Original Article
CDCA5 overexpression is an indicator of poor prognosis in patients with urothelial carcinomas of the upper urinary tract and urinary bladder

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Abstract: *Aims*: Urothelial carcinoma (UC) is the most common tumor involving upper urinary tract (UTUC) and urinary bladder (UBUC) whose molecular survival determinants remains obscured. By computerizing a public transcriptomic database of UBUCs (GSE32894), we identified *cell division cycle associated 5 (CDCA5)* as the most significantly upregulated gene among those associated with G1-S transition of the mitotic cell cycle (GO:0000082). We therefore analyzed the clinicoptaological significance of *CDCA5* expression in our well-characterized UC cohort. *Methods and results*: Quantigene assay was used to detect *CDCA5* transcript levels in 36 UTUCs and 30 UBUCs. We used immunohistochemistry evaluated by H-scores to determine CDCA5 protein expression in 295 UBUCs and 340 UTUCs, respectively. CDCA5 expression was further correlated with clinicopathological features and disease-specific survival (DSS) and metastasis-free survival (MeFS). For both groups of UCs, increments of *CDCA5* transcript levels were associated with higher pT status, CDCA5 protein overexpression was also significantly associated with advanced pT status, nodal metastasis, high histological grade, vascular invasion, and frequent mitoses. CDCA5 overexpression was predictive for worse DSS and MeFS in univariate and multivariate analysis. *Conclusions*: CDCA5 overexpression is associated with advanced clinical features of UC, suggesting its potential value as a prognostic biomarker and a novel therapeutic target.

Keywords: CDCA5, *cell division cycle associated 5*, urinary bladder, upper tract, urothelial carcinoma

Introduction

Urothelial carcinoma (UC) is the most common histological type of urinary tract malignancy. It may occur anywhere along the urinary tract, from the upper urinary tract (UT; including renal pelvis and ureter) to the urinary bladder (UB). Bladder cancer is ranked the ninth most common cancer and the thirteenth most common cause of cancer mortality among both sexes in the world [GLOBOCAN 2012 http://globocan.

iarc.fr]. In contrast, upper tract urothelial carcinoma (UTUC) is uncommon and accounts for only 5% to 10% of all urothelial carcinomas [1]. Etiologically, all UCs—arising from both anatomical locations—are caused by common carcinogens, such as cigarette smoking and occupational exposure to aromatic amines, such as benzidine and β-naphtylanine, etc [2-4]. Nevertheless, certain populations are more susceptible to UTUC than urothelial carcinomas of the urinary bladder (UBUC), e.g., Balkans endemic

nephropathy [5, 6], Chinese herb nephropathy [6, 7], and analgesic nephropathy [8]. In spite of more common promoter hypermethylation in UTUC than UBUC, as revealed by a previous study [9], gene expression profiling of UCs from both anatomical sites shows they are akin to each other [10]. Although UTUCs tend to have higher stage and grade than UBUCs, there is no difference in tumor behavior with respect to location after controlling for the effects of grade and stage [11]. This imply that carcinogenesis in UC occurring anywhere in the urinary tract may share a common molecular pathway.

Alteration in cell cycle control is one of the most important events in carcinogenesis, especially at the checkpoint pathways [12]. There are three main checkpoints, one at the G1-S transition and two at S-phase and G2-M transition [13]. They maintain genomic integrity. Mutations in checkpoint pathways usually permit the survival or the continued growth of cells with genomic abnormalities, thereby enhancing the opportunity for malignant transformation. Of these, the G1-S transition is known to be the restriction point beyond which a cell is committed to dividing [14]. Deregulation in the G1-S transition of the mitotic cell cycle is commonly encountered in oncogenesis, however, the genes associated with this phenomenon have not been systemically evaluated in UC. By performing data mining on documented transcript expression profiles in the Gene Expression Omnibus (GEO, National Center Biotechnology information (NCBI), Bethesda, Maryland, USA) with a special focus on the G1-S transition of the mitotic cell cycle (GO:0000082), we identified that the transcription of the *cell division cycle associated 5 (CDCA5)* gene was most significantly upregulated from early tumor development and associated stepwise with tumor progression, suggesting it plays a role in tumorigenesis and its progression.

The *CDCA5* gene encodes CDCA5 protein (A.K.A. Sororin), a master regulator of sister chromatid cohesion and separation [15]. CDCA5 (Sororin) maintains sister chromatid cohesion by stabilizing the cohesion complex [16]. It ensures accurate chromosome partitioning in both meiotic and mitotic cells, and plays an important role in DNA repair. In one study, CDCA5 was overexpressed in the majority of non-small cell and small cell lung cancers at both mRNA and protein levels [17]. In the

same study, positive immunostaining for CDCA5 in 262 non-small cell lung cancer samples was significantly associated with poor prognosis [17]. However, to our best knowledge, no prior report has evaluated CDCA5 expression in UC. We therefore aimed to comprehensively analyze CDCA5 expression and its association with clinicopathological factors and survival in our well-characterized cohort of UC patients.

