

Bladder Cancer

Concordance and prognostic value of antibody drug conjugate and checkpoint inhibition targets in matched bladder cancer specimens from transurethral resection of the bladder, radical cystectomy and lymphadenectomy

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Abstract

**Background:** Concordance of NECTIN-4 gene and protein expression and clinic-pathologic associations in matched bladder cancer (BC) triplets, defined as histological specimens from transurethral resection of the bladder (TUR-B), radical cystectomy (RC), and lymphadenectomy (LA) from the same patient, remains unknown. With Enfortumab Vedotin (EV) potentially moving into earlier treatment settings, understanding expression of NECTIN-4 and other targets in archival tissue samples is important. This study characterized protein expression of NECTIN-4 and gene expression of *NECTIN4*, *TACSTD2*, *ERBB2*, *PD-L1* and *PD1* in matched BC triplet specimens and correlated findings with survival endpoints.

**Methods:** This retrospective study analyzed NECTIN-4 protein expression and gene expression of 6 target genes using immunohistochemistry and quantitative real-time polymerase chain reaction in matched TUR-B, RC, and LA tissue samples from 27 patients with BC. *NECTIN-4* was validated in 3 independent BC cohorts. Protein and gene expression were correlated with clinic-pathologic characteristics. Prognostic associations with survival were analyzed using Kaplan-Meier estimates, log-rank tests and Cox proportional hazard models.

**Results:** Median NECTIN-4 protein expression differed significantly across tissues types with highest levels in TUR-B, lower levels in RC, and intermediate levels in LA specimens. Quantitative analysis confirmed significant differences in H-scores across specimens. Expression of *NECTIN-4*, *TACSTD2*, *PD-L1* and *ERBB2* varied significantly across triplets. NECTIN-4 protein expression was not associated with overall or progression-free survival, whereas *NECTIN-4* gene expression showed significant but contradictory survival associations in different BC cohorts.

**Conclusion:** NECTIN-4 expression differs by tissue type, limiting prognostic value and supporting site-specific biomarker assessment in BC. © 2026 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

**Key words:** Bladder cancer; NECTIN-4; Immunohistochemistry; Radical cystectomy, matched bladder cancer triplets

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## 1. Introduction

Bladder cancer (BC) is one of the most common cancer types in western countries with an estimated 84,870 new cancer cases and 17,420 new deaths in the United States in 2025 [1].

Recent advances in drug development have expanded the landscape of BC treatment options culminating in a paradigm shift in the first line treatment of patients with metastatic BC [2].

The combination of the antibody-drug-conjugate (ADC) Enfortumab Vedotin (EV) and the immune checkpoint inhibitor (ICI) Pembrolizumab (*P*, combination therapy referred to as EVP) nearly doubled the median overall survival (OS) compared to the control arm of standard chemotherapy in the EV-302 trial and is a new standard of care in metastatic BC [2]. EV acts through binding to the protein NECTIN-4 and subsequently releases a cytotoxic payload. The predominant expression of NECTIN-4 on tumor cells, particularly on urothelial cancer cells [3,4] has led to the development and approval of EV as a third line monotherapy option in patients with metastatic BC [5]. However, currently no biomarker stratification for NECTIN-4 or PD-L1 using immunohistochemistry (IHC) is performed prior to receiving EVP or EV monotherapy.

Despite an impressive 37.1% progression-free survival (PFS) rate at 24 months not all patients respond and treatment options beyond this point are severely limited [6]. As EVP and potentially other ADC combinations move further into the perioperative setting new questions arise. In this context, optimally stratifying patients for these treatments and selecting the most appropriate tissue source for testing the target expression will become particularly important. This is amplified by the current expansion of ADC treatment options in BC with approved ADC such as TROP2 and potential approvals such as Disitamab Vedotin which targets HER-2 and has already been granted conditional approval in HER2-expressing, locally advanced or metastatic BC following platinum-based chemotherapy in China based on phase II data with an ongoing phase III study [7].

To understand the use of novel ADCs in earlier therapy lines this retrospective study sought to characterize (i) the prevalence of the NECTIN-4 protein and gene expression in BC matched triplets, i.e., in different histological tissue specimens from the same patient obtained at transurethral resection of the bladder (TUR-B), radical cystectomy (RC) and lymphadenectomy (LA) to sample archival tissue from lymph node metastases. Additionally, the protein and mRNA level of *NECTIN-4* were compared and the gene expression of other ADC and ICI targets was analyzed in matched BC triplets. Expression information was (ii) associated with clinicopathological characteristics and (iii) correlated with

survival of patients with BC. NECTIN-4 expression was validated in 3 independent cohorts.

## 2. Methods

### 2.1. Patient cohorts and sample characteristics

The protein expression of NECTIN-4 and the gene expression of the target genes *ERBB2* (encoding gene for HER-2), *PD1*, *PD-L1*, *NECTIN-4*, *TACSTD2* (encoding gene for TROP2), and *MKI67* was retrospectively analyzed in the Mannheim cohort 1 using matched Formalin-Fixed Paraffin-Embedded tissue (FFPE) samples and clinic-pathologic data from 27 patients who underwent TUR-B and RC with concomitant LA between 2015 and 2020 at the Department of Urology and Uro-surgery at the University Medical Centre Mannheim, University Hospital Heidelberg – UK Mannheim, Medical Faculty Mannheim, University of Heidelberg.

A second, different cohort of 160 patients with muscle-invasive BC (MIBC), the Mannheim cohort 2, who underwent RC at the Department of Urology and Uro-surgery at the University Medical Centre Mannheim (University Hospital Heidelberg – UK Mannheim) between 2008 and 2014 was used to assess the prognostic impact of the *NECTIN-4* gene expression.

Findings from the Mannheim cohort 2 were validated in 2 independent cohorts of patients with BC. The first validation cohort was comprised of MIBC patients with FFPE tissue samples from RC from The Cancer Genome Atlas (TCGA), a publicly available multi-institutional repository of gene expression and clinical data [8]. The second validation cohort was a cohort of BC patients that underwent RC and LA at Chungbuk University hospital with RNA expression measured on FFPE tissue using microarrays [9].

