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Research Paper

Necdin Overexpression Predicts Poor Prognosis in Patients with Urothelial Carcinomas of the Upper Urinary Tract and Urinary Bladder

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Abstract

Background and Aims: Oncogenesis is a multistep process, resulting from the accumulations of multiple mutations. Of these mutations, self-sufficiency in growth signals, *i.e.*, disruption of cell growth regulation, is the first episode. Nonetheless, the genes associated with cell growth dysregulation have seldom been systematically evaluated in either urothelial carcinomas of upper urinary tract (UTUC) or urothelial carcinomas of urinary baldder (UBUC). By data mining a published transcriptomic dataset of UBUCs (GSE31684), we identified the *NDN* gene as one of the most significant of those associated with the regulation of cell growth and found this gene is associated with advanced tumor status and metastatic disease (GO:0001558). Accordingly, we analyzed *NDN* transcript and protein expression with their clinicopathological significance.

Materials and Methods: We used real time RT-PCR to detect *NDN* transcript levels in 27 UTUCs and 27 UBUCs, respectively. Immunohistochemical study was performed to determine NDN protein (a.k.a. Necdin) expression evaluated by H-score method in 340 UTUCs and 295 UBUCs. NDN expression was further correlated with clinicopathological features and disease-specific survival (DSS) and metastasis-free survival (MeFS).

Results: *NDN* transcriptional level was significantly higher in UCs of both sites with stepwise more advanced pT statuses. Through immunohistochemistry, we found NDN protein expression was significantly associated with adverse clinicopathological parameters, e.g., advanced pT status, nodal metastasis, high grade histological patterns, and frequent mitotses (all $P<0.05$). In univariate analysis, NDN overexpression not only predicted worse DSS and MeFS in both the UTUC and UBUC groups, it also served as an independent prognostic factor for DSS and MeFS in multivariate analysis (all $P<0.05$).

Conclusions: NDN may play an important role in tumor progression in UC and could serve as a prognostic biomarker and a potential novel therapeutic target in UC.

Key words: *NDN* gene, Necdin, Urothelial carcinoma, Prognosis.

Introduction

Urothelial carcinoma (UC) is the most prevalent histological type of malignancy throughout the urinary tract, from the upper urinary tract (UT) to the urinary bladder (UB) [1]. The former consists of the renal pelvis and the ureter. In contrast to urothelial carcinoma of the urinary bladder (UBUC), which is the seventh most common cancer in the United States [2], urothelial carcinoma of the upper urinary tract (UTUC) is much uncommon and accounts for only 5% to 10% of all UCs [3]. Nevertheless, the incidence of UTUC in Taiwan is unusually high, especially in southern Taiwan and areas of endemic "Black-foot disease" [4-6]. Arsenic-contaminated drinking water may contribute to the prevalence of UTUC in Taiwan [6]. Etiologically, all UCs, regardless of anatomical location, are attributed to identical carcinogens, such as cigarette smoking, and aromatic amines, *e.g.* benzidine, β-naphtylanine [7-9]. However, certain populations are particularly predisposed to UTUC. For instance, patients with analgesic nephropathy [10], Chinese herb nephropathy [11, 12], and Balkans nephropathy [11, 13] are more susceptible to UTUC than UBUC. In spite of the fact that UTUCs usually have higher stage and grade than UBUCs, their clinical behavior is similar after balancing the stages and grades [14]. In addition, the previous study revealed that gene expression profiles of both UBUCs and UTUCs are much alike [15]. These findings indicate that carcinogensis of UCs from both anatomical locations may participate in a common molecular pathway.

Cancer is essentially a disease of regulation of cell growth; genes that regulate cell growth must be altered in order to transform normal cells into cancer cells [16]. Oncogenesis is a multistep process, resulting from the accumulations of multiple mutations. Of these mutations, self-sufficiency in growth signals, *i.e.*, disruption of cell growth regulation is the first episode [17]. Nonetheless, the genes associated with cell growth dysregulation have seldom been systematically evaluated in either UTUC or UBUC. By data mining documented transcript expression profiles (GSE31684) in the Gene Expression Omnibus (GEO, National Center for Biotechnology Information (NCBI), Bethesda, MD, USA) with a special focus on growth regulation (GO:0001558), we found that transcription of the *NDN* gene was significantly upregulated from early tumor development and associated stepwise with tumor progression. This evidence suggests that the *NDN* gene plays an important role in tumorigenesis and its progression.

The *NDN* gene encodes Necdin protein, a member of the melanoma-associated antigen gene (MAGE) family [18] that was first identified in neurally differentiated mouse stem cells of P19 embryonal carcinoma cell lines treated with retinoic acid [19]. Necdin was originally recognized as a suppressor of cell proliferation in postmitotic neurons, leading the differentiated neurons into permanent withdrawal from the cell cycle due to constitutive and lifelong expression of Necdin [20]. Moreover, *NDN* transcripts and their encoded proteins showed downregulation or low expression in previous UBUC cell lines and human UBUC tissue studies [21]. Hence, we conducted this study to elucidate the expression of Necdin protein and *NDN* mRNA in UCs from both anatomical sites, as well as their association with clinicopathological parameters and clinical outcomes.