Materials and methods

Data mining on the GEO to identify overexpressed transcripts in UCs

We carried out data mining on the GEO (National Center Biotechnology Information). We identified one data set, GSE32894 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32894) analyzing transurethral resection specimens from 308 patients with UBUC using Affymetrix U133 Plus 2.0 Array. Statistical software Nexus Expression 3 (BioDiscovery, El Segundo, California, USA) was used to analyze all probe sets without preselection or filtering. We performed supervised comparative analysis to examine the statistical significance of differentially expressed transcripts on the basis of primary tumor (pT) status and the development of metastatic events. Transcriptomes of high-stage (pT2-pT4) UCs with developed metastases and low-stage (pTa-pT1) UCs devoid of metastasis were used to perform functional profiles, focusing on those related to the G1-S transition of the mitotic cell cycle (GO:0000082). Further survival analysis was performed by dichotomizing all cases into high-expression and low-expression clusters, in order to computerize the prognostic impact of selected genes.

Patients and tumor specimens

This study was approved by the institutional review board (IRB10302015) of Chi Mei Medical Center. Informed consent has been obtained for those enrolled into BioBank. For imunohistochemical study and statistical analysis, we retrieved urothelial carcinoma cases from the archives of Chi Mei Medical Center between 1996 and 2004. A total of 635 consecutively treated well-characterized urothelial carcinomas, not otherwise specified, were enrolled including 340 tumors originating from the upper urinary tract and 295 arising from
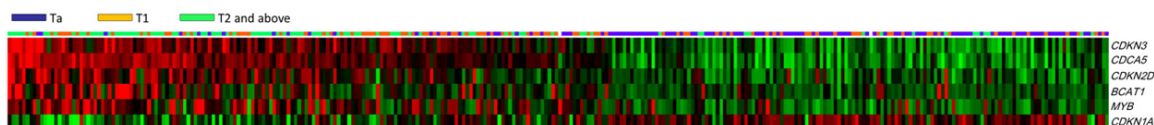
Figure 1. Analysis of gene expression in urothelial carcinoma from a published transcriptomic dataset (GSE32894). Clustering analysis of genes focusing on the G1-S transition of the mitotic cell cycle (GO:0000082) revealed *CDCA5* is the most significantly up-regulated gene associated with increments of pT status. Tissue specimens from tumors with different pT statuses are indicated on top of the heatmap, and expression levels of up-regulated and down-regulated genes are represented as a spectrum of brightness or red and green, respectively. Those unaltered in mRNA transcriptional level are coded black.

the urinary bladder. Other histological variants were excluded. All patients were treated initially by surgical intervention with curative intent. As a rule, patients with urinary bladder urothelial carcinoma (UBUC) with pT3 or pT4 tumors or with nodal involvement received cisplatin-based adjuvant chemotherapy. However, only 29 of 106 pT3 or pT4 and nodal positive patients with upper tract urothelial carcinoma (UTUC) received cisplatin-based adjuvant chemotherapy. The criteria for clinicopathological evaluation were essentially identical to those in our previous works [18]. Two pathologists (IWC & CFL) re-evaluated hematoxylin-eosin sections of all cases. By testing a series of cutoff values, the high mitotic activity was defined as the mitotic rate no less than 10 high power fields which showed most prognostic relevance. For the quantification of CDCA5 transcript levels, fresh tissue from an independent cohort of 36 UTUCs and 30 UBUCs were selected and CDCA5 mRNA was detected with ABI StepOnePlus™ System. Of these, 21 and 15 UTUCs were of pTa-pT1 and pT2 to pT4, respectively; and 15 and 15 UBUCs were of pTa-pT1 and pT2 to pT4, respectively.

Transcriptional level of CDCA5 gene

The materials for molecular genetic examination were harvested from macrodissection of fresh tumor tissue. For quantification of *CDCA5* mRNA expression, we extracted total RNAs, quantified them, and submitted them for reverse-transcription. Using pre-designed TaqMan assay reagents (Applied Biosystems), we measured mRNA abundance of *CDCA5* (Hs-01591589_m1) with the ABI StepOnePlus™ System, as previous described [19]. The fold expression of *CDCA5* relative to normal urothelium was calculated by comparative Ct method, after normalization to POLR2A (Hs01108291_m1) as the internal control.

Immunohistochemical staining and scoring of CDCA5

Tissue sections underwent standard procedures for deparaffinization, rehydration and antigen retrieval. Subsequently, the sections were incubated with a primary antibody targeting CDCA5 (NBP1-89530, rabbit polyclonal, Novus Biologicals, CO, USA, dilution 1:100) for an hour. Scoring of CDCA5 immunoreactivity was evaluated based on the combination of the percentage and intensity of positively stained tumoral nuclei to generate an H-score, which was calculated using the following equation: H-score = ΣP_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 equals to 100% of tumor cells being negative and 400 equals 100% of tumor cells strongly stained (3+) [20, 21].