Patients with no histologic evidence of residual disease after RC, non-muscle invasive BC, non-urothelial histologic subtype, distant metastases at RC or missing detection of the reference Ygene *Calmodulin 2* (*Calm2*) were excluded from subsequent analyses (Supplementary Fig. 1, A). Pathological TNM staging and grading was performed using the 2004 and 2016 World Health Organization (WHO) classifications and the Tumor, Node, Metastasis (TNM) Classification (2017, 8th edition and 2009, 7th edition). A local ethics approval under the number 2015-549N-MA in line with the standards of the Declaration of Helsinki was already in place before the start of this study. All patients provided written informed consent to participate in this study.

### 2.2. In silico validation of findings

Findings from the Mannheim cohort 2 were validated in 2 independent cohorts of patients with MIBC from The Cancer Genome Atlas (TCGA) and the Chungbuk University hospital [8,9]. Supplementary Fig. 1, B and C, show patient exclusion criteria for the TCGA and the Chungbuk

cohort. All TCGA data were downloaded from public repositories and have been produced in earlier analyses. CNA and gene expression data were downloaded from the Xenabrowser (<https://xenabrowser.net>) [10]. Clinical data were downloaded from cBioPortal (<https://www.cbioportal.org>) [11,12]. CNA data in the TCGA cohort were generated using Affymetrix SNP6.0 arrays and mRNA gene expression data were profiled using RNA Sequencing on an Illumina HiSeq. Data underwent further bioinformatic processing. CNA data were curated with the GISTIC2.0 algorithm [13] and gene expression data quantified and normalized using RNA-Seq by Expectation Maximization (RSEM) and expressed as  $\log_2$ . CNA data ranged from  $-2$  to  $2$ , with the following nomenclature applied:  $-2$ : 2 copy del;  $-1$ : 1 copy del;  $0$ : no change,  $1$ : amplification,  $2$ : high amplification. For the purposes of this study a GISTIC2.0 value of  $2$ , or high amplification defined NECTIN-4 amplification. All other gene-level events ( $-2$  to  $1$ ) were defined as non-amplification.

Clinical and genomic data for the Chungbuk cohort were obtained through the Gene Expression Omnibus database (GSE13507) and are included in the [supplementary material](#) [9]. Gene expression in the Chungbuk cohort was measured on Illumina Human-6 BeadChip microarrays using tissue from RC [9].

### 2.3. Histopathology and IHC

Histopathological assessment and IHC for NECTIN-4 and PD-L1 expression were performed by a trained pathologist (BR). NECTIN-4 IHC staining was performed on BC triplets, while PD-L1 was only stained on RC specimens, mirroring current clinical practice.

The NECTIN-4 antibody used for IHC staining was a recombinant monoclonal antibody procured from Abcam [EPR15613-68] (ab192033). PD-L1 was stained using the clone CAL-10 from Biocare Medical (AVI 3171 G). Staining was performed according to the manufacturer's instructions using a VENTANA BenchMark ULTRA autostainer at the recommended dilution of  $1/3000$  (Nectin-4) and  $1/25$  (PD-L1). For Nectin-4 both membranous and cytoplasmic expression were investigated. However, both stains were almost always congruent.

A histochemical scoring system (H-score) was used to classify the staining reaction of NECTIN-4. Briefly, the H-score is the sum of the product of the staining intensity ( $0; 1; 2; 3$ ) and the percentage of cells stained at each level of intensity ( $0-100$ ) [14]. The final score ranges from  $0$  to  $300$  and gives rise to 4 different categories: negative ( $0$ ; H-score,  $0-14$ ), low ( $1+$ ; H-score,  $15-99$ ), medium ( $2+$ ; H-score,  $100-199$ ) and high ( $3+$ ; H-score,  $200-300$ ) [14].

NECTIN-4 protein expression was stratified into low and high expression by applying 3 different cut-off values. First, an H-score level of  $100$  as employed by Klümper et al. [15] and Challita-Eid et al. [16] (negative/weak versus moderate/strong) was used. Next, the cut-off level of  $150$  from the EV-101 trial

was employed [5]. As a third option, NECTIN-4 expression was divided based on an H-score with a cut-off of  $200$  [17,18].

PD-L1 expression was assessed using the tumor proportion score (TPS), the immune cell score (IC) and the combined positivity score (CPS). The TPS measures PD-L1 on viable tumor cells, the IC assesses PD-L1 on immune cells, and the CPS combines both the TPS and the IC score. The IC score was divided into the following categories: IC  $0 < 1\%$ , IC 1  $1-5\%$ , IC 2  $5-10\%$ , and IC 3  $> 10\%$ . Specimens with a CPS  $\geq 10\%$  and/or an IC score  $\geq 2$  were classified as PD-L1 positive [19].

### 2.4. qRT-PCR

RNA was extracted from  $10 \mu\text{m}$  FFPE sections with the magnetic-based XTRAKT FFPE kit (Stratifyer, Cologne, Germany) according to the manufacturer's instructions. For the qRT-PCR analyses, RNA was reversely transcribed into cDNA with sequence-specific reverse primers (reference gene *Caln2* and target genes *ERBB2*, *PDI*, *PD-L1*, *NECTIN-4*, *TACSTD2* and *MKI67*). Superscript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and supporting reagents were incubated at  $55^\circ\text{C}$  for  $120$  min with a subsequent enzyme deactivation step at  $70^\circ\text{C}$  for  $15$  min. The resulting cDNA was amplified through  $40$  cycles at  $95^\circ\text{C}$  for  $3$  s and  $60^\circ\text{C}$  for  $30$  s on a StepOne-Plus qRT-PCR cycler (Applied Biosystems, Waltham, MA, USA). *Caln2* was used as a reference gene for normalization and gene expression determined using the  $40-(\Delta\text{Ct})$ -method [20]. [Supplementary Table 1](#) shows the primers and probes used in this study.