Materials and Methods

Data mining the GEO to identify the most altered transcripts in UCs

We performed data mining of the GEO of NCBI, identifying dataset GSE31684 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31684) in order to analyze radical cystectomy specimens from 93 patients with UBUC using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. To analyze all probe sets, we used Nexus Expression 3 statistical software (BioDiscovery, El Segundo, CA, USA) without preselection or filtering. Under supervision, our comparative analysis examined the statistical significance of differentially expressed transcripts on the basis of primary tumor status (pT) and the development of metastatic events. We performed functional profiling using transcriptomes of high-stage UCs (pT2-pT4) with metastases and low-stage UCs (pTa-pT1) devoid of metastasis, focusing on those related to the regulation of cell growth (GO:0001558). We further analyzed survival patterns by dichotomizing all cases into high-expression and low-expression clusters for computing the prognostic significance of the selected genes.

Patients and tumor specimens

The Institutional Review Board of Chi Mei Medical Center approved this study (IRB971006). For immunohistochemical study and survival analysis, we enrolled 635 consecutive cases diagnosed as conventional UC between 1996 and 2004, from the archives of the Department of Pathology, Chi-Mei Medical Center. Of these cases, 340 tumors originated from the UT and 295 arose from the UB. Other histological classifications as well as variants of UC were excluded. The criteria for clinicopathological evaluation were essentially identical to those in our previous work [22]. Detailed information is provided in the online supplementary material.

Transcriptional levels of the *NDN* gene

The materials for genetic examination were gathered from macrodissection of fresh tumor tissue. For quantification of *NDN* mRNA expression, we extracted total RNAs, quantified them, and submitted them for reverse-transcription from 27 UTUCs and 27 UBUCs (both were composed of 9 cases of pTa; 9 pT1; 9 pT2-4), respectively. Using pre-designed TaqMan assay reagents (Applied Biosystems, Waltham, MA, USA), we measured mRNA abundance of *NDN* (Hs00267349_s1) with the ABI StepOnePlus™ System, as previously described [23]. We calculated the fold expression of *NDN* relative to normal urothelium by comparative Ct method, after normalization to POLR2A (Hs01108291_m1) as the internal control.

Immunohistochemical staining and scoring of NDN

Tissue sections underwent standard procedures for deparaffinization, rehydration and antigen retrieval. Afterwards, the sections were incubated with a primary antibody targeting NDN (TA506975, 1:150; OriGene, Rockville, MD, USA) for an hour. Normal brain tissue with or without incubation of primary antibody were run in parallel as positive and negative control, respectively. We scored NDN immunoreactivity based on the combination of the percentage and intensity of positivity of the immunostain in tumoral nuclei in order to generate an H-score, which was calculated using the following equation: H-score=$\Sigma P_i(i+1)$, where i is the intensity of stained tumor cells (0-3+), and Pi is the percentage of stained tumor cells for each intensity varying from 0% to 100%. This formula produced a score range from 100-400, where 100 indicated that 100% of tumor cells were negative and 400 indicated 100% of tumor cells were strongly stained (3+) [24, 25].

Statistical analysis

We performed statistics using SPSS V.14.0 software (SPSS Inc., Chicago, IL, USA). The median H-score of NDN immunoreactivity was used as the cut-off to dichotomize the study cohort, separating cases into high expression and low expression groups. We used Pearson's χ^2 test to compare NDN expression status and various categorical clinicopathological parameters. Mann-Whitney U test was used to compare NDN expression and numerical ones, *i.e.*, mitotic rate. The end points analyzed were disease-specific survival (DSS) and metastasis-free survival (MeFS), calculated from the date of tumor resection to the date the event developed. Patients lost to follow-up were censored on the latest follow-up date. We performed univariate survival analyses using Kaplan-Meier plots and compared them by log-rank test. Those parameters with univariate $P<0.1$ were enrolled into multivariate tests using Cox proportional hazards regression. For all analyses, we used two-sided tests of significance, with $P<0.05$ considered statistically significant.

Results

NDN is a significant differentially upregulated transcript linked with regulation of cell growth in UBUCs

From the transcriptomic profiling of 93 UBUC cases (GSE31684), which contains 78 high-stage (T2-T4) and 15 low-stage (Ta-T1) cases, 28 with metastatic disease and 49 without metastasis, we identified six probes covering two transcripts associated with regulation of cell growth (GO:0001558) (**Figure 1**). As shown in **Table 1**, both transcripts, *i.e.*, *IGFBP5* and *NDN*, were significantly upregulated with a log2 ratio of 0.337 to 2.6399-fold and 0.7821-fold upregulation, respectively, comparing high-stage to low-stage (all $P<0.005$). *IGFBP5* and *NDN* upregulation also showed statistically significant association with metastatic disease in all probes (all $P\leqq0.0001$). Comparing high-expression (n=37) to low-expression (n=56) clusters, high expression levels of *NDN* transcripts significantly predicted worse disease-specific survival (**Figure S1**, $P=0.0059$). We had investigated *IGFBP5* in a previous study [26]; however, *NDN* has not previously been systematically studied in UCs. Hence, we further characterized the endogenous expression levels and clinical significance of both *NDN* transcript and its protein in UC.