Statistical analysis

Statistics were performed using SPSS V.14.0 software (SPSS Inc., Chicago, Illinois, USA). The median H-score of CDCA5 was used as the cutoff to dichotomize the study cohort, separating cases into high expression and low expression groups. The Chi-square test was used to compare CDCA5 expression status and various clinicopathological parameters. The end points analyzed were disease-specific survival (DSS) and metastasis-free survival (MeFS), calculated from the starting date of resection of tumor to the date the event developed. Patients lost to follow-up were censored on the latest follow-up date. Univariate survival analyses were performed using Kaplan-Meier plots and compared by the log-rank test. Those parameters with univariate $p < 0.1$ were enrolled into multivariate tests performed using Cox proportional

Table 1. Summary of differentially expressed genes associated with Transition of G1-S transition of mitotic cell cycle and showed stepwise alterations during cancer progression in the transcriptome of urothelial carcinoma of urinary bladder

Probe	Comparing T2-4 to Ta		Comparing T1 to Ta		Comparing T2-4 to T1		Gene symbol	Biological process	Molecular function
	log ratio	p-value	log ratio	p-value	log ratio	p-value			
ILMN_1683450	1.5233	< 0.0001	1.0594	< 0.0001	0.4639	< 0.0001	CDCA5	G1/S transition of mitotic cell cycle, cell cycle, cell division, mitosis, mitotic chromosome condensation, mitotic metaphase plate congression	chromatin binding, protein binding
ILMN_1666305	0.8475	< 0.0001	0.5026	< 0.0001	0.3449	0.0011	CDKN3	G1/S transition of mitotic cell cycle, cell cycle, cell cycle arrest, dephosphorylation, negative regulation of cell proliferation, regulation of cyclin-dependent protein kinase activity	Hydrolase activity, protein binding, protein serine/threonine phosphatase activity, protein tyrosine phosphatase activity, protein tyrosine/serine/threonine phosphatase activity
ILMN_1711894	0.5142	< 0.0001	0.2972	0.0198	0.217	0.0389	MYB	G1/S transition of mitotic cell cycle, calcium ion transport, regulation of transcription; DNA-dependent	DNA binding, protein binding, transcription activator activity, transcription factor activity
ILMN_1748883	0.455	< 0.0001	0.1736	0.0038	0.2815	< 0.0001	CDKN2D	DNA synthesis during DNA repair, G1/S transition of mitotic cell cycle, anti-apoptosis, autophagic cell death, cell cycle arrest, negative regulation of caspase activity, negative regulation of cell cycle, negative regulation of cell growth, negative regulation of cell proliferation, negative regulation of phosphorylation, regulation of cyclin-dependent protein kinase activity, response to UV, response to retinoic acid, response to vitamin D	Cyclin-dependent protein kinase inhibitor activity, protein kinase binding
ILMN_1766169	0.8965	< 0.0001	0.1708	0.0173	0.7258	< 0.0001	BCAT1	G1/S transition of mitotic cell cycle, branched chain family amino acid biosynthetic process, cell proliferation, metabolic process	Branched-chain-amino-acid transaminase activity, transferase activity
ILMN_1784602	-0.6946	< 0.0001	-0.3761	0.0017	-0.3185	0.0073	CDKN1A	G1/S transition of mitotic cell cycle, G2/M transition of mitotic cell cycle, cell cycle arrest, cellular response to extracellular stimulus, goid 43071, induction of apoptosis by intracellular signals, negative regulation of apoptosis, negative regulation of cell growth, negative regulation of cell proliferation, negative regulation of cyclin-dependent protein kinase activity, negative regulation of phosphorylation, positive regulation of B cell proliferation, positive regulation of fibroblast proliferation, response to DNA damage stimulus, response to UV	Cyclin binding, cyclin-dependent protein kinase inhibitor activity, metal ion binding, protein kinase inhibitor activity, zinc ion binding
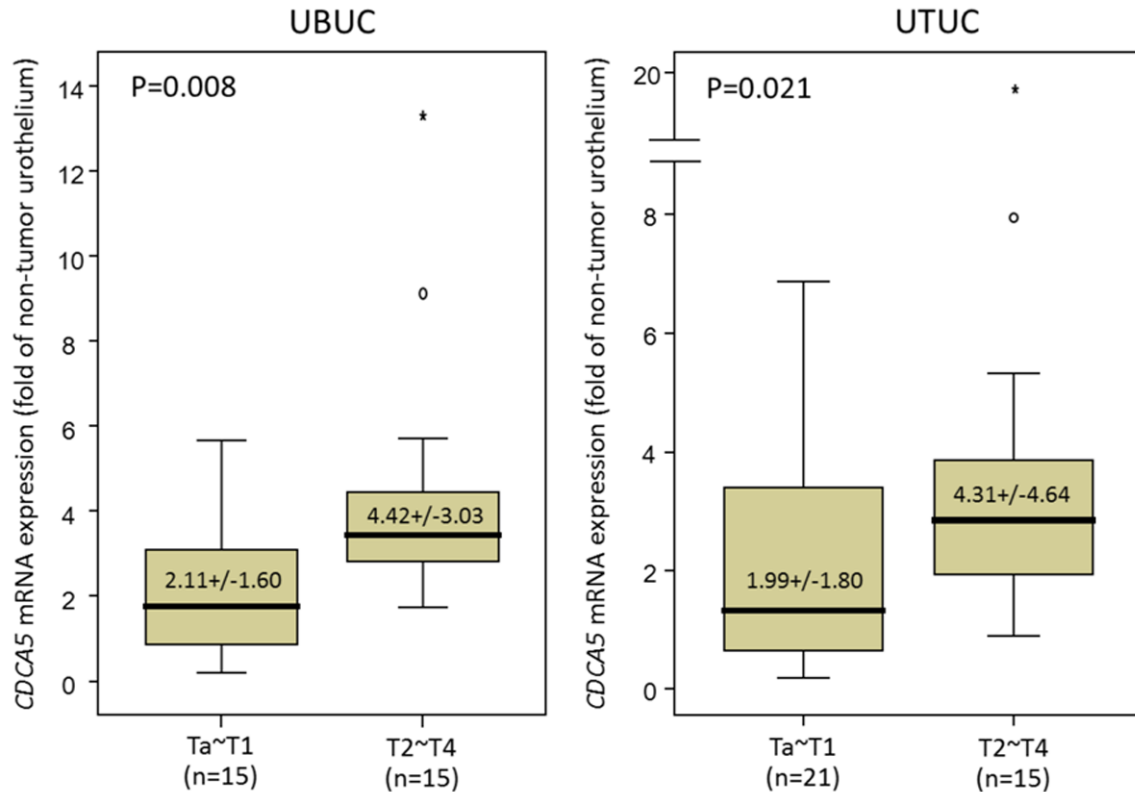
Figure 2. Quantitative real-time PCR (qPCR) analysis showed that CDCA5 mRNA expression was significantly increased in both UBUCs (*left* panel) and UTUCs (*right* panel) with advanced primary pT status. (p = 0.008 and p = 0.021, respectively).

