# Metastatic bladder cancer expression and subcellular localization of Nectin-4 and Trop-2 in variant histology: a rapid autopsy study

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## Abstract

**Background**—Nectin-4 and Trop-2 are transmembrane targets of FDA-approved antibody-drug conjugates (ADC) Enfortumab-vedotin (EV) and Sacituzumab govitecan (SG), respectively, for the treatment of metastatic urothelial carcinoma (mUC). The expression and role of Nectin-4 and Trop-2 in mUC variant histology is poorly described.

**Objective**—We evaluate membranous and cytoplasmic protein expression, and mRNA levels of Nectin-4 and Trop-2 within matched primary and metastatic mUC samples to determine heterogeneity of ADC targets in mUC variants.

**Results**—Patients with mUC were consented for rapid autopsy immediately after death. Tissues from matched primary and metastatic lesions were collected. A total of 67 specimens from 20 patients were analyzed: 27 were UC, 17 plasmacytoid (PUC), 18 UC with squamous differentiation (UCSD), and 5 neuroendocrine (NE); 10 from primary and 57 from metastatic sites. All histology except NE expressed moderate-high levels of Nectin-4 and Trop-2 by both immunohistochemistry and RNAseq. Nectin-4 demonstrated prominent cytoplasmic staining in metastatic PUC and UCSD. Trop-2 demonstrated strong cytoplasmic and membrane staining in primary and metastatic tumors. Interestingly, Nectin-4 and Trop-2 expression are positively correlated at both mRNA and protein levels.

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**Conclusion—**UC and non-NE variants express notable level of Nectin-4 and Trop-2 in both primary and metastatic lesions. Membrane staining of Nectin-4 and Trop-2 is present but cytoplasmic staining is a more common event in both mUC and mUC variant histology. These findings support evaluation of EV and SG in heavily treated variant histology BC and urge attention on the clinical relevance of cytoplasmic localization of ADC targets.

## MicroAbstract

We sought to characterize the expression level and subcellular localization of Nectin-4 and Trop-2, targets of novel antibody-drug conjugates in metastatic urothelial cancer, within variant bladder cancer from matched primary and metastatic sites. We find significant levels of the targets in histologic variants. Additionally, high levels of cytoplasmic rather than membrane staining is seen, which may have important clinical implications.

### Keywords

## Introduction

Patients with advanced bladder cancer (BC) have a grim prognosis despite advances in systemic therapy[1]. Recently, FDA approved two humanized antibody-drug conjugates (ADC) targeting membrane molecules in metastatic urothelial carcinoma (mUC). Enfortumab vedotin (EV) targeting Nectin-4, and Sacituzumab govitecan (SG) targeting Trop-2 have demonstrated improved survival in patients with mUC[2,3]. However, their potential utility to mUC histologic variants is unclear.

Variant histology BC is aggressive with low response rates to systemic therapy and poor outcomes. Moreover, their relative rarity adds significant challenges for conducting much-needed clinical trials. Standard of care treatments for variants has historically been extrapolated from non-variant UC studies, which partly contributed to the low response rate. Studies of Nectin-4 and Trop-2 expression to date in BC have focused on primary tumor[4–6]. However, genomic and phenotypic heterogeneity across metastatic sites are frequent and key to treatment resistance in advanced BC[7]. As such, characterizing the expression of Nectin-4 and Trop-2 in the aggressive but understudied UC histologic variants, as well as across metastatic sites, will allow understanding of potential activity of ADC in these challenging patient groups.

In this study, we evaluate expression level and report subcellular localization of Nectin-4 and Trop-2 in histologic variants of BC within matched primary and metastatic specimens acquired from 2015–2020 from the urothelial cancer rapid autopsy program[7].

## Materials and methods

### Patients and tissue specimens

Specimens were obtained from patients who died of metastatic urothelial cancer in 2015–2020, and the rapid autopsy was typically performed within 6 hours of death[7]. All

procedures involving human subjects were approved by the Institutional Review Board (IRB) of the University of Washington. All 20 patients have signed written informed consent for the rapid autopsy. Metastases and the primary tumor (if present) were identified and biopsied. Biopsies of all bone sites were obtained using a drill with an attached trephine. Rib metastasis was acquired by a Dyke cutter. All visceral metastases and matched primary tumor were embedded in Optimal Cutting Temperature compound (OCT; Tissue-Tek, Sakura Finetek, CA) or formalin-fixed and embedded in paraffin. Bone metastases were either snap frozen or decalcified in 10% formic acid before paraffin embedding.