Higher *NDN* mRNA transcriptional levels in UBUCs and UTUCs with advanced pT stage

In the examined 27 UBUCs and 27 UTUCs, *NDN* transcripts were significantly more abundant in tumors with stepwise more advanced pT status, from pTa to pT1 to pT2-4 (all $P<0.05$), suggesting it plays a role in tumor progression (**Figure 2**).

Clinicopathological findings on UTUCs and UBUCs

Clinicopathological features of the patients with UTUCs and UBUCs are listed in **Table 2**. In the UTUC group, no obvious sex predilection was noted despite there being slightly more women (n=182, 53.5%). In contrast, the majority of patients with UBUC were male (n=216, 73.2%). For both types of UC, most patients' age at diagnosis was older than 65 years (n=202, 59.4% for UTUC and n=174, 59.0% for UBUC). In the UTUC group, synchronous multifocal tumors occurred in 62 patients (18.2%); both the renal pelvis and ureter were involved in 49 (14.4%) of these. In both UC groups, most tumors were classified as high

histological grade (n=284, 83.5% for UTUC and n=239, 81% for UBUC). Advanced pT stages (pT2-T4) were seen in 46.8% (n=159) and 41.7% (n=123) of patients with UTUCs and UBUCs, respectively. Nodal metastasis was detected in 8.2% (n=28) and 9.8% (n=29) of UTUC and UBUC patients, respectively. Vascular invasion was noted in 31.2% of UTUC (n=106) and 16.6% of UBUC cases (n=49); while perineurial invasion was observed in 5.6% of UTUC (n=19) and 6.8% of UBUC cases (n=20). In addition, about half of the tumors showed ten or more mitoses per ten high power fields (n=167, 49.1% for UTUC and n=156, 52.9% for UBUC).

Correlations between immunoreactivity of NDN and clinicopathological parameters in UTUCs and UBUCs

NDN showed variable nuclear expression in UCs from both sites. The median H-score for each group was 105 and 370 for the UTUCs and UBUCs, respectively. As **Table 2** demonstrates, after dichotomizing tumors into low and high NDN expression, increased NDN expression in UC of both anatomical sites was significantly associated with stepwise increases of pT status (**Figure 3**, UTUC, P=0.003; UBUC, P<0.001), lymph node metastasis (UTUC, P =0.002; UBUC, P=0.012), high grade histological patterns (UTUC, P=0.019; UBUC, P=0.003) and higher mitotic rate (UTUC, P=0.004; UBUC, P=0.005). Increased NDN expression was significantly associated with vascular invasion and perineural invasion only in the UBUC group (P=0.008 and 0.001, respectively).

Survival analysis for UTUC and UBUC patients

The univariate and multivariate analyses of relationships between clinical outcomes and miscellaneous clinicopathological parameters in both UTUC and UBUC patients are illustrated in **Tables 3-4**. In multivariate analysis, multifocality (P=0.007), nodal metastasis (P<0.001), high histological grade (P=0.007) and perineurial invasion (P=0.001) are independent prognostic factors for poor DSS in the UTUC group. Similarly, inferior MeFS was significantly associated with multifocality (P=0.002), nodal metastasis (P=0.001), high histological grade (P=0.030), vascular invasion (P<0.001) and perineurial invasion (P=0.014) in patients with UTUCs. In UTUCs, advanced pT status was significantly associated with worse DSS and MeFS in univariate (P<0.0001) but not in multivariate analyses. Tumor location and vascular invasion in UTUC group correlated with poorer patient DSS in univariate analysis only (P=0.0079 and P<0.0001, respectively).

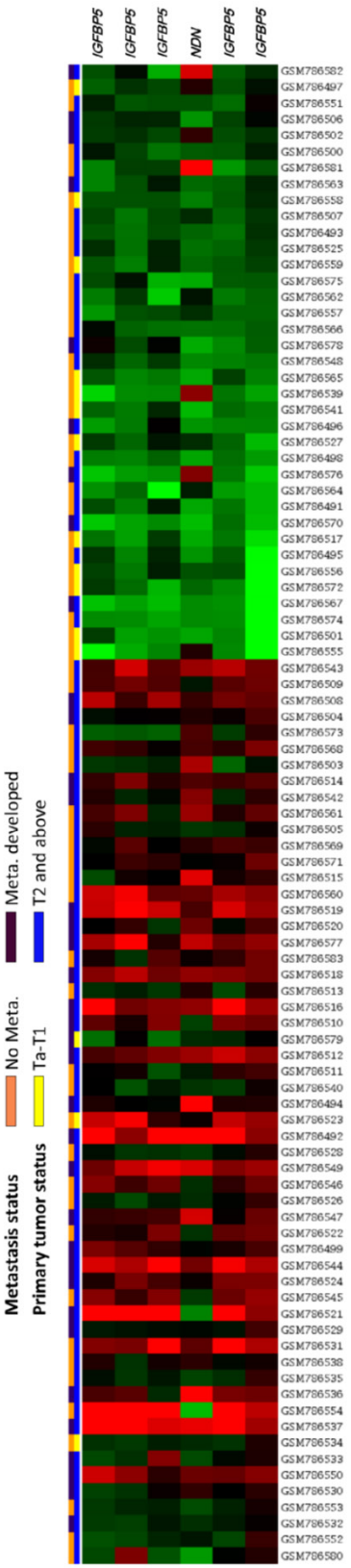

Figure 1. Analysis of gene expression in urothelial carcinoma using a published transcriptomic dataset (GSE31684). Clustering analysis of genes focused on the regulation of cell growth (GO:0001558) revealed *NDN* is one of the most significantly upregulated genes associated with increments of pT status and metastatic disease. Tissue specimens from tumors with different pT statuses are indicated on top of the heatmap, and expression levels of upregulated and downregulated genes are represented as a spectrum of brightness or red and green, respectively. Those with unaltered mRNA transcriptional levels are coded black.