hazards regression. For all analyses, we used two-sided tests of significance with p < 0.05 considered as significant.

Results

CDCA5 identified as a significant differentially upregulated transcript implicating dysregulation of the G1-S transition of mitotic cell cycle in UBUC

From the transcriptomic profile of 93 high-stage (T2-T4) and 213 low-stage (Ta-T1) cases of UBUC, six probes covered six transcripts associated with regulation the G1-S transition of the mitotic cell cycle (GO:0000082) were found (**Figure 1**). The log2 ratios of one transcript met the selection criteria of >1.5 increase (p < 0.0001), *i.e.*, *CDCA5*, with log2 ratio of 0.4639 to 1.5233-fold up-regulation (**Table 1**). That *CDCA5* has not been systemically studied in UCs prompted us to further characterize the endogenous expression levels and clinical significance of CDCA5 protein in UC.

CDCA5 mRNA transcriptional levels are higher in UBUC and UTUC with advanced pT stage

In the tested 30 UBUCs and 36 UTUCs, *CDCA5* transcriptional level was significantly higher in tumors with higher pT status (p = 0.008 and p = 0.021, respectively), suggesting it plays a role in tumor progression (**Figure 2**).

Clinicopathological findings regarding UTUC

The clinicopathological characteristics of the UTUC patients are listed in **Table 2**. No sex predilection was noted. The patients' age at diagnosis ranged from 34 to 87 years (median, 68 years). Sixty-two (18.2%) cases had multiple foci of tumors; of these, 49 (14.4%) cases involved both the renal pelvis and ureter. The majority of cases (n = 284, 83.5%) were of high histological grade. Advanced pT stages (pT2-T4) were seen in 159 (46.8%) of cases. About half (n = 167, 49.1%) of the cases showed frequent mitosis. Vascular invasion and perineurial invasion were observed in 106 cases (31.2%) and

Table 2. Correlations between CDCA5 Expression and other important clinicopathological parameters in urothelial carcinomas

Parameter	Category	Upper urinary tract urothelial carcinoma				Urinary bladder urothelial carcinoma			
		Case no.	CDCA5 expression		p-value	Case no.	CDCA5 expression		p-value
			Low	High			Low	High	
Gender	Male	158	74	84	0.277	216	104	112	0.339
	Female	182	96	86		79	43	36	
Age (years)	< 65	138	73	65	0.377	121	62	59	0.686
	≥ 65	202	97	105		174	85	89	
Tumor location	Renal pelvis	141	60	81	0.055	-	-	-	-
	Ureter	150	85	65		-	-	-	-
	Renal pelvis & ureter	49	25	24		-	-	-	-
Multifocality	Single	278	140	138	0.779	-	-	-	-
	Multifocal	62	30	32		-	-	-	-
Primary tumor (T)	Ta	89	78	11	< 0.001*	84	62	22	< 0.001*
	T1	92	45	47		88	46	42	
	T2-T4	159	47	112		123	39	84	
Nodal metastasis	Negative (N0)	312	165	147	< 0.001*	266	138	128	0.033*
	Positive (N1-N3)	28	5	23		29	9	20	
Histological grade	Low grade	56	44	12	< 0.001*	56	41	15	< 0.001*
	High grade	284	126	158		239	106	133	
Vascular invasion	Absent	234	142	92	< 0.001*	246	129	117	0.045*
	Present	106	28	78		49	18	31	
Perineural invasion	Absent	321	167	154	0.002*	275	141	134	0.066
	Present	19	3	16		20	6	14	
Mitotic rate (per 10 high power fields)	< 10	173	101	72	0.002*	139	85	54	< 0.001*
	≥ 10	167	69	98		156	62	94	

*Statistically significant.

19 cases (5.9%), respectively. Nodal metastasis was detected in 28 (8.2%) patients.

Clinicopathological findings regarding UBUC

The majority of UBUC patients were male (n = 216, 73.2%) and were older than 60 years (n = 214, 72.5%). As shown in **Table 2**, most (n = 239, 81%) were of high histological grade and 123 (41.7%) of them were in advanced stages (pT2-T4) at diagnosis. High mitotic activity (≥ 10 per high power fields) was found in 156 cases (52.9%). Lymph node metastasis was observed in 23.6% of patients (n = 29). In addition, vascular invasion and perineurial invasion had been observed in 49 cases (16.6%) and 20 cases (6.8%), respectively.