### Tissue microarray construction

Human tissue microarrays (TMA) were constructed for this bladder cancer rapid autopsy series. Formalin-fixed, paraffin-embedded specimens of paired primary (if available) and 2–5 metastatic specimens were used per patient, and TMA were constructed as previously described[8]. The TMA represented a total of 216 cores made from triplicate 1-mm cores taken from tumor regions of 72 specimens, as defined by two genitourinary pathologists (FVL and MPR).

### Immunohistochemistry and analyses

Five-μm TMA sections were deparaffinized and rehydrated in sequential xylene and graded ethanol series. Antigen retrieval was performed in Tris/EDTA buffer (pH 9.0). Endogenous peroxidase and avidin/biotin were blocked (Vector Laboratories Inc., Burlingame, CA) and TMAs were incubated with 5% normal goat-horse-chicken serum for 1 hour at room temperature. Sections were incubated with rabbit monoclonal primary antibody to Nectin-4[5,9,10](EPR15613–68; Abcam, ab192033; 1:250) or to Trop-2[11] (EPR20043; Abcam, ab214488; 1:500) at 4°C overnight, followed by biotinylated secondary antibody (Vector Laboratories Inc.) and ABC reagent (Vector Laboratories Inc.). Of note, the anti-Nectin-4 antibody used differs from the Astellas and Seattle Genetics proprietary anti-Nectin-4 antibody (clone M22–321b41.1[12]) which is not available. We utilized the publicly available alternative anti-Nectin-4 antibody employed by various other groups examining Nectin-4 in urothelial and other malignancies[5,9,10]. Staining was detected with stable DAB (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. All sections were counterstained with hematoxylin and mounted with Cytoseal XYL (Richard Allan Scientific, San Diego, CA). Rabbit IgG were used as negative controls.

Staining for both membrane and cytoplasm was graded in a blinded fashion by two pathologists (FKL and MPR). For the variant histology specimens in this study, all the variants are either pure (PUC) or predominant (neuroendocrine [NE] and UC with squamous differentiation [UCSD], >50–95% variant), and only the variant component was scored. Primary and metastatic specimen had concordant histology except for in one patient, 19–022, which was pure UC primary but one of 3 metastases (H2-Adrenal) was UCSD. Signal intensity (0–3: 0 for negative stain, 1 for faint/equivocal stain, 2 for moderate stain, and 3 for intense stain) and percentage of signal coverage (0–100) of each core were scored, and the product of the intensity and coverage was represented as an H-score (0–300) as previously described[8,13]. Nectin-4 and Trop-2 was considered positive when the H-score is 30 and 100, respectively. Owing to tissue loss during the immunohistochemistry (IHC) process,

a total of 201 cores from 67 bladder cancer specimens were evaluated. The score for each specimen was an average of triplicated cores.

### RNA sequencing and analyses

Primary or metastatic specimens selected for sequencing were macro-dissected to represent greater than 80% tumor cellularity by genitourinary pathology review (FVR and MPR). RNA was extracted from 75 OCT-embedded specimens representing 31 UCC, 16 PUC, 21 UCSD, and 7 NE specimens. Briefly, RNA was extracted using RNA STAT 60 (Tel-Test, Friendswood, TX) and RNeasy Mini kit, followed by DNase digestion in solution prior to purification (Qiagen, Germantown, MD) as described before[7]. RNA integrity number was determined using the Agilent Bioanalyzer (Agilent, Santa Clara, CA). Total RNA (300 ng) was used to construct RNA-seq libraries using the Illumina TruSeq RNA Exome Sample Prep Kit according to the manufacturer's protocol (Illumina, San Diego, CA). Barcoded libraries were pooled and sequenced using Illumina HiSeq 2500 system to obtain 50-bp paired-end reads (Illumina, San Diego, CA). The reads were aligned to the human hg38 using STAR v2.1.0[14]. Gene level abundance was quantitated from the filtered human alignments in python using HTSeq-count[15].

### Statistical Analyses

Statistical comparisons were conducted by Mann-Whitney U test for continuous variables, and Chi-square test for categorical variables based on normality of data assessment using Shapiro-Wilk test ($p<0.05$ across all groups). Central tendency data are presented as medians with interquartile range (IQR). Kaplan-Meier survival curves were estimated, and log-rank tests were used to compare survival between high and low expression groups. Pearson correlation analysis was performed for all correlations between RNA and protein levels. All statistical analyses were performed using Statistical Package for the Social Sciences, Version 22.0 (IBM Corp., 2020, Armonk, NY) and figures were generated using GraphPad Prism version 9.4.0 (GraphPad Software, San Diego, CA).

