMA = tissue microarray; TILs = tumor infiltrating immune cells; TCs = tumor cells

TABLE 3. **Concordance between the two anti PD-L1 antibodies (28.8 and E1L3N)**

Cells analyzed	1% positive cut off	5% positive cut off
TCs	0.2	0.3
TILs	0.4	0.4

TCs = tumor cells; TILs = tumor-infiltrating immune cells

between 1% and 5% cut offs for positivity, given that the Kappa coefficient was 0.8 for both, Table 2a and 2b.

Evaluation of PD-L1 expression

For evaluation of PD-L1 expression on tumor cells, the best concordance with whole tissue sections was observed using the 28.8 antibody on 2 mm-core TMAs at a cut off of 5% for positivity, given that the Kappa coefficient was 0.8, Table 2b. Similar results were found with 0.6 mm-core TMAs for the evaluation of PD-L1 expression on TILs, although the Kappa coefficient was slightly lower (0.7, Table 2b). In addition, concordance between PD-L1 28.8 and E1L3N antibodies was low on both tumor cells and TILs from whole tissue sections using either 1% or 5% cut off, given that the Kappa coefficient ranged from 0.2 to 0.4, Table 3, Figure 3.

Discussion

Although it is well-established that TMA enables immunohistochemical analyses of multiple cases at the same time by providing uniform slides staining for many cancers,[11,12] this technique, along other methodological aspects, needs to be validated for routine evaluation of PD-1 and PD-L1 expression in UTUC. Thus, we sought to compare diagnostic performances of 0.6 mm- and 2 mm-core TMAs to those obtained from whole tissue sections considered as the gold standard method, using different PD-1 and PD-L1 antibodies with 1% and 5% cut offs on both TILs and tumor cells. Based on 22 UTUC patients, our results suggest that the best concordance with whole tissue sections could be obtained using the NAT 105 and 28.8 antibodies on 2 mm-core TMAs with a 5% cut off for positivity of PD-1 and PD-L1 expression on TILs and tumor cells, respectively. This method is likely to save time and cost of reagents.

With regards to TMAs size, we observed that 2 mm cores were more accurate than 0.6 mm cores for evaluation of PD-1 and PD-L1 expression. Logically, these results could be explained by the size of the area covered by a 2 mm core (3.14 mm^2), which is greater than that covered by three 0.6 mm cores (0.85 mm^2). It is likely

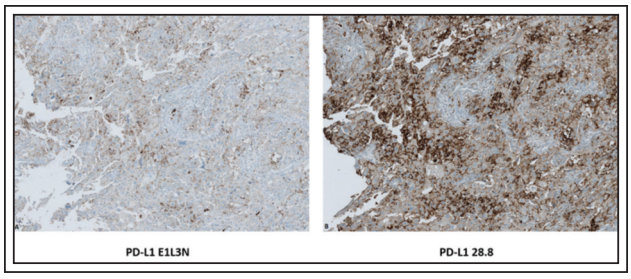

Figure 3. Whole slides (scan) x 20. PD-L1 E1L3N antibody staining of 5% of tumor cells **(A)**. PD-L1 28.8 antibody staining of 50% of tumor cells **(B)**.

that a larger area would give a better estimate of the percentage of positive cells, in particular when labeling is heterogeneous and varies from one tumor region to another, as observed in our study and as previously reported.[20] This could partly explain the discordance between the results observed in the literature using either 0.6 mm-core TMAs[7] or whole tissue sections.[8,10]

With regards to antibodies, only NAT105 was available for the evaluation of PD-1 expression. In contrast to Hayakawa et al,[9] we did not observe any PD-1 staining on tumor cells with this antibody. However, it is noteworthy that staining of intra-tumoral T-cells or associated stroma was considered as positive tumor cells for PD-1 expression by the authors. For the evaluation of PD-L1 expression, we found that the 28.8 antibody was more accurate than the E1L3N antibody. Notably, the PD-L1 28.8 antibody was characterized by the presence of diffuse cytoplasmic granular staining in some of our samples that we considered as negative, as previously described in peritoneal malignant mesothelioma.[21] In addition, we performed a direct comparison of diagnostic performance of PD-L1 28.8 and E1L3N antibodies on tumor cells and TILs from whole tissue sections, and we found that there was low concordance between them for both cell types. To our knowledge, this is the first report to examine such concordance in UTUC. The heterogeneous nature of urothelial tissue is likely to explain our results by making interpretation of staining difficult. Another explanation could be that the epitope for PD-L1 28.8 antibody is intracellular whereas that for PD-L1 E1L3N antibody is extracellular. Interestingly, although the findings of the present study contrast with those from other cancers showing high concordance between several PD-L1 antibodies,[22,23] they are consistent with others from lung cancer.[14] This suggests tissue-specific expression of PD-L1, with heterogeneity of labeling according to cancers and could partly explain the discordance between the results observed in the literature using either PD-L1 E1L3N[7] or 5H1[10] antibody.

With regards to cut offs for positivity, we observed that the best concordance with whole tissue sections was observed with 5% for evaluation of both PD-1 and PD-L1 expression on tumor cells and TILs, respectively. For PD-L1, this cut off has been widely used in previous studies on UTUC[8,10] or urothelial carcinoma of the bladder.[1,2,24] We also evaluated the 1% cut off, which provided lower concordance of 2 mm-core TMAs with whole tissue sections for the evaluation of all antibodies on tumor cells and TILs, except for E1L3N.

When comparing the results from our overall methodology to the available literature, we observed lower PD-1 expression using the 5% cut off for NAT105 antibody on TILs from 2 mm-core TMAs (10%) than that previously reported for both UTUC (37%-57%)[7,9] and urothelial carcinoma of the bladder (42%-43%).[1,2] Similarly, despite equivalent results in several studies,[1,8] PD-L1 expression (10%) was lower than that previously reported in others for both UTUC (23%-26.0%)[7,10] and urothelial carcinoma of the bladder (20%-30%)[2,3,24,25] using the 5% cut off for 28.8 antibody on tumor cells from 2 mm-core TMAs. Many methodological aspects could have participated to these differences. For example, in the study by Hayakawa et al,[9] the cut off for PD-1 expression was based on a low number of positive TILs (10 per field) and not the percentage of labeled TILs. In addition, patient selection could partly explain heterogeneity in PD-1 and PD-L1 expression.

Conclusions

To conclude, the best concordance with whole tissue section for PD-1 and PD-L1 expression in UTUC was observed with 2 mm-core TMAs using NAT105 and 28.8 antibodies with a 5% cut off for positivity on TILs and tumor cells, respectively. This is likely to represent the most accurate methodology for routine evaluation of PD-1 and PD-L1 expression in UTUC. □

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