Correlations between immunoactivity of CDCA5 and parameters in UTUC and UBUC

CDCA5 showed variable nuclear expression in UC of both sites. The medium value of H-score

as the cut-off was 210 and 215 for UTUC and UBUC, respectively. After dichotomizing tumors into low and high CDCA5 expression, as **Table 2** demonstrates, increased CDCA5 expression in urothelial carcinoma of both sites was significantly associated with increment of pT status (**Figure 3**, p < 0.001), lymph node metastasis (UTUC, p < 0.001; UBUC, p = 0.033), higher histological grade (p < 0.001, both), vascular invasion (UTUC, p < 0.001; UBUC, p = 0.045) and frequent mitosis (UTUC, p = 0.002; UBUC, p < 0.001). Increased CDCA5 expression was statistically significantly (p = 0.002) associated with perineurial invasion in UTUC only.

Survival analysis for UTUC

The univariate and multivariate analyses of associations between clinical outcomes and various clinicopathological parameters in UTUC cases are demonstrated in **Table 3**. Multivariate analysis showed that poor DSS was significantly associated with multifocality (p = 0.005),
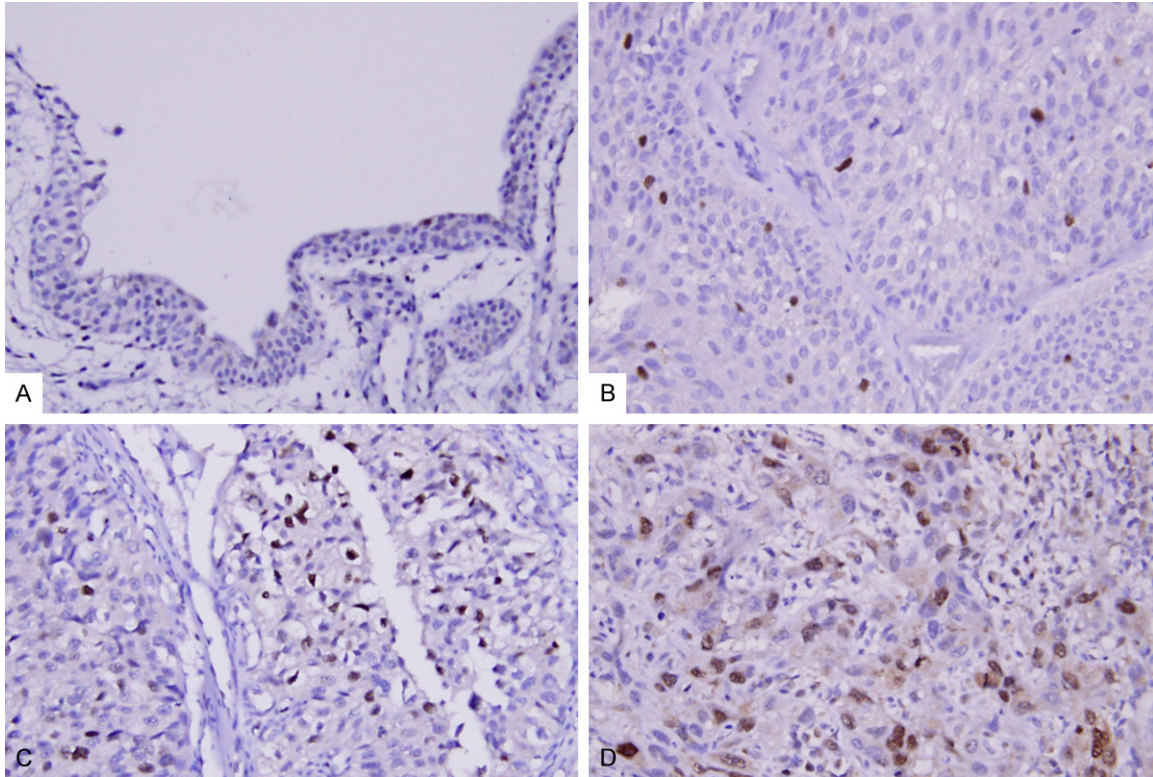
Figure 3. CDCA5 immunostaining on representative sections of non-tumor urothelium (A), non-invasive urothelial carcinoma of low grade (B) and high grade (C) and high-grade infiltrating urothelial carcinoma (D), respectively, exhibits stepwise increased CDCA5 expression.

nodal metastasis (p < 0.001), high histological grade (p = 0.043), and perineurial invasion (p = 0.001). Similar results were also seen with MeFS. In UTUC, pT stage (**Figure 4A**, **4B**) and vascular invasion were significantly associated with worse DSS and MeFS in univariate (p < 0.0001) but not in multivariate analyses. UTUC tumor location correlated with poor patient DSS in univariate analysis only (p = 0.0079).