In the UBUC group, multivariate analysis revealed advanced pT status and higher mitotic rate were significantly associated with both dismal DDS and MeFS (all P<0.05). Lymph node metastasis was significantly associated with poor MeFS (P=0.037) in multivariate analysis, as well. In UBUCs, high histological grade, and vascular and perineural invasion were significantly associated with both adverse DSS and MeFS in univariate analysis (all P<0.005), but not in multivariate analysis.

Prognostic significance of NDN expression in UC

As shown in **Tables 3-4** and **Figure 4**, in univariate analysis, either the UTUC or UBUC group with NDN overexpression had significantly worse DSS and MeFS (P<0.005 for all). Notably, in multivariate analysis, high NDN nuclear expression was still an independent prognosticator predicting more dismal DSS and MeFS for all UCs (P<0.05 for all).

Table 1. Summary of differentially expressed genes associated with regulation of cell growth (GO:0001558) and showed positive associations to cancer invasiveness and metastasis in the transcriptome of urothelial carcinoma of urinary bladder (GSE31684).

Probe	Comparing T2-4 to Ta-T1		Comparing Meta. to Non-Meta.#		Gene Symbol	Biological Process	Molecular Function
	log ratio	p-value	log ratio	p-value			
1554741_s_at	0.4313	<0.0001	0.3919	<0.0001	*IGFBP5*	regulation of cell growth, signal transduction	growth factor binding, insulin-like growth factor binding
201666_at	1.1435	0.0001	1.0825	<0.0001	*IGFBP5*	regulation of cell growth, signal transduction	growth factor binding, insulin-like growth factor binding
205131_x_at	0.337	0.0008	0.2968	0.0001	*IGFBP5*	regulation of cell growth, signal transduction	growth factor binding, insulin-like growth factor binding
205168_at	0.7821	0.0044	1.0228	<0.0001	*NDN*	axon extension involved in development, axonal fasciculation, axonogenesis, central nervous system development, glial cell migration, negative regulation of cell proliferation, nerve growth factor receptor signaling pathway, nervous system development, neuron development, neuron migration, regulation of cell growth, regulation of progression through cell cycle, regulation of transcription; DNA-dependent, respiratory gaseous exchange, sensory perception of pain, transcription	DNA binding, gamma-tubulin binding, protein binding
205782_at	0.8325	0.0003	0.8815	<0.0001	*IGFBP5*	regulation of cell growth, signal transduction	growth factor binding, insulin-like growth factor binding
207426_s_at	2.6399	<0.0001	1.4678	<0.0001	*IGFBP5*	regulation of cell growth, signal transduction	growth factor binding, insulin-like growth factor binding

#, Meta., distal metastasis developed during follow-up; Non-Meta.: no metastatic event developed.

Table 2. Correlations between NDN expression and other important clinicopathological parameters in urothelial carcinomas.