### 2.5. Statistical analysis

Statistical analyses were performed with JMP<sup>®</sup> 16.0.0. (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism version 10.4.1 (GraphPad Software, San Diego, CA, USA). Continuous outcomes were reported with median and range. Categorical variables were provided with absolute and relative frequencies. Group differences between categorized variables were assessed with the Chi-Square Test, Fisher's Exact Test and the Bannard Test. Differences in continuous variables were investigated with the Wilcoxon signed rank test, the Friedman-Test and the Kruskal-Wallis test. Survival rates were calculated with the Kaplan-Meier method and subgroups were compared using the log-rank test. Cut-off values for high and low gene expression groups were provided by using the partition test, with each group representing at least  $20\%$  of the total cohort. Correlation analyses were conducted using the Spearman correlation coefficient method, applying specific cut-off values to interpret correlation strength (weak:  $<0.39$ , moderate:  $0.4-0.69$ , strong:  $0.7-0.89$ , very strong  $0.9-1.0$ ) [21].

Statistical significance of prognostic variables in univariable and multivariable analyses was investigated using Cox proportional hazard models. Multivariable analyses

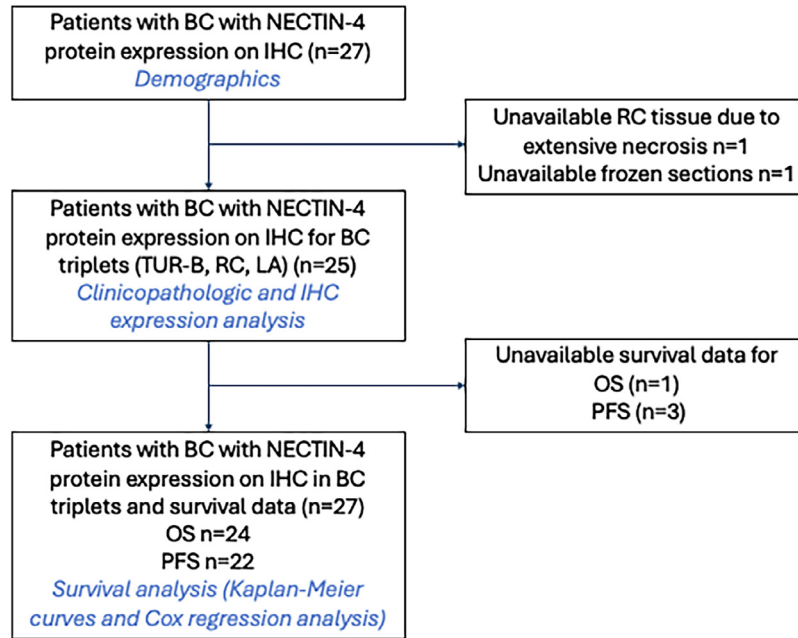


Fig. 1. REMARK diagram of the Mannheim I cohort. Immunohistochemical (IHC) expression of Nectin-4 was available in 27 bladder cancer (BC) patients. Two of these patients were excluded, with 25 BC patients remaining for subsequent analysis stages (indicated in blue). Nectin-4 IHC expression was correlated with clinicopathologic data and overall survival (OS) and progression-free survival analyses were performed.

considered factors with a  $p$  value  $\leq .2$  on univariable analysis. A 2-sided  $p$  value  $\leq .05$  was considered statistically significant.

### 3. Results

#### 3.1. Clinicopathological characteristics of the Mannheim cohort 1 are comparable to other contemporary RC cohorts

From the 27 cases with matched TUR-B, RC and LA samples 25 cases had complete staining of the NECTIN-4 protein in each histological tissue specimen.

Although this represents one of the largest available matched triplet cohorts, the statistical power is reduced by the limited sample size. Fig. 1 shows the exclusion criteria and different analysis stages for the Mannheim cohort 1. Clinical and pathological characteristics of the Mannheim cohort 1 are presented in Table 1. Two patients received inductive/neoadjuvant chemotherapy. Demographics were comparable to large contemporary cohorts of RC reported in the literature [22].

#### 3.2. NECTIN-4 protein expression varies between TUR-B, RC and LA samples in the Mannheim cohort 1

Typical patterns of NECTIN-4 IHC expression in BC triplets are shown in Fig. 2.

Table 1  
Patient demographics and histopathologic data of the Mannheim cohort 1.

Characteristic	Outcome parameter (n)
Age, Years <sup>±</sup>	75 (65; 78)
Sex <sup>°</sup>	
Male	21 (78)
Female	6 (22)
Pathologic Stage	
Primary tumor TUR-B (n = 25) <sup>°</sup>	
Ta	2 (8)
T1	4 (16)
Tis	4 (16)
T2	11 (44)
T3	3 (12)
T4	1 (4)
Grading (WHO 2016) (n = 25) <sup>°</sup>	
High grade	24 (96)
Primary tumor RC (n = 27) <sup>°</sup>	
pT1	3 (11)
pTis	3 (11)
pT2	2 (7)
pT3	13 (48)
pT4	6 (22)
Pathological N-stage <sup>°</sup>	
pN1	11 (41)
pN2	16 (59)
Surgical margin status (n = 26) <sup>°</sup>	
Negative	24 (92)
Positive	2 (8)

<sup>±</sup> median (interquartile range) <sup>°</sup> absolute frequency (percentage).