## Results

### Patient population and specimen histology

A total of 67 specimens from 20 patients were analyzed, consisted of 27 UC, 17 plasmacytoid (PUC), 18 UC with squamous differentiation (UCSD), and 5 neuroendocrine (NE); 10 specimens were collected from primary tumor and 57 from metastases. Histology in metastases was consistent with that of the primary tumor. Thirteen of 20 patients (65%) received systemic chemotherapy and 14 (70%) received immunotherapy. Specific chemotherapy regimens can be seen in Table 1. None of the patients received EV or SG. Clinicopathologic data and biospecimen information are shown in Fig. 1A and 1B, and Supplementary Table 1.

### *PVRL4* and *TACSTD2* mRNA expression is correlated in patient specimens

We first evaluated the mRNA expression of *PVRL4* (Nectin-4) and *TACSTD2* (Trop-2) in UC and its variants and determine the expression heterogeneity across metastases within an individual. RNA sequencing results showed a high expression of *PVRL4* and *TACSTD2* in

UC and histological variants PUC and UCSD, but not in NE (Fig. 2A). The high expression of both *PVRL4* and *TACSTD2* were largely conserved across multiple metastases within an individual showing conventional UC histology or variant histology (Fig. 2A). Interestingly, a significant positive correlation was noted for *PVRL4* and *TACSTD2* in each specimen (Fig. 2B and C; r = 0.81, $p < 0.001$).

### Cytoplasmic Nectin-4 and Trop-2 is prominent in UC and its variants

Next, we assessed the membrane protein expression for Nectin-4 and Trop-2 based on their approved utility as targets for ADCs. Pathological evaluation noted a remarkable cytoplasmic presence of both Nectin-4 and Trop-2, in addition to their anticipated membranous localization. As such, all specimens were independent evaluated by our GU pathologists for membrane and cytoplasmic staining separately. IHC staining for Nectin-4 and Trop-2 proteins revealed both prominent membranous and cytoplasmic staining in UC and its variants, except NE (Fig. 3A–B, Supplementary Fig. 1A). Nectin-4 was expressed heterogeneously and at moderate levels in conventional UC samples (Fig. 3B), with modest enrichment in the cytoplasm (56%) compared to the membrane (30%, $p = 0.03$; Fig. 3B and Fig. 4A). However, UC variants PUC and UCSD demonstrated significantly enriched cytoplasmic staining (cNectin-4) when compared to membranous (mNectin-4; PUC: 94% cytoplasmic vs. 6% membranous, $p < 0.001$; UCSD: 83% cytoplasmic vs. 44% membranous, $p = 0.01$; Fig. 3A–B and Fig. 4A). NE had no detectable Nectin-4 (Fig. 3A–B and Fig 4A).

Trop-2 was detected at high levels both on the membrane and within the cytoplasm in UC and its histologic variants, except for NE (Fig. 3A–B and Fig. 4B). Despite a few of the histological variant PUC showed very weak membrane Trop-2 (mTrop-2) staining at metastases, most specimens (UC and its variants) showed moderate – high level of mTrop-2 staining (Fig. 3B–C), suggesting Trop-2 is a relatively conserved membrane target for UC from primary to metastasis.

### Correlation of Nectin-4 and Trop-2 mRNA and protein levels

In view of the notable membrane and cytoplasmic protein expression of both NEctin-4 and Trop-2, we questioned whether mRNA was useful in predicting localization of the proteins. For Nectin-4, we observed the *PVRL4* mRNA level was significantly correlated with cNectin-4 (Supplementary Fig. 2A; r = 0.55, $p < 0.001$) but not with mNectin-4 protein (r = 0.10, $p = 0.45$). For Trop-2, *TACSTD2* mRNA level was significantly correlated with both membranous Trop-2 (mTrop-2; Supplementary Fig. 2A; r = 0.36, $p = 0.01$) and cytoplasmic Trop-2 protein (cTrop-2; r = 0.42, $p = 0.002$). These correlations, despite statistically significant, are of moderate strength. Interestingly, a significant positive correlation between mNectin-4 and mTrop-2 (Supplementary Fig 2B; r = 0.42, $p = 0.002$) and cNectin-4 and cTrop-2 (r = 0.59, $p < 0.001$) was observed.