Parameter	Category	Upper Urinary Tract Urothelial Carcinoma				Urinary Bladder Urothelial Carcinoma			
		Case No. (%)	NDN Expression		p-value	Case No. (%)	NDN Expression		p-value
			Low (%)	High (%)			Low (%)	High (%)	
Gender	Male	158 (46.5)	86 (54.4)	72 (45.6)	0.128	216 (73.2)	109 (50.5)	107 (49.5)	0.719
	Female	182 (53.5)	84 (46.2)	98 (53.8)		79 (26.8)	38 (48.1)	41 (51.9)	
Age (years)	< 65	138 (40.6)	70 (50.7)	68 (49.3)	0.825	121 (41.0)	59 (48.8)	62 (51.2)	0.759
	≥ 65	202 (59.4)	100 (49.5)	102 (50.5)		174 (59.0)	88 (50.6)	86 (49.4)	
Tumor location	Renal pelvis	141 (41.5)	71 (50.4)	70 (49.6)	0.685	-	-	-	-
	Ureter	150 (44.1)	72 (48.0)	78 (52.0)		-	-	-	-
	Renal pelvis & ureter	49 (14.4)	27 (55.1)	22 (44.9)		-	-	-	-
Multifocality	Single	278 (81.8)	138 (49.6)	140 (50.4)	0.779	-	-	-	-
	Multifocal	62 (18.2)	32 (51.6)	30 (48.4)		-	-	-	-
Primary tumor (pT)	pTa	89 (26.2)	56 (62.9)	33 (37.1)	0.003*	84 (28.5)	62 (73.8)	22 (26.2)	<0.001*
	pT1	92 (27.0)	49 (53.3)	43 (46.7)		88 (29.8)	48 (54.5)	40 (45.5)	
	pT2-pT4	159 (46.8)	65 (40.9)	94 (59.1)		123 (41.7)	37 (30.0)	86 (70.0)	
Nodal metastasis	Negative (pN0)	312 (91.8)	164 (52.6)	148 (47.4)	0.002*	266 (90.2)	139 (52.3)	127 (47.7)	0.012*
	Positive (pN1-pN3)	28 (8.2)	6 (21.4)	22 (78.6)		29 (9.8)	8 (27.6)	21 (72.4)	
Histological grade	Low grade	56 (16.5)	36 (64.3)	20 (35.7)	0.019*	56 (19.0)	38 (67.9)	18 (32.1)	0.003*
	High grade	284 (83.5)	134 (47.2)	150 (52.8)		239 (81.0)	109 (45.6)	130 (54.4)	
Vascular invasion	Absent	234 (68.9)	122 (52.1)	112 (47.9)	0.242	246 (83.4)	131 (53.3)	115 (46.7)	0.008*
	Present	106 (31.1)	48 (45.3)	58 (54.7)		49 (16.6)	16 (32.7)	33 (67.3)	
Perineural invasion	Absent	321 (94.4)	164 (51.1)	157 (48.9)	0.098	275 (93.2)	144 (52.4)	131 (47.6)	0.001*
	Present	19 (5.6)	6 (31.6)	13 (68.4)		20 (6.8)	3 (15.0)	17 (85.0)	
Mitotic rate (per 10 high power fields)#		340	9.66+/-7.315	14.96+/-15.324	0.004*	295	11.41+/-10.286	17.38+/-16.469	0.005*

#, Mann-Whitney U test; *, Statistically significant.

Table 3. Univariate log-rank and multivariate analyses for Disease-specific and Metastasis-free Survivals in upper urinary tract urothelial carcinoma.

Parameter	Category	Case No. (%)	Disease-specific Survival					Metastasis-free Survival				
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
			No. of event (%)	p-value	R.R.	95% C.I.	p-value	No. of event (%)	p-value	R.R.	95% C.I.	p-value
Gender	Male	158 (46.5)	28 (17.7)	0.8286	-	-	-	32 (20.3)	0.7904	-	-	-
	Female	182 (53.5)	33 (18.1)		-	-	-	38 (20.9)		-	-	-
Age (years)	< 65	138 (40.6)	26 (18.8)	0.9943	-	-	-	30 (21.7)	0.8470	-	-	-
	≥ 65	202 (59.4)	35 (17.3)		-	-	-	40 (19.8)		-	-	-
Tumor side	Right	177 (52.1)	34 (19.2)	0.7366	-	-	-	38 (21.5)	0.3074	-	-	-
	Left	154 (45.3)	26 (16.9)		-	-	-	32 (20.8)		-	-	-
	Bilateral	9 (2.6)	1 (11.1)		-	-	-	0 (0)		-	-	-
Tumor location	Renal pelvis	141 (41.5)	24 (17.0)	0.0079*	1	-	0.840	31 (22.0)	0.0659	-	-	-
	Ureter	150 (44.1)	22 (14.7)		0.876	0.472-1.625		25 (16.7)		-	-	-
	Renal pelvis & ureter	49 (14.4)	15 (30.6)		1.286	0.607-7.438		14 (28.6)		-	-	-
Multifocality	Single	278 (81.8)	48 (17.3)	0.0026*	1	-	0.007*	52 (18.7)	0.0127*	1	-	0.002*
	Multifocal	62 (18.2)	18 (29.0)		2.821	1.321-6.024		18 (29.0)		2.402	1.381-4.4178	
Primary tumor (pT)	pTa	89 (26.2)	2 (2.2)	<0.0001*	1	-	0.071	4 (4.5)	<0.0001*	1	-	0.227
	pT1	92 (27.0)	9 (9.8)		3.042	0.646-14.324		15 (16.3)		2.344	0.756-7.267	
	pT2-pT4	159 (46.8)	50 (31.4)		5.159	1.150-23.153		51 (32.1)		2.497	0.795-7.846	
Nodal metastasis	Negative (pN0)	312 (91.8)	42 (13.5)	<0.0001*	1	-	<0.001*	55 (17.6)	<0.0001*	1	-	0.001*
	Positive (pN1-pN3)	28 (8.2)	19 (67.9)		5.071	2.753-9.340		15 (53.6)		2.888	1.552-5.372	
Histological grade	Low grade	56 (16.5)	4 (7.1)	0.0215*	1	-	0.007*	3 (5.4)	0.0027*	1	-	0.030*
	High grade	284 (83.5)	57 (20.0)		3.853	1.441-10.298		67 (23.6)		3.818	1.139-12.792	
Vascular invasion	Absent	234 (68.9)	24 (10.3)	<0.0001*	1	-	0.059	26 (11.1)	<0.0001*	1	-	<0.001*
	Present	106 (31.1)	37 (34.9)		1.774	0.978-3.218		44 (41.5)		2.960	1.625-5.392	
Perineural invasion	Absent	321 (94.4)	50 (15.6)	<0.0001*	1	-	0.001*	61 (19.0)	<0.0001*	1	-	0.014*
	Present	19 (5.6)	11 (57.9)		3.463	1.661-7.222		9 (47.4)		2.562	1.208-5.434	
Mitotic rate (per 10 high power fields)	< 10	173 (50.9)	27 (15.6)	0.167	-	-		30 (17.3)	0.0823	-	-	
	>= 10	167 (49.1)	34 (20.4)		-	-		40 (24.0)		-	-	
NDN expression	Low	170 (50.0)	17 (10.0)	0.0002*	1	-	0.032*	24 (14.1)	0.0027*	1	-	0.033*
	High	170 (50.0)	44 (25.9)		1.893	1.058-3.385		46 (27.1)		1.731	1.044-2.869	

* Statistically significant.