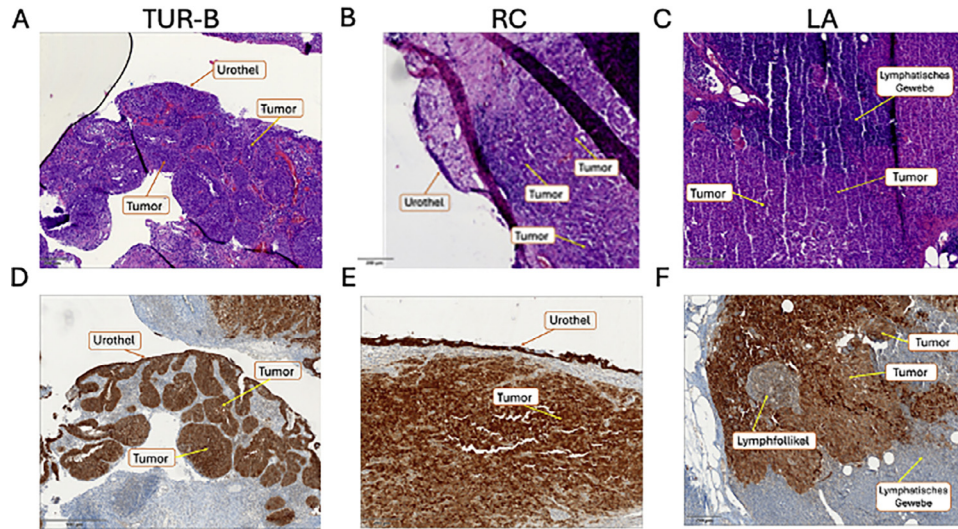


Fig. 2. Typical staining patterns of immunohistochemical (IHC) Nectin-4 expression in bladder cancer triplets. (A-C) TUR-B, RC and LA with hematoxylin eosin (HE) staining and (D-F) Nectin-4 IHC staining.

Most samples showed a medium (H-score 100–199) or high NECTIN-4 expression (H-score 200–300) [14]. The NECTIN-4 H-score decreased from TUR-B specimens to RC and LA samples (Supplementary Table 2). Comparison of the median H-scores of these different tissue types revealed a significant overall difference in NECTIN-4 expression (Friedmann-Test:  $p < .0001$ , Supplementary Table 2). Pairwise comparisons of NECTIN-4 between tissue types showed a significant differential expression (Wilcoxon signed rank test for each comparison:  $p < .0001$ ).

For a potentially more realistic application of a cutoff for the NECTIN-4 protein expression in the clinic, the 4 categories of the H-score were combined into low and high expression, relying on the 3 different cut-off-levels described in section (Histopathology and IHC) and shown in Supplementary Table 3.

Group sizes of low and high expression varied between the 3 defined cut-off levels. At a cut-off level of 100 or 200, RC and LA samples showed almost similar group sizes for low and high NECTIN-4 expression. An exploratory comparison of low and high expression between archival tissue sample types revealed an almost identical and statistically significant distribution of low and high expression groups between RC and LA (Supplementary Table 4: cut-off value 100,  $P = .04$  and Supplementary Table 6: cut-off value 200,  $P = .03$ ).

Further investigation into the relationship of the NECTIN-4 protein expression between different archival tissue specimens revealed a significant and moderately high correlation between RC and TUR-B as well as RC and LA (Supplementary Table 7, Spearman correlation:  $r = 0.6$ ,  $P = .0004$  and  $r = 0.65$ ,  $P = .0004$ ). PD-L1 protein

expression data were available for analysis in 21 RC tissue specimens, of which only 2 had a positive PD-L1 status as indicated by a CPS  $\geq 10\%$  and none of the tumor samples was PD-L1 positive as per IC score definition ( $>2\%$ ) (Supplementary Table 8).

While the observed differences reached statistical significance, they should be interpreted as hypothesis-generating given the small cohort size and multiple exploratory comparisons performed.

### 3.3. Gene expression of NECTIN-4, PD-1, PD-L1 and selected ADC targets in the Mannheim I cohort

A total of 6 candidate genes were measured in the Mannheim cohort 1. Of those the ADC targets *ERBB2* and *TACSTD2* as well as the proliferation marker *MKI67* showed the highest median expression overall (Supplementary Table 9, Fig. 3). To detect significant differences in the mean or median expression of these genes between archival tissue sample types, oneway ANOVA analysis and the Kruskal-Wallis test were performed, based on the distribution of the data. Significant differences in the ANOVA analysis could be detected for *PD-L1* ( $p = .0114$ ) and *NECTIN-4* ( $p = .0416$ ) expression. The Kruskal-Wallis test revealed significant variation in the median gene expression of *ERBB2* ( $p = .0032$ ) and *TACSTD2* ( $p = .0001$ ) (Fig. 3). Post hoc analysis using the Tukey test showed significant differences for *NECTIN-4* between TUR-B and RC specimens ( $p = .0431$ ), while *PD-L1* was significantly different between RC and LA ( $p = .0113$ ). *NECTIN-4* expression was highest in TUR-B material, while *PD-L1* showed the greatest

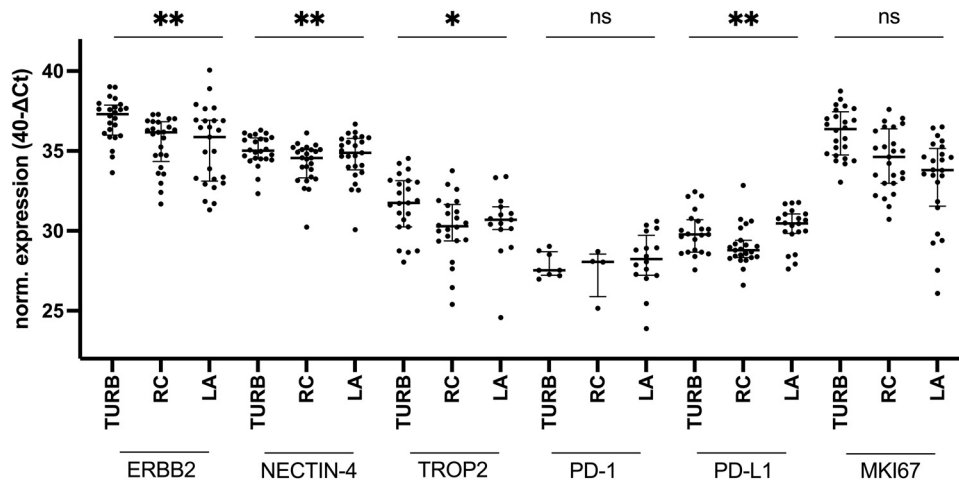


Fig. 3. Gene expression of the 6 target genes in the Mannheim I cohort. The 40-ΔCt method was used for normalization. Significant variation in the median gene expression results of the Kruskal-Wallis test or ANOVA are indicated with a \* for a *p*-value of ≤.05 and \*\* for a *p*-value of ≤.01. *P*-values for the respective genes: *ERBB2* (*p* = .0032), *TROP2* (*p* = .0001), *NECTIN-4* (*p* = .0416) and *PD-L1* (*p* = .0114).