### Nectin-4 and Trop-2 protein expression in UC metastases

In query of Nectin-4 and Trop-2 protein expression in metastatic tumors, we observed similar levels in metastatic compared to primary sites, though Nectin-4 was enriched in the cytoplasm in metastases (Fig. 3C and Fig. 4A, p<0.0001). Trop-2 was highly expressed in

primary and metastatic sites (Fig. 3B–C and Fig. 4B), except for NE histology. Despite the small sample size, we found no significant difference in Nectin-4 or Trop-2 protein expression in BC vs upper tract UC (data not shown), and in specimens post-chemotherapy or immunotherapy (vs naïve; Supplementary Fig. 3).

## Discussion

Our study reports for the first time prominent cytoplasmic staining of both Nectin-4 and Trop-2, in addition to their anticipated membrane staining in UC. Specifically, Nectin-4 is enriched in the cytoplasm within non-NE histologic variants in BC, especially within metastatic specimens. Trop-2 proteins (membrane and cytoplasmic) are expressed at moderate-high levels in both primary and metastatic specimens within UC and non-NE histologic variants. These data support prospective trials of ADC targeting Nectin-4 and Trop-2 in mUC variant histology.

Nectin-4 and Trop-2 protein expression have been reported in primary UC and its variants, as well as some metastatic UC. Nectin-4 expression is high/moderate in 60% of primary UC[16] and its expression is heterogeneous in primary UC histologic variants[5,6]; whereas Trop-2 is expressed at high level in most primary UC[17]. However, these important observations on clinical specimens were exclusively focused on total protein staining. Although Nectin-4 protein expression was detected for all UC specimens available in the EV-201 trial, the objective response rate (ORR) is 44–52%[12,18]. Likewise, Trop-2 is thought to be commonly and highly expressed in UC[19] and therefore staining was not included as an inclusion criteria for TROPHY-U-01 trial, where the ORR is only 27%[3]. These results demonstrating total target protein expression (without differentiating membrane or cytoplasm) argue many of the patients expressing Nectin-4 or Trop-2 did not respond to ADCs. This discordance between abundant target expression and limited therapeutic response of ADCs raised questions regarding the subcellular localization of these otherwise membrane protein targets.

In our study, cytoplasmic staining of Nectin-4 and Trop-2 is a common event in addition to the anticipated membrane staining. A recent report found that low mNectin-4 was associated with reduced progression-free survival on EV[20], supporting the relevance of confirming membrane presence of the protein. The same study reported a decrease in mNectin-4 within metastatic specimens compared to primary[20]. However, we observed the expression of both Nectin-4 and Trop-2 are largely conserved from primary to multiple metastatic sites within an individual in patients within conventional UC and non-NE variants. Additionally, despite the heavily treated nature in this cohort, Nectin-4 and Trop-2 protein expression did not appear to be impacted by prior chemotherapy or immune checkpoint inhibitor (ICI) exposure.

The clinical implications of subcellular localization of ADC targets have been scantly described in other malignancies. One study identified primarily cytoplasmic localization of Nectin-4 in hepatocellular carcinoma, which is associated with worse progression-free and overall survival[21]. In contrast, mNectin-4 expression predicted a shorter disease-free and metastasis-free survival compared to cNectin-4 expression in patients with breast cancer[22].

A study in breast cancer identified that mTrop-2 was associated with a significantly higher risk of relapse and death[23]; and loss of mTrop-2 was associated with SG resistance in triple-negative breast cancer[24]. In our limited cohort, we did not observe differences in overall survival based on localization of either protein (data not shown) and none of our patients received either ADCs.

Previous studies have focused on primary UC and its variants, and our study brings in perspectives on Nectin-4 and Trop-2 expression in more aggressive metastatic variants which treatments are essentially non-existing. While UC and UCSD has reasonable levels of mNectin-4 staining, we find the mNectin-4 staining within PUC is rare. Conversely, Trop-2 is a more commonly (over 90%) expressed targets in both membrane and cytoplasm for both UC and UCSD, and was robustly observed in PUC (membrane 59% and cytoplasmic 100%). In view of the prominent cytoplasmic staining for both Nectin-4 and Trop-2, it may be reasonable to ascertain the presence of membrane staining before commencing ADC therapy and evaluate the biological implication for cytoplasmic retention of the protein.

Our study reports strong correlation between Nectin-4 and Trop-2 expression at both mRNA and protein levels. Clinically, responses with SG were noted after progression on EV in advanced UC[3]. As such, studies evaluating the combination (NCT04724018) or the optimal sequence of ADCs are of clinical importance. Our study showed mRNA levels were associated with cNectin-4, but weak surrogates for mNectin-4. In light of the potential importance of mNectin-4 on durability of EV response[20], it is logical to evaluate membrane presence of Nectin-4, rather than mRNA levels, within ongoing trials.