Table 4. Univariate log-rank and multivariate analyses for Disease-specific and Metastasis-free Survivals in urinary bladder urothelial carcinoma.

Parameter	Category	Case No. (%)	Disease-specific Survival					Metastasis-free Survival				
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
			No. of event (%)	p-value	R.R.	95% C.I.	p-value	No. of event (%)	p-value	R.R.	95% C.I.	p-value
Gender	Male	216 (73.2)	41 (19.0)	0.4446	-	-	-	60 (27.8)	0.2720	-	-	-
	Female	79 (26.8)	11 (13.9)		-	-	-	16 (20.3)		-	-	-
Age (years)	< 65	121 (41.0)	17 (14.0)	0.1136	-	-	-	31 (25.6)	0.6875	-	-	-
	≥ 65	174 (59.0)	35 (20.1)		-	-	-	45 (25.9)		-	-	-
Primary tumor (pT)	pTa	84 (28.5)	1 (1.4)	<0.0001*	1	-	<0.0001*	4 (4.8)	<0.0001*	1	-	0.011*
	pT1	88 (29.8)	9 (10.2)		3.817	1.748-8.333		23 (26.1)		4.631	1.311-16.364	
	pT2-pT4	123 (41.7)	42 (34.1)		21.277	2.823-200.00		49 (39.8)		6.688	1.889-23.677	
Nodal metastasis	Negative (pN0)	266 (90.2)	41 (15.4)	0.0002*	1	-	0.387	61 (22.9)	<0.0001*	1	-	0.037*
	Positive (pN1-pN3)	29 (9.8)	11 (37.9)		1.364	0.675-2.758		15 (51.7)		1.939	1.042-3.607	
Histological grade	Low grade	56 (19.0)	2 (3.6)	0.0013*	1	-	0.889	5 (8.9)	0.0007*	1	-	0.711
	High grade	239 (81.0)	50 (20.9)		0.895	0.188-4.266		71 (29.7)		0.809	0.264-2.481	
Vascular invasion	Absent	246 (83.4)	37 (15.0)	0.0024*	1	-	0.185	54 (22.0)	0.0001*	1	-	0.835
	Present	49 (16.6)	15 (30.6)		0.629	0.317-1.249		22 (44.9)		1.067	0.580-1.964	
Perineural invasion	Absent	275 (93.2)	44 (16.0)	0.0001*	1	-	0.131	66 (24.0)	0.0007*	1	-	0.347
	Present	20 (6.8)	8 (40.0)		1.908	0.824-4.420		10 (50.0)		1.429	0.679-3.009	

Parameter	Category	Case No. (%)	Disease-specific Survival					Metastasis-free Survival				
			Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
			No. of event (%)	p-value	R.R.	95% C.I.	p-value	No. of event (%)	p-value	R.R.	95% C.I.	p-value
Mitotic rate (per 10 high power fields)	< 10	139 (47.1)	12 (8.6)	<0.0001*	1	-	0.011*	23 (16.5)	<0.0001*	1	-	0.019*
	>= 10	156 (52.9)	40 (25.6)		2.409	1.219-4.761		53 (34.0)		1.871	1.109-3.156	
NDN expression	Low	147 (49.8)	10 (6.8)	<0.0001*	1	-	0.012*	21 (14.3)	<0.0001*	1	-	0.021*
	High	148 (50.2)	42 (28.4)		2.477	1.220-5.028		55 (37.2)		1.852	1.098-3.123	

* Statistically significant.