expression in LA specimens. Post hoc analysis of differences detected by the Kruskal-Wallis test using Dunn’s test revealed significant differences between TUR-B and RC samples (*ERBB2*: *p* = .0075; *TACSTD2*: *p* = .0122) and TUR-B and LA samples (*ERBB2*: *p* = .0134; *TACSTD2*: *p* = .0001).

Investigating the gene expression of these 6 genes in the Mannheim cohort 1 stratified by high or low *NECTIN-4* protein expression according to cut-offs described in section (Histopathology and IHC), only revealed *ERBB2* to be significantly higher expressed in patients with a high *NECTIN-4* expression (cut-off 150, supplementary material) in their RC (high: 36.80 [34.13 – 37.24] vs. low: 34.99 [32.05 – 36.89] *p* = .0118) or their LA specimens (high: 36.49 [32.84 – 39.36] vs. low: 33.59 [31.36 – 36.93] *p* = .0458).

### 3.4. *NECTIN-4* gene expression in RC tissue specimens from patients with MIBC in the Mannheim cohort 2 with validation in the TCGA and Chungbuk cohorts

*NECTIN-4* gene expression could be analyzed in 131 patients with histologically confirmed urothelial MIBC (median age: 72, range: 64 – 78, 30% female patients, 76% locally advanced carcinomas (T3/4)) in the Mannheim cohort 2, after excluding 29 patients (from initially 160 patients). Validation of findings in this cohort was performed on data from 361 patients from the TCGA MIBC cohort (median age: 69, range: 60 – 77, 27% female patients, 68% T3/4 tumors) and the Chungbuk University hospital cohort (55 patients with MIBC, median age 66, IQR 60 – 73.5 years, 21% female). Demographic and clinic-pathologic data of these cohorts are shown in Table 2.

Table 2  
Demographics of patients in the Mannheim cohort 2, the TCGA and the Chungbuk cohort.

Characteristic	MA cohort ( <i>n</i> = 131) <i>n</i> (%)	TCGA cohort ( <i>n</i> = 360) <i>n</i> (%)	Chungbuk cohort ( <i>n</i> = 61) <i>n</i> (%)
Age (years)*	72 (64 – 78)	69 (60 – 77)	66 (60 – 73.5)
Gender			
Male	92 (70%)	263 (73%)	48 (21%)
Female	39 (30%)	97 (27%)	13 (79%)
Pathological T-stage			
T2	31 (24%)	116 (32%)	31 (51%)
T3	79 (60%)	188 (52%)	19 (31%)
T4	21 (16%)	56 (16%)	11 (18%)
Lymph node metastases			
negative	91 (76%)	216 (64%)	46 (77%)
positive	29 (24%)	122 (36%)	14 (23%)
NA	11	22	1

\* Median (range 25th to 75th percentile); NA: not available.

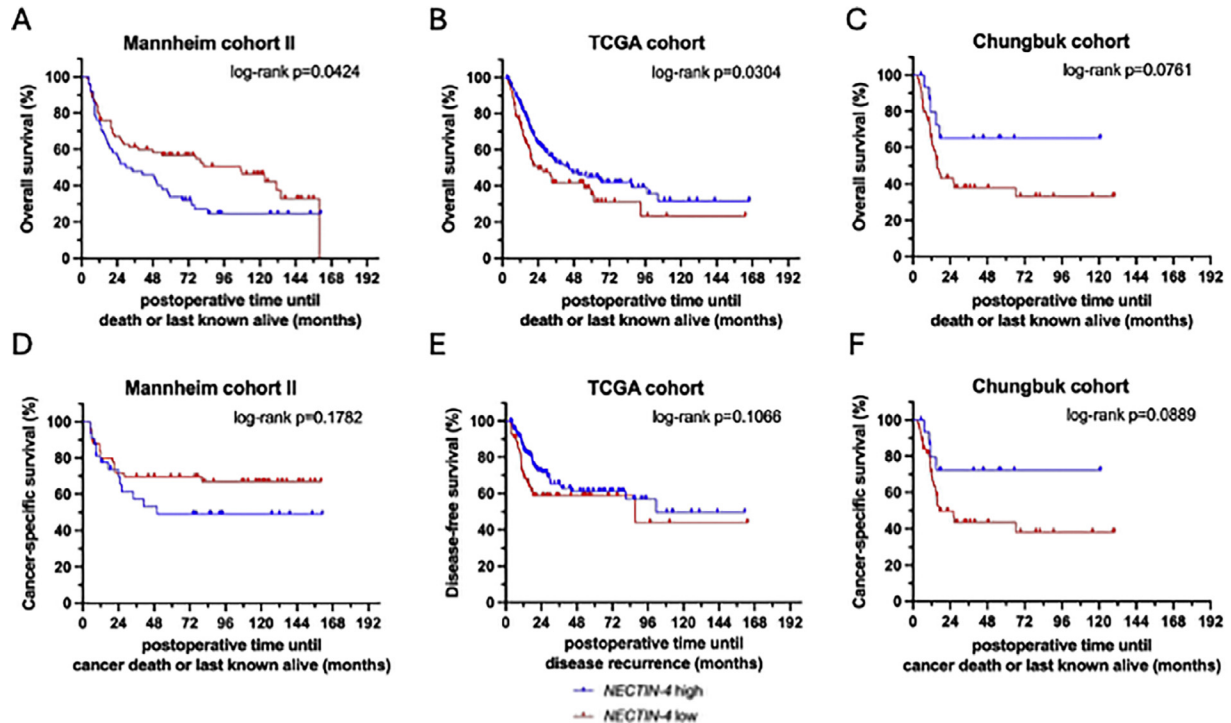


Fig. 4. Kaplan-Meier analyses of overall survival (OS) in (A) the Mannheim II, (B) the TCGA and (C) the Chungbuk cohort according to *NECTIN-4* expression status (high vs. low). Kaplan-Meier analyses of cancer-specific survival (CSS) in (D) the Mannheim II, (E) disease-free survival (DFS) in the TCGA and (F) CSS in the Chungbuk cohort according to Nectin-4 expression status (high vs. low). The blue line represents the high Nectin-4 gene expression, and the red line represents the low Nectin-4 gene expression group.