Our study is limited by the small sample size which prevents robust clinical correlations and comparison across different metastatic sites. We did not observe significant differences based on tumor site (BC vs UTUC), chemotherapy, and immunotherapy, yet larger sample sizes are needed to confirm this. While our research identifies prominent cytoplasmic staining of both Nectin-4 and Trop-2, the biological significance of such enrichment or retention remains to be evaluated. It would be clinically important to observe if the loss of membrane staining (or the retention of cytoplasmic staining), not the mRNA level in general, is associated with treatment resistance.

To our knowledge, this unique rapid autopsy cohort represents a comprehensive mRNA and protein expression, and subcellular localization of novel ADC targets in matched primary and metastatic UC and its variants. Nectin-4 and Trop-2 are expressed within UC and non-NE variants and correlate strongly with each other at both mRNA and protein levels. PUC and UCSD were enriched with cytoplasmic Nectin-4, especially in metastatic sites. Trop-2 was expressed highly in both the membrane and cytoplasm in UC and non-NE variants. The clinical impact of Nectin-4 and Trop-2 localization and expression warrants further investigation. Taken together, the moderate to high expression of Nectin-4 and Trop-2 in variant histology BC (except in NE type), at both primary and metastatic sites, provides evidence supporting clinical trials evaluating ADCs in BC variants.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

**Conflict of interest:**

Evan Yu:

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Other authors declare no potential conflicts of interest.