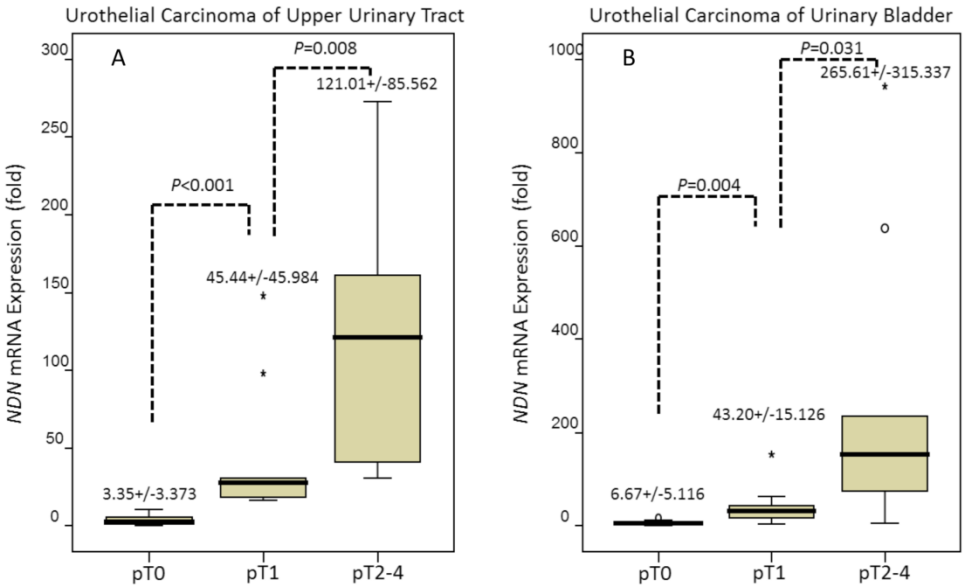

Figure 2. Quantitative real-time PCR (qPCR) analysis showed that *NDN* mRNA expression was significantly increased stepwise in both urothelial carcinomas of the upper urinary tract (*left* panel) and urinary bladder (*right* panel) along with more advanced primary pT status, from pTa to pT1 and pT1 to pT2-4 (all *P*<0.05).

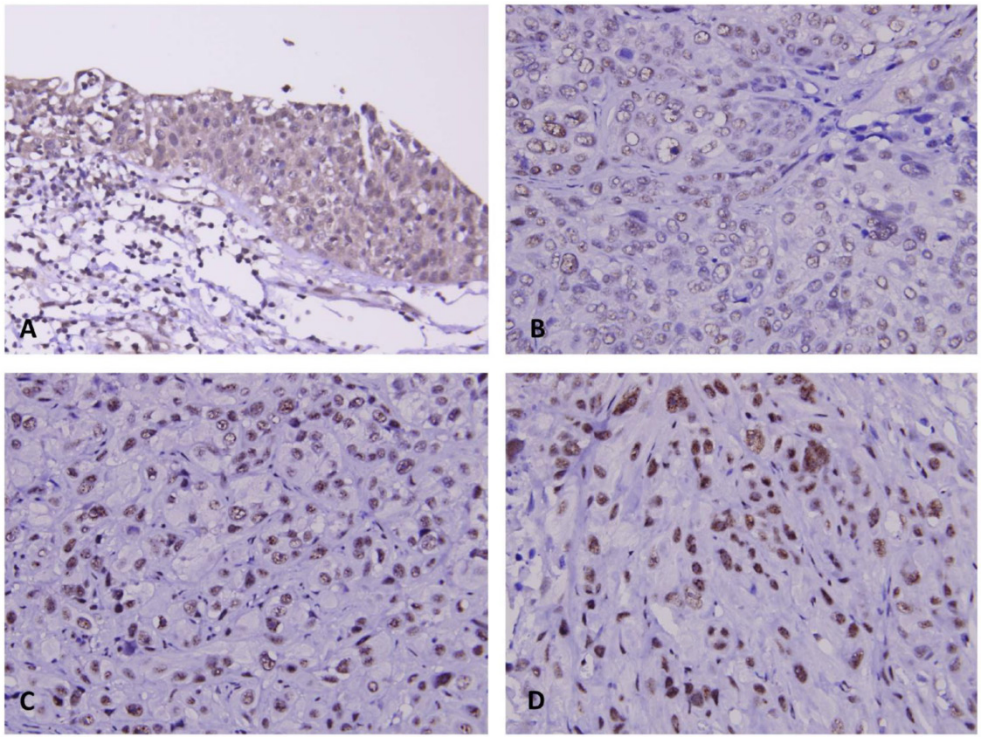

Figure 3. NDN immunostaining on representative sections revealed stepwise increase of NDN expression from non-tumoral urothelium **(A)**, non-invasive **(B)**, superficially infiltrating (pT1) **(C)** and deeply infiltrating (pT2-4) urothelial carcinoma **(D)**.