Survival analysis for UBUC

As demonstrated in **Table 4**, both univariate and multivariate analyses revealed advanced pT stage was significantly associated with both dismal DDS and MeFS (both p < 0.0001 for univariate analysis, **Figure 4E**, **4F**; p < 0.001 and p = 0.015 for multivariate analyses, respectively). Perineural invasion and mitotic rate were also significantly associated with poor DDS (p = 0.025 and p = 0.032, respectively). Lymph node metastasis was associated with poor MeFS (p = 0.040) in multivariate analysis, as well. Histological grade and vascular invasion in UBUC are associated with both worse DDS and

MeFS in univariate analysis to a statistically significant degree (all p < 0.005), but not in multivariate analysis.

Prognostic significance of CDCA5 expression in UC

As shown in **Tables 3**, **4**, in univariate analysis, patients with either UTUC or UBUC exhibiting high CDCA5 nuclear expression had significantly worse DSS and MeFS (p < 0.0001 for all, **Figure 4C**, **4D**, **4G**, **4H**). Remarkably, in multivariate analysis, CDCA5 overexpression remained an independent prognosticator predicting dismal DSS and MeFS for all UCs (**Tables 3**, **4**). To further confirm the prognostic significance of CDCA5 expression, we performed subgroup analysis. As shown in Figure S1, in four groups namely UTUC of low- and high-pT status and UBUC of low- and high-pT status, CDCA5 over-exression was still associated with poor DSS and MeFS. Of three of these four groups (UTUC-Low- and high-stage and UBUC-Low stage), the log-rank test revealed statistical significance for both DSS and MeFS (all p < 0.05).
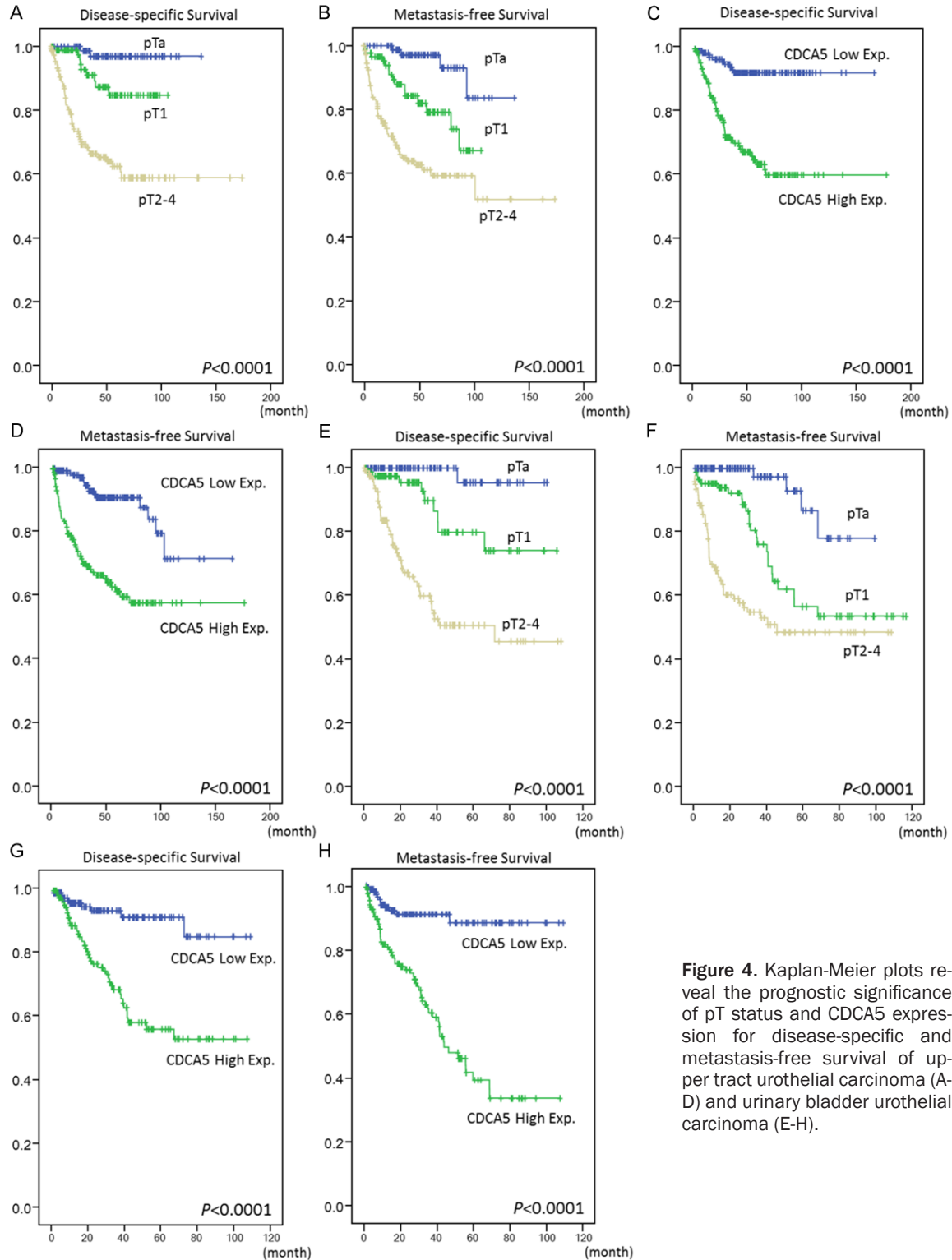
Table 3. Univariate log-rank and multivariate analyses for Disease-specific and Metastasis-free Survivals in Upper urinary tract urothelial carcinoma

| 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	28	0.8286	-	-	-	32	0.7904	-	-	-
	Female	182	33		-	-	-	38		-	-	-
Age (years)	< 65	138	26	0.9943	-	-	-	30	0.8470	-	-	-
	≥ 65	202	35		-	-	-	40		-	-	-
Tumor side	Right	177	34	0.7366	-	-	-	38	0.3074	-	-	-
	Left	154	26		-	-	-	32		-	-	-
	Bilateral	9	1		-	-	-	0		-	-	-
Tumor location	Renal pelvis	141	24	0.0079*	1	-	0.868	31	0.0659	1	-	0.907
	Ureter	150	22		0.814	0.438-1.513		25		0.913	0.379-3.763	
	Renal pelvis & ureter	49	15		1.266	0.352-4.555		14		0.799	0.326-3.658	
Multifocality	Single	273	48	0.0026*	1	-	0.005*	52	0.0127*	1	-	0.009*
	Multifocal	62	18		2.985	1.381-6.454		18		2.780	1.288-6.003	
Primary tumor (T)	Ta	89	2	< 0.0001*	1	-	0.065	4	< 0.0001*	1	-	0.578
	T1	92	9		2.202	0.451-10.754		15		1.796	0.560-5.762	
	T2-T4	159	50		3.679	0.774-17.488		51		1.855	0.562-6.124	
Nodal metastasis	Negative (N0)	312	42	< 0.0001*	1	-	< 0.001*	55	< 0.0001*	1	-	0.001*
	Positive (N1-N2)	28	19		5.087	2.733-9.469		15		2.952	1.570-5.549	
Histological grade	Low grade	56	4	0.0215*	1	-	0.043*	3	0.0027*	1	-	0.023*
	High grade	284	57		3.073	1.038-9.096		67		4.007	1.214-13.222	
Vascular invasion	Absent	234	24	< 0.0001*	1	-	0.083	26	< 0.0001*	1	-	0.002*
	Present	106	37		1.684	0.934-3.035		44		2.639	1.444-4.823	
Perineural invasion	Absent	321	50	< 0.0001*	1	-	0.001*	61	< 0.0001*	1	-	0.015*
	Present	19	11		3.567	1.711-7.435		9		2.579	1.200-5.539	
Mitotic rate (per 10 high power fields)	< 10	173	27	0.167	-	-	-	30	0.0823	1	-	0.285
	≥ 10	167	34		-	-	-	40		0.989	0.969-1.009	
CDCA5 expression	Low	170	10	< 0.0001*	1	-	0.016*	15	< 0.0001*	1	-	0.006*
	High	170	51		2.415	1.175-4.966		55		2.354	1.273-4.353	

*Statistically significant.

Figure 4. Kaplan-Meier plots reveal the prognostic significance of pT status and CDCA5 expression for disease-specific and metastasis-free survival of upper tract urothelial carcinoma (A-D) and urinary bladder urothelial carcinoma (E-H).

Discussion

When the cells receive growth-promoting stimuli, the ultimate outcome is the entry of quiescent cells into the cell cycle. The regulation of the cell cycle is extremely important to the cell; because the cell cycle dysregulated by mutations or amplification of related genes leads the

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	41	0.4446	-	-	-	60	0.2720	-	-	-
	Female	79	11		-	-	-	16		-	-	-
Age (years)	< 65	121	17	0.1136	-	-	-	31	0.6875	-	-	-
	≥ 65	174	35		-	-	-	45		-	-	-
Primary tumor (T)	Ta	84	1	< 0.0001*	1	-	< 0.001*	4	< 0.0001*	1	-	0.015*
	T1	88	9		4.621	0.501-42.641		23		3.798	1.105-13.060	
	T2-T4	123	42		17.283	1.978-105.997		49		4.941	1.451-16.828	
Nodal metastasis	Negative (N0)	266	41	0.0002*	1	-	0.352	61	< 0.0001*	1	-	0.040*
	Positive (N1-N2)	29	11		1.397	0.691-2.822		15		1.903	1.030-3.515	
Histological grade	Low grade	56	2	0.0013*	1	-	0.802	5	0.0007*	1	-	0.581
	High grade	239	50		1.214	0.266-5.528		71		1.333	0.480-3.702	
Vascular invasion	Absent	246	37	0.0024*	1	-	0.273	54	0.0001*	1	-	0.803
	Present	49	15		0.685	0.348-1.348		22		1.077	0.601-1.929	
Perineural invasion	Absent	275	44	0.0001*	1	-	0.025*	66	0.0007*	1	-	0.117
	Present	20	8		2.531	1.121-5.714		10		1.780	0.866-3.662	
Mitotic rate (per 10 high power fields)	< 10	139	12	< 0.0001*	1	-	0.032*	23	< 0.0001*	1	-	0.077
	≥ 10	156	40		2.100	1.067-4.133		53		1.599	0.951-2.688	
CDCA5 expression	Low	147	9	< 0.0001*	1	-	0.016*	14	< 0.0001*	1	-	< 0.001*
	High	148	43		2.467	1.181-5.152		62		3.030	1.657-5.540	

*Statistically significant.