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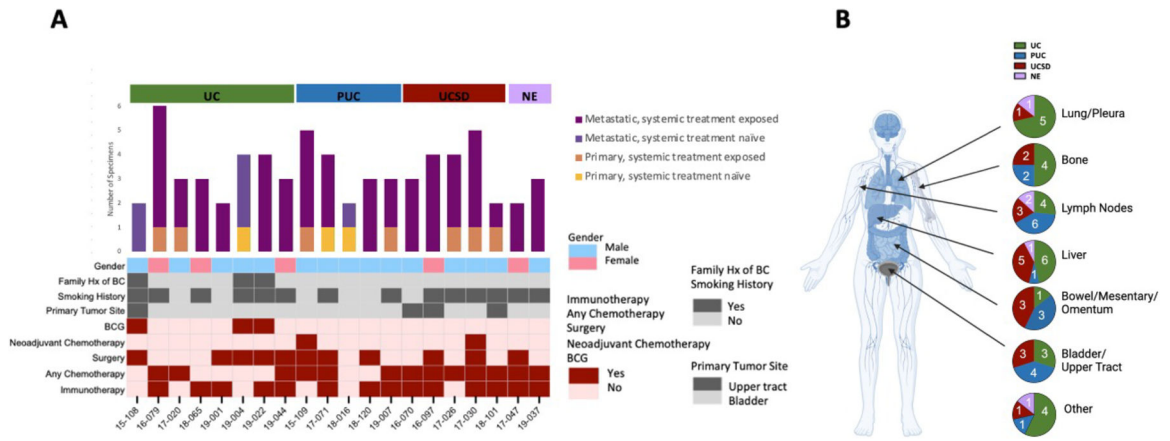
**Fig. 1. Clinical and specimen information for the bladder cancer rapid autopsy series.**
A) Demographics, treatment history, and number of specimens (n=67) analyzed for each patient. B) Tissue source and histology of specimens analyzed.
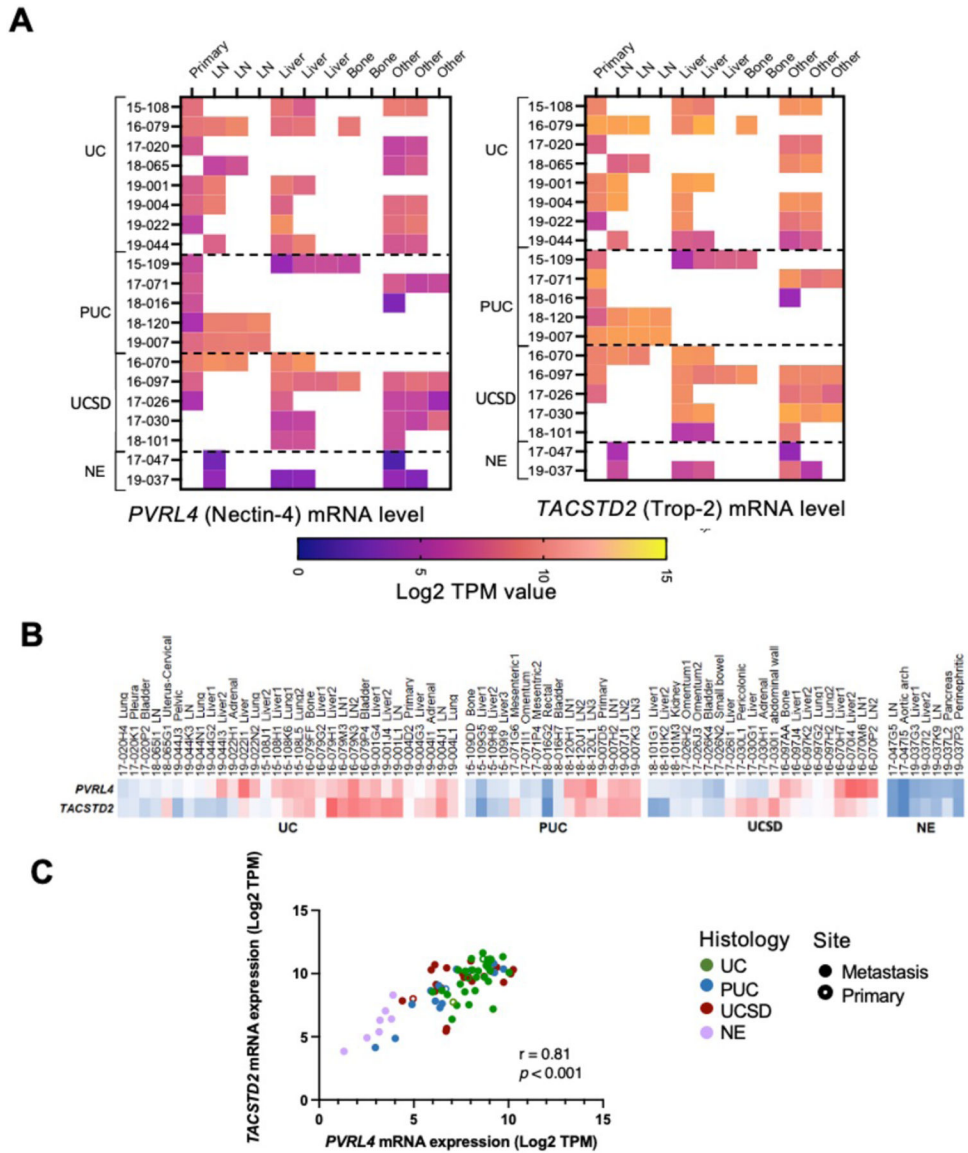
**Fig. 2. RNA expression by specimens in the bladder cancer rapid autopsy series.**
A) Absolute RNA expression represented by log2TPM (Transcripts Per Million) value for PVRL4 (Nectin-4) and TACSTD2 (Trop-2). LN denotes lymph node, 'other' includes lung, pleura, omentum, mesentery, bowel, vasculature, adrenal gland, pancreas, and abdominal wall. B) Mean-centered RNA expression comparison among the 76 specimens from A). Red denotes higher gene expression; blue denotes lower gene expression. C) Correlation analysis of PVRL4 and TACSTD2 with Pearson correlation coefficient, r = 0.81, $p < 0.001$.