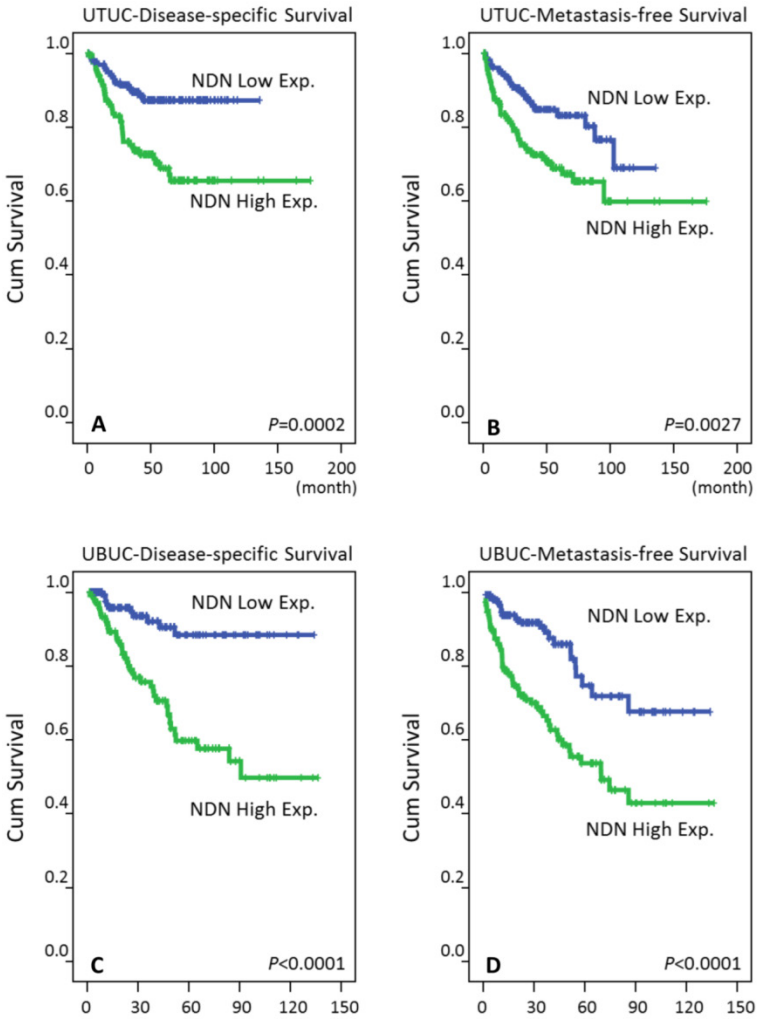

Figure 4. Kaplan-Meier plots disclosed the predictive value of NDN immunoreactivity for disease-specific survival (DSS) and metastasis-free survival (MeFS) in both groups of UTUC (**A** and **B** for DSS and MeFS, respecitvely) and UBUC (**C** and **D** for DSS and MeFS, respectively) (all *P*<0.005).

Discussion

Urothelial carcinoma is prevalent worldwide and has high recurrence rates [27]. Pathological staging is still the most decisive prognosticator of outcomes for bladder cancer, where five-year survival rates are approximately 60% to 85% for T2 bladder cancers after cystectomy, but decrease rapidly to 40% to 60% for T3 tumors [28]. In spite of the introduction of cisplatin-based combination chemotherapy, the prognosis is still poor for advanced stage bladder cancers; so far bladder cancer is ranked as the thirteenth most common cause of cancer death in the world (GLOBOCAN 2012 http://globocan.iarc.fr). Therefore, developing more potent therapeutic regimens is of crucial importance for high-risk patients.

Every cancer has ten hallmarks: sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, and deregulating cellular energetics [29]. Among these characteristics, sustaining proliferative signaling, that is, cell growth dysregulation, serves as the first step of oncogenesis.

The maternally imprinted gene, *NDN*, is associated with Prader-Willi syndrome [30]. Necdin, encoded by the *NDN* gene, is a 325-amino acid protein belonging to the MAGE family [18, 19]. The function of Necdin protein is similar to Rb, which has been shown to interact with viral oncogenes such as simian virus 40 (SV40) large T antigen and adenovirus E1A, as well as E2F1 and p53 [31, 32]. Nevertheless, Necdin also bears oncogenic properties. Necdin's inhibitory effect on p53-mediated growth arrest and suppressive impact on p53-dependent apoptosis have also been demonstrated to be oncogenic functions [33, 34]. In line with this, there have been divergent results for Necdin expression in different cancers of each study. The scarcity of Necdin expression in brain tumor cell lines [35], as well as its decreased expression in mel-

anomas [36] and bladder cancer cell lines and tumors [21] suggest *NDN* may be a tumor suppressor gene. Conversely, upregulation and loss of imprinting of Necdin in pancreatic cancers have been demonstrated by more recent studies [37, 38]. The paradoxical results of experiments designed to recognize the role of Necdin in oncogenesis indicate that it has complex functions, which may be due to different microenvironments and cellular contexts [34]. Our current investigation proves the possible oncogenic role of *NDN* in UCs, which is not consistent with a previous study that found *NDN* was downregulated in bladder cancer cell lines and tumors [21]. A study by Chapman, *et al.* [21] first observed that *NDN* was induced to be downregulated in normal human urothelial cells transducted by hTERT, the catalytic subunit of telomerase. After that, the authors found *NDN* transcripts were downregulated in 26 of 28 (92.9%) bladder cancer cell lines and 35 of 58 (60%) bladder cancer tumors. However, the researchers neither correlated the *NDN* transcription levels with clinicopathological significance nor analyzed their association with survival. In addition, to the best of our knowledge, the current investigation is the first to inspect the relationship between NDN expression of both mRNA and protein levels, along with clinical outcomes in UTUC.

Conclusion

In sum, our work demonstrates that *NDN* gene and NDN protein (a.k.a. Necdin) plays an influential role in tumor progression of UCs. High expression of Necdin in both UTUC and UBUC is associated with worse clinicopathological parameters. Overexpression of Necdin is also an independent prognosticator of inferior DSS and MeFS in both groups. Further research to elucidate the details of the biological role of the *NDN* gene and Necdin in carcinogenesis of UC is essential for exploring the potential of NDN-targeted therapy for UCs, as we illustrated the hopeful targets for new strategies for UC therapy lately [39-41].

Supplementary Material

Supplementary methods and Figure S1.
http://www.jcancer.org/v07p0304s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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