*NECTIN-4* gene expression was significantly higher in patients in whom lymphovascular invasion (LVI,  $p = .0044$ ) was present and in patients with lymph node metastases ( $N+$ ,  $p = .0004$ ) in the Mannheim cohort 2. In the TCGA cohort, male patients ( $p = .0008$ ),  $N+$  patients ( $N+$ ,  $p = .0257$ ) and patients with a *NECTIN-4* gene amplification ( $p < .0001$ ) showed a significantly higher *NECTIN-4* expression. No other differences in the *NECTIN-4* expression between different clinic-pathologic characteristics were detected (Supplementary Table 10).

### 3.5. Survival association of *NECTIN-4* protein expression in the Mannheim cohort 1

The median follow-up time after RC was 12 months (IQR 8 - 26.5). At the time of the last follow-up appointment, 15 (55.6%) patients were still alive, while 12 (44.4%) patients had died. Assessment of the prognostic value of the *NECTIN-4* protein expression (high vs. low) on OS ( $n = 24$ ) and PFS ( $n = 22$ ) was performed using the 3 cut-off levels (100, 150 and 200) described in section (Histopathology and IHC) (Supplementary Figs. 2-4, Supplementary Table 11.)

A high *NECTIN-4* protein expression ( $\geq 100$ ) on archival RC and LA tissue was associated with an improved OS (RC: Hazard ratio (HR): 0.67 [0.18; 2.55]  $p = .56$ ; LA: 0.92 [0.19; 4.33]  $p = .91$ ) and PFS (RC: HR: 0.75 [0.2; 2.84]  $p = .68$ ; LA: 0.86 [0.23; 3.18]  $p = .82$ ). Except for a

decrease in OS based on the *NECTIN-4* expression in the RC specimen (HR: 1.49 [0.45; 4.92]  $p = .51$ ) the observed associations persisted when increasing the IHC cut-off level to 150. A further increase to 200 reversed the observed survival associations (Supplementary Table 11). These inconsistent associations likely reflect limited statistical power and cutoff sensitivity in a small exploratory cohort rather than true biological reversal.

Multivariable analysis including variables with a  $p$ -value of  $\leq .2$  on univariable analysis could only be built for PFS (Supplementary Tables 12-14). None of the included variables reached statistical significance.

### 3.6. Survival association of the *NECTIN-4* gene expression in the Mannheim cohort 2, TCGA and Chungbuk cohorts

In the Mannheim cohort 2, a higher *NECTIN-4* gene expression was associated with a significantly shorter OS ( $p = .0424$ ; Fig. 4A). On the contrary, in the TCGA cohort ( $p = .0304$ ; Fig. 4B) and the Chungbuk cohort ( $p = .0761$ ; Fig. 4C) a higher *NECTIN-4* gene expression correlated with a longer OS. While similarly opposing patterns were also observed for the CSS in the Mannheim cohort 2 ( $p = .1782$ ; Fig. 4D) and in contrast to that DFS in the TCGA ( $p = .1066$ ; Fig. 4E) and CSS in the Chungbuk cohort ( $p = .0889$ ; Fig. 4F), none of these reached statistical significance.

Table 3

Uni- and multivariable cox regression analyses of gene expression and clinicopathological parameters regarding overall survival (OS) in patients with MIBC after radical cystectomy (RC) in the Mannheim cohort 2.

Parameter	Univariable analysis		Multivariable analysis	
	<i>P</i> value	HR (95% CI)	<i>P</i> value	HR (95% CI)
Gender (male vs. female)	.5133	0.85 (0.52 – 1.39)	-	-
Age (>75 years vs. ≤75 years)	<b>.0023</b>	1.97 (1.27 – 3.06)	.0052	2.10 (1.25 – 3.53)
T stage (T3/4 vs. T2)	<b>.0007</b>	2.82 (1.55 – 5.11)	<b>.0024</b>	2.82 (1.44 – 5.52)
N stage (positive vs. negative)	.0652	1.61 (0.97 – 2.68)	<b>.0236</b>	2.21 (1.11 – 4.38)
LVI (yes vs. no)	.1937	1.35 (0.86 – 2.14)	.4134	0.78 (0.43 – 1.42)
<i>NECTIN-4</i> expression (high vs. low)	.0457	1.59 (1.01 – 2.50)	.3166	1.30 (0.78 – 2.19)

Table 3 gives an overview of the HR of *NECTIN-4* gene expression in the uni- and multivariable analyses of these cohorts. The full models with all variables can be found in the [supplementary material](#). In the multivariable analysis of the Mannheim cohort 2 a high *NECTIN-4* gene expression did not remain a significant prognostic factor of worse OS (Table 3). However, in the TCGA cohort a high *NECTIN-4* gene expression persisted as an independent predictor of a favorable OS (Table 4). Overall, *NECTIN-4* showed diverging survival results between the Mannheim cohort 2 and the validation cohorts (Table 3, 4).

#### 4. Discussion

This retrospective study investigated gene and protein expression of *NECTIN-4* in matched tissue triplets from BC patients, consisting of TUR-B, RC and LA specimens derived from each individual patient. By directly comparing

spatially (different origin of BC tissue) and temporally (initial diagnosis at TUR-B and subsequent surgical removal with RC and LA) distinct tumor samples within the same patient we were able to quantify intra-patient target heterogeneity across clinically meaningful specimen types.