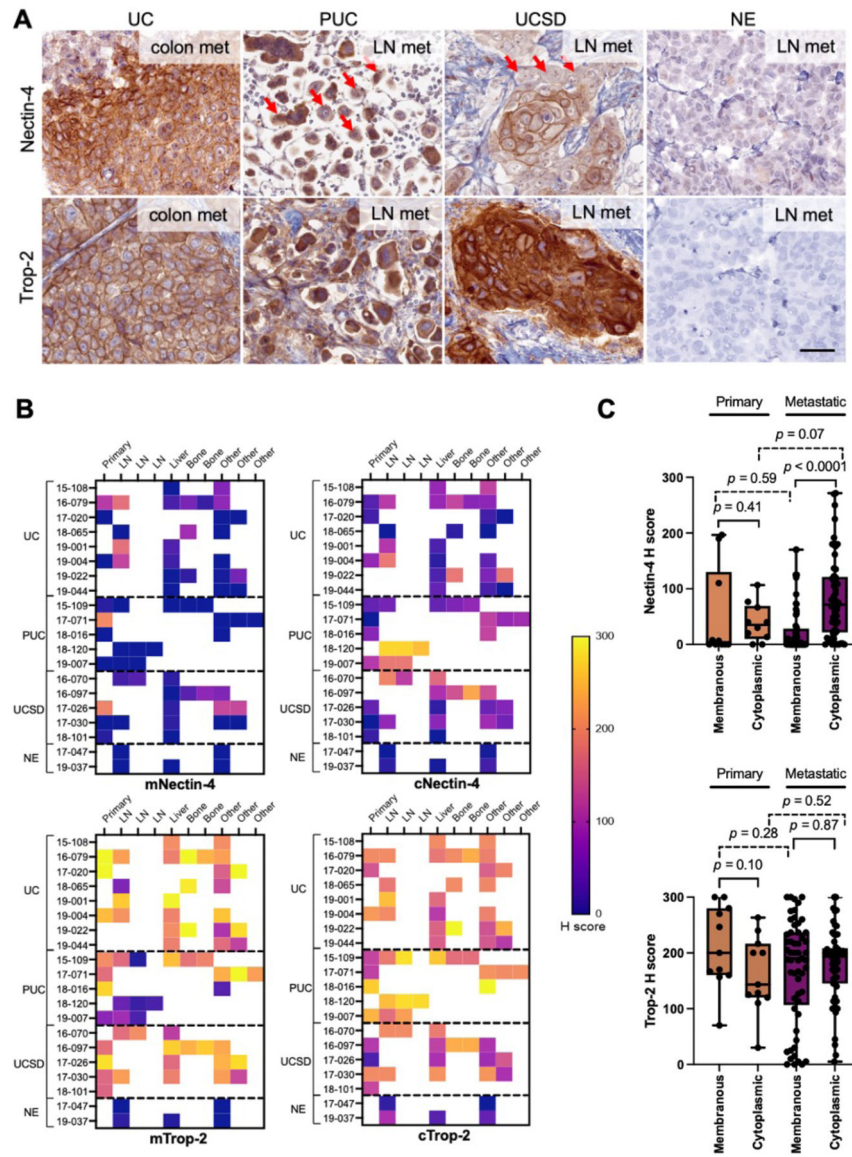
**Fig. 3. Nectin-4 and Trop-2 expression in primary and metastatic BC variants.**
A) Representative Nectin-4 and Trop-2 IHC images by histology. Both Nectin-4 and Trop-2 showed prominent membranous and cytoplasmic staining in non-NE variants. In PUC and UCSD, cells with predominantly cytoplasmic staining were noted for Nectin-4 (red arrows). Magnification:400×, scale bar = 50μm. B) H score by specimen (n=67), separated for membranous and cytoplasmic staining. LN denotes lymph node, 'other' includes lung, pleura, omentum, mesentery, bowel, vasculature, adrenal gland, pancreas, and abdominal wall. C) Box-and-whisker plot of primary and metastatic H scores, separated by membranous and cytoplasmic subcellular localization (n=67 each). The P value from Mann-Whitney U test was shown.