cell to grow autonomously, a characteristic of cancers. The cell cycle in eukaryotic cells is regulated by a complex set of molecules, including cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors, as well as retinoblastoma (RB) protein, and p53. Dysregulation of cell cycle control is one of the most important events in carcinogenesis, especially the checkpoint pathways [12]. There are three main checkpoints: the G1-S, S-phase and G2-M checkpoints [13]. The G1-S checkpoint checks for DNA damage; if DNA damage is present, the DNA-repair mechanisms that arrest the cell cycle will be activated. The delay in cell cycle progression gives the cell time to repair its DNA. If the damage is unrepairable, apoptotic pathways will be stimulated. Therefore, the G1-S checkpoint prevents cells with defective DNA from replicating and perpetuating mutations or chromosomal breaks in the cell's descendants [22]. When the cell suffers from genotoxic insult, the intra-S-phase checkpoint is activated, and then transiently and reversibly inhibits the replication-origin firing and protects the integrity of the stalled replication forks [22]. The G2-M checkpoint monitors the completion of DNA replication and checks the safety of both the initiation of mitosis and the separation of sister chromatids. Defects in the G2-M checkpoint give rise to chromosomal abnormalities [23, 24]. The most important of these is the G1-S checkpoint, known as the restriction point, after which the cell is committed to DNA replication and cell division [13]. Dysregulation in the G1-S transition of the mitotic cell cycle occurs commonly in carcinogenesis [25]. Nevertheless, the expressions of these genes have not been systemically evaluated in UC until now. By performing data mining on the published transcript expression profiles of UBUCs (GSE32894) in the GEO, with a special focus on the G1-S transition of the mitotic cell cycle (GO:0000082), we identified the gene, *cell division cycle associated 5 (CDCA5)*, as the most significantly upregulated gene associated stepwise with disease progression.

The *CDCA5* gene encodes CDCA5 protein (A.K.A. Sororin). CDCA5 was initially recognized as a substrate of the anaphase-promoting complex [26] and as a master regulator of sister chromatid cohesion [27] in cells. CDCA5 is degraded through anaphase-promoting complex-dependent ubiquitination in the G1 phase, and is required for stabilizing binding of the

cohesion complex to sister chromatids [27]. However, there is only one study describing the significance of the activation of CDCA5 in carcinogenesis of the human lung [17]. In that study, the authors identified *CDCA5* as an up-regulated gene in the majority of lung carcinomas by analyzing gene expression profiles using cDNA microarray containing 27,648 genes or expressed sequence tags. In a immunohistochemical study of 262 non-small cell lung carcinoma cases, 192 cases (73.3%) were judged as positive and 70 (26.7%) as negative. CDCA5 positivity was also significantly associated by log-rank test (p = 0.0143) with shorter tumor-specific survival periods. Furthermore, multivariate analysis demonstrated that CDCA5 status was an independent prognostic factor for surgically treated lung cancer (p = 0.0244) [17]. They also conducted *in vitro* MTT cell proliferation assay to evaluate the growth-promoting effect of CDCA5. First, when CDCA5 expression was knocked down by *CDCA5*-specific siRNAs, the growth of the two lung cancer cell lines, A549 and LC319 both with high CDCA5 expression levels, was significantly suppressed compared with the controls (p < 0.0001). Next, when COS-7 cells, a fibroblast-like cell line derived from monkey kidney tissue, were transfected by plasmids containing full-lengths of the *CDCA5* gene, the growth of COS-7—CDCA5 cells was promoted compared with the controls, to a significant degree (p < 0.005) [17]. This study also proved that CDCA5 was phosphorylated by ERK at two phosphorylation sites, Ser79 and Ser209. The authors suggested that transactivation of CDCA5 and its phosphorylation by ERK play a pivotal role in lung carcinogenesis [17].

In the present study, CDCA5 overexpression was associated with advanced pT status, nodal metastasis, high histological grade, vascular invasion and frequent mitoses in both UTUC and UBUC to a statistically significant degree (all p < 0.05). Along with other important clinicopathological parameters, such as pT status, nodal metastasis, and histological grade, CDCA5 overexpression also predicted worse DSS and MeFS in patients with urothelial carcinoma from both anatomical sites, both in univariate and multivariate analyses, with statistical significance (all p < 0.05). When we divided our cohort into four groups: UTUC/UBUC of low- or high-stage, we still found high CDCA5 expression was associated with dismal DSS and

MeFS. In addition, in three groups of these—UTUC of low- and high-stage and UBUC of low stage, CDCA5 overexpression predicted poor DSS and MeFS with statistical significance (all p < 0.05). Higher *CDCA5* transcriptional level was also significantly associated with higher pT status in both UTUC and UBUC (p = 0.021 and p = 0.008, respectively).

Conclusion

In summary, our study demonstrates that CDCA5 plays a significant role in tumor progression in both UTUC and UBUC. In patients with UCs, CDCA5 over-expression and up-regulation in both protein and mRNA levels are predictive of poor DSS and MeFS. Further investigation to elucidate the biological significance of CDCA5 protein expression in UC is essential for exploring the potential of CDCA5-targeted therapy for UC.

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Abbreviations

UC, Urothelial carcinoma; UBUC, Urinary bladder urothelial carcinoma; UTUC, upper urinary tract urothelial carcinoma; GEO, Gene Expression Omnibus; CDCA5, cell division cycle associated 5; DSS, Disease-specific survival; MeFS, metastasis-free survival; CDK, cyclin-dependent kinase; RB: retinoblastoma.

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