*NECTIN-4* IHC scores varied significantly within different archival tissue types from the same patients. This finding is clinically relevant, as *NECTIN-4* is used as the therapeutic target for EV. Our data indicate that biomarker results derived from one tissue type – particularly from early diagnostic TUR-B samples – may not reliably reflect target expression in the residual BC or lymph node metastases, which are currently the cancer that is treated systemically. While Klümper et al. found a decrease in membranous *NECTIN-4* expression during metastatic spread of BC [15] which supports our observed discordance, Challita-Eid and colleagues reported similar *NECTIN-4* IHC expression in primary tumors and metastases,

Table 4

Uni- and multivariable cox regression analyses of gene expression regarding overall survival (OS) and cancer-specific survival/disease-free survival in patients with MIBC after radical cystectomy in the Mannheim cohort 2, the TCGA MIBC and the Chungbuk cohort. Detailed descriptions of multivariable parameters are described in the [supplementary material](#).

Parameter	Overall survival				Cancer-specific survival/Disease-free survival			
	Univariable analysis		Multivariable analysis		Univariable analysis		Multivariable analysis	
	<i>P</i> value	HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value	HR (95% CI)
Mannheim II <i>NECTIN-4</i> expression (high vs. low)	<b>.0457</b>	1.59 (1.01 – 2.50)	.3166	1.30 (0.78 – 2.19)	.1244	1.78 (0.85 – 3.69)	.9243	1.04 (0.46 – 2.37)
TCGA Age (>75 years vs. ≤75 years)	.0503	0.70 (0.48 – 1.00)	.0330	0.61 (0.39 – 0.96)	.2300	0.77 (0.51 – 1.18)	n.a.	n.a.
Chungbuk <i>NECTIN-4</i> expression (high vs. low)	.4280	1.37 (0.63 – 3.02)	n.a.	n.a.	.6022	1.21 (0.59 – 2.52)	n.a.	n.a.

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indicating a stable expression pattern [16]. However, in the later study only 25 metastases, which were not matched to primary tumor samples, compared to 499 primary tumor specimens were investigated, which limits the ability of the study to detect clinically relevant intra-patient discordance. In contrast, our matched triplet approach demonstrates that NECTIN-4 expression can change substantially between primary and metastatic sites within the same patient, supporting the need for site-specific biomarker assessment.

The observed decrease in NECTIN-4 expression from TUR-B to RC and LA samples suggests that biomarker testing performed on diagnostic TUR-B specimens may overestimate target availability at the actual time point when systemic therapy is started. Conversely, some patients exhibited preserved or even higher expression in samples obtained at LA, which suggests that metastatic tissue sometimes reflects therapeutic vulnerability even better than the primary tumor. Thus, whenever feasible the most recent and clinically relevant tumor tissue – particularly metastatic tissue or samples post-cystectomy should be prioritized for NECTIN-4 testing for therapy selection with ADC.

Observed differences in NECTIN-4 expression between the studies by Challita-Eid et al. and Klümper et al. and our cohort should be interpreted with caution and in the context of different antibody clones that were used. In this study and in the seminal investigation by Klümper and colleagues [15], the anti-NECTIN-4 antibody EPR15613–68 (#ab251110, Abcam) was used. For the EV-101 study and the work by Challita-Eid et al. the anti-NECTIN-4 antibody clones M22–321b41.1 (used for EV-101) and M22–244b3 were used. Both clones were manufactured by Agensys Inc. and cannot be acquired commercially, patent ID CA3065514A1 [5,16,23].

Most samples in the Mannheim cohort 1 showed a medium (H-score 100–199) or high NECTIN-4 expression (H-score 200–300) with a marked decrease in the NECTIN-4 H-score from TUR-B specimens to RC and LA samples. Interestingly, the NECTIN-4 expression in our RC cohort was higher (median H-score = 140; interquartile range (IQR), 120 - 200) compared to the combined cohort of primary tumor samples analyzed by Klümper et al. (median H-score = 110; IQR, 25–200). Additionally, a 4 times higher NECTIN-4 expression was observed in the LA group (median H-score = 160; IQR, 115-190) compared to the expression in the corresponding matched metastatic tissue samples observed by Klümper et al. (median H-score = 40; IQR, 0–140), which also included LA tissue. Another contrasting finding to the analyses by Klümper et al. and Bahlinger et al. was the fact that only one patient showed a negative NECTIN-4 expression in the LA group. Discrepancies in the observed NECTIN-4 expression in LA tissue from this study and the works of Klümper et al. should be interpreted with caution in the context of variations in staining protocols and interrater variability.

Assessment of the gene expression of the established ADC targets *NECTIN-4* and *TACSTD2*, as well as *ERBB2*

and the ICI targets *PD-L1* and *PD1*, showed that *ERBB2* and *TACSTD2* were most highly expressed across triplet specimens. High mRNA levels of *TACSTD2* were also reported in the study from Bahlinger et al. who analyzed NECTIN-4 and TROP2 protein expression as well as *NECTIN-4* and *TACSTD2* gene expression in the TCGA MIBC cohort as well as a German single center cohort.

Like the heterogenous expression pattern of NECTIN-4 observed in the BC matched triplets - defined as TUR-B, RC and LA specimens obtained from the same patient - mRNA of *NECTIN-4*, *TACSTD2*, *ERBB2* and *PD-L1* varied significantly across the different tissue origins. This further reinforces the concept of biomarker instability across disease stages and anatomical sites. While *NECTIN-4* expression was significantly higher in TUR-B compared to RC specimens, *PD-L1* expression was significantly higher in LA specimens compared to RC samples, indicating that the optimal tissue choice for ICI versus ADC selection may differ.