**A**
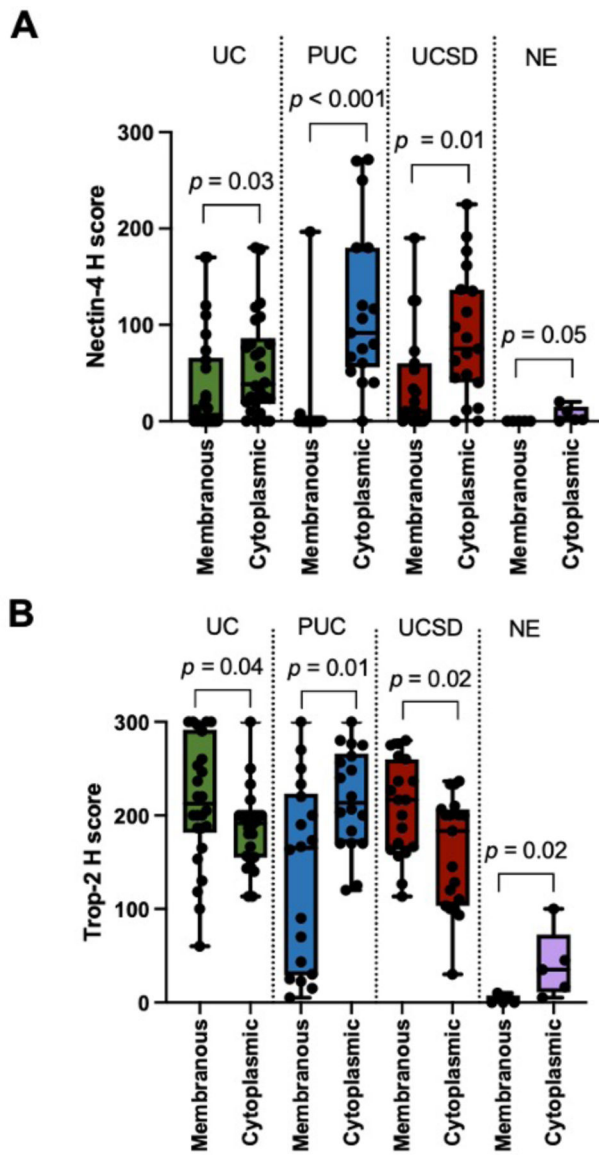


| | | Nectin-4 | |
|---|---|---|---|
| **Histology** | **n** | **Membranous** | **Cytoplasmic** |
| UC | 27 | 8 (30%) | 15 (56%) |
| PUC | 17 | 1 (6%) | 16 (94%) |
| UCSD | 18 | 8 (44%) | 15 (83%) |
| NE | 5 | 0 (0%) | 0 (0%) |
| **Site** | | | |
| Primary | 10 | 3 (30%) | 6 (60%) |
| Metastatic | 57 | 14 (25%) | 40 (70%) |

**B**



| | | Trop-2 | |
|---|---|---|---|
| **Histology** | **n** | **Membranous** | **Cytoplasmic** |
| UC | 27 | 25 (93%) | 27 (100%) |
| PUC | 17 | 10 (59%) | 17 (100%) |
| UCSD | 18 | 18 (100%) | 17 (94%) |
| NE | 5 | 0 (0%) | 1 (20%) |
| **Site** | | | |
| Primary | 10 | 9 (90%) | 9 (90%) |
| Metastatic | 57 | 56 (98%) | 52 (91%) |

**Fig. 4. Protein expression level and box-and-whisker plot for each histology and specimen site for A) Nectin-4 and B) Trop-2.**
The P value from Mann-Whitney U test is shown in the box-and-whisker plot.

**Table 1.**

Treatment information for individual bladder cancer patient in the rapid autopsy cohort.

| Patient | Gender | Race (C:Caucasian, A: Asian) | Surgery | BCG | Chemotherapy | PD-1/PD-L1-based Immunotherapy | Radiation (site) | Other treatment(s) |
|---|---|---|---|---|---|---|---|---|
| 15–108 | M | C | Y | Y | None | None | Lung | |
| 15–109 | M | C | Y | N | Gemcitabine and cisplatin (GC), Docetaxel | None | No | Rapamycin |
| 16–070 | M | C | N | N | Gemcitabine and cisplatin (GC), Docetaxel, Gemcitabine and Paclitaxel | Atezolizumab | No | OGX427–02, IMC-18F1, Pemetrexed, AGS-15E, Eribulin |
| 16–079 | F | C | N | N | Gemcitabine and carboplatin, Docetaxel | Atezolizumab | No | |
| 16–097 | F | C | Y | N | Gemcitabine and cisplatin (GC) | Atezolizumab | No | AGS-15E |
| 17–020 | M | A | N | N | Gemcitabine and carboplatin, Docetaxel | None | No | |
| 17–026 | M | C | N | N | Gemcitabine and cisplatin (GC) | None | Bladder and pubic ramus | |
| 17–030 | M | C | Y | N | Gemcitabine and cisplatin (GC) | Atezolizumab | | Rapamycin, Pemetrexed |
| 17–047 | F | C | Y | N | None | Atezolizumab | Pelvis | |
| 17–071 | M | C | Y | N | Gemcitabine and cisplatin (GC), Cisplatin | Pembrolizumab | Pelvis | |
| 18–016 | M | C | N | N | None | None | | |
| 18–065 | F | C | N | N | None | Pembrolizumab | | |
| 18–101 | M | C | N | N | Gemcitabine and cisplatin (GC) | Pembrolizumab | | |
| 18–120 | M | C | Y | N | accelerated MVAC (aMVAC) | Pembrolizumab | | |
| 19–001 | M | C | Y | N | None | Atezolizumab | | |
| 19–004 | M | C | Y | Y | None | None | Bladder | |
| 19–007 | M | C | N | N | Gemcitabine and cisplatin (GC) | Atezolizumab | Bladder, brain | |
| 19–022 | M | C | Y | Y | None | Pembrolizumab | Lung | |
| 19–037 | M | C | N | N | Etoposide and Cisplatin (EP), Paclitaxel | Pembrolizumab | Brain, spine | |
| 19–044 | F | C | Y | N | Gemcitabine and cisplatin (GC) | Atezolizumab | | |

M: Male, F: Female

C:Caucasian, A: Asian

Y: Yes, N: No