In clinical practice, this means that relying on a single archival specimen to guide both ICI and ADC treatment strategies may be suboptimal. Like Bahlinger et al. we found a higher *TACSTD2* expression compared to *NECTIN-4* across the TUR-B, RC and LA groups. While TROP2 protein expression was not specifically looked at, the higher *TACSTD2* expression observed in the Mannheim cohort 1 could be indicative of the observed plentiful expression of *TACSTD2*/TROP2 compared to *NECTIN-4*/NECTIN-4, with the later showing distinct expression patterns such as a decrease in membranous expression during metastatic spread.

The observation that *ERBB2* was the only of the 6 genes that showed a significantly higher expression in patients with a high NECTIN-4 staining result (H-score  $\geq 150$ ) indicates a possible use for anti-Her2 agents. However, we did not specifically investigate the Her2 protein expression in this study; thus, this finding remains descriptive and associative in nature.

Similar to the survival analyses performed in the studies by Klümper et al. and Bahlinger et al. [24] this study could not detect any prognostic associations of NECTIN-4 with OS and PFS. The fact that only 2 out of 21 patients had a positive PD-L1 status (CPS  $\geq 10\%$ ) in the respective RC specimens precluded us from performing additional analyses into these associations. However, Bahlinger and colleagues did not find a correlation between PD-L1 expression and NECTIN-4 or TROP2 on tumor and immune cells.

Contrary to the findings of Bahlinger et al. who could not detect significant associations of *NECTIN-4* expression with clinic-pathologic variables in 2 independent cohorts, we found *NECTIN-4* to be significantly higher expressed in patients with MIBC with LVI or N+ who underwent RC at our institution. In contrast to the study of Bahlinger et al., whose TCGA data analysis showed no clinicopathologic associations [24], the analysis of the TCGA data in this

study discovered a significant association of male gender, *N* + disease and *NECTIN-4* amplification with higher *NECTIN-4* expression. However, this discrepancy must be interpreted with caution. Differences in TCGA data analyses can be attributed to different exclusion criteria, the observed contrasting findings between the Mannheim cohort 2 and the CCC-EMN cohort, investigated by Bahlinger et al., could be due to variations in patient populations [24].

While Bahlinger and colleagues did not find any associations of the *NECTIN-4* expression, when applying tertile splits of both *TACSTD2* and *NECTIN-4* in the TCGA and CCC-EMN cohort, in our study a higher *NECTIN-4* gene expression was associated with a significantly shorter OS in the Mannheim cohort 2. However, in the 2 validation cohorts employed in our study a higher *NECTIN-4* expression correlated with better OS, which also persisted as an independent predictor of a better OS in the TCGA cohort.

Differences in the observed survival associations need to be discussed considering variations in the analyzed clinical cohorts, different gene expression measurement and analyses techniques as well as varying cut-off values. This study also did not look at predictive associations of protein and gene expression of Nectin-4, as none of the patients in this study received EV or EVP.

Beyond the biological and clinical implications, the observed discordance of *NECTIN-4* and other ADC and ICI targets across matched TUR-B, RC and LA specimens have further important health-economic relevance. ICI and ADC-based therapies are a major and growing cost burden in BC care, with only a proportion of patients deriving durable clinical benefit. Recent health-economic analyses have demonstrated that indiscriminate ICI use has a substantial impact on healthcare sustainability [25]. This emphasizes the need for biomarker-driven patient selection approaches. Our research suggests that selecting inaccurate or outdated tissue samples for biomarker testing can negatively affect clinical decision-making and result in inefficient allocation of expensive treatments. Relying on early TUR-B specimens that overestimate *NECTIN-4* or underestimate PD-L1 expression could result in inappropriate treatment assignments, leading to ineffective therapy and unnecessary toxicity while contracting excessive healthcare expenditures. In contrast, prioritizing metastatic or post-cystectomy tissue for biomarker testing can enhance both clinical outcomes and cost-effectiveness by more accurately aligning target expression with treatment options.

Possible limitations of this study include its retrospective nature, inherent uncertainties in *NECTIN-4* biology and potential limitations in the IHC and qRT-PCR methodology. Even though representative tissue samples were used, inhomogeneous *NECTIN-4* expression in general cannot definitively be ruled out. The IHC methodology and its scoring and interpretation system carry a certain risk of error susceptibility. Nevertheless, to our knowledge this study is the first to investigate *NECTIN-4* expression in

matched triplets from patients with BC. However, the triplet cohort comprised only 25 evaluable patients, which represents a major limitation. While the study design provides unique biological insight into intra-patient heterogeneity, its small sample size limits statistical power and precludes definitive prognostic assumptions. By explicitly acknowledging these limitations, we aim prevent an overinterpretation of the triplet-based analyses while highlighting their value in understanding spatial and temporal target heterogeneity in BC.

## 5. Conclusion

In summary, this study shows that *NECTIN-4* and other therapeutically relevant targets demonstrate clinically meaningful discordance across matched bladder cancer specimens. These findings support a shift towards site- and time-specific biomarker assessment to optimize patient selection for targeted therapies and immunotherapy in BC.

## Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the local ethics review board II of the University of Heidelberg under the number 2015-549N-MA. All patients provided written informed consent for this study.

## Data availability

The raw data that support the results and conclusions of this study are available through the corresponding author, DU, upon reasonable request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRedit authorship contribution statement

**Daniel Uysal:** Writing – review & editing, Writing – original draft, Validation, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Bruno Reible:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lukas Wildner:** Methodology, Data curation. **Annette Steidler:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Manuel Stöth:** Writing – review & editing, Methodology. **Frederik Wessels:** Writing – review & editing. **Malin Nientiedt:** Writing – review & editing. **Thomas Stefan Worst:** Writing – review & editing. **Philipp Erben:** Writing – review & editing. **Luisa Egen:** Writing – review & editing. **Katja Nitschke:**

Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Christoph Brochhausen:** Writing – review & editing, Supervision, Methodology. **Maurice Stephan Michel:** Writing – review & editing, Supervision. **Philipp Nuhn:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Karl-Friedrich Kowalewski:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization.

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## Supplementary materials

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.urolonc.2026.111095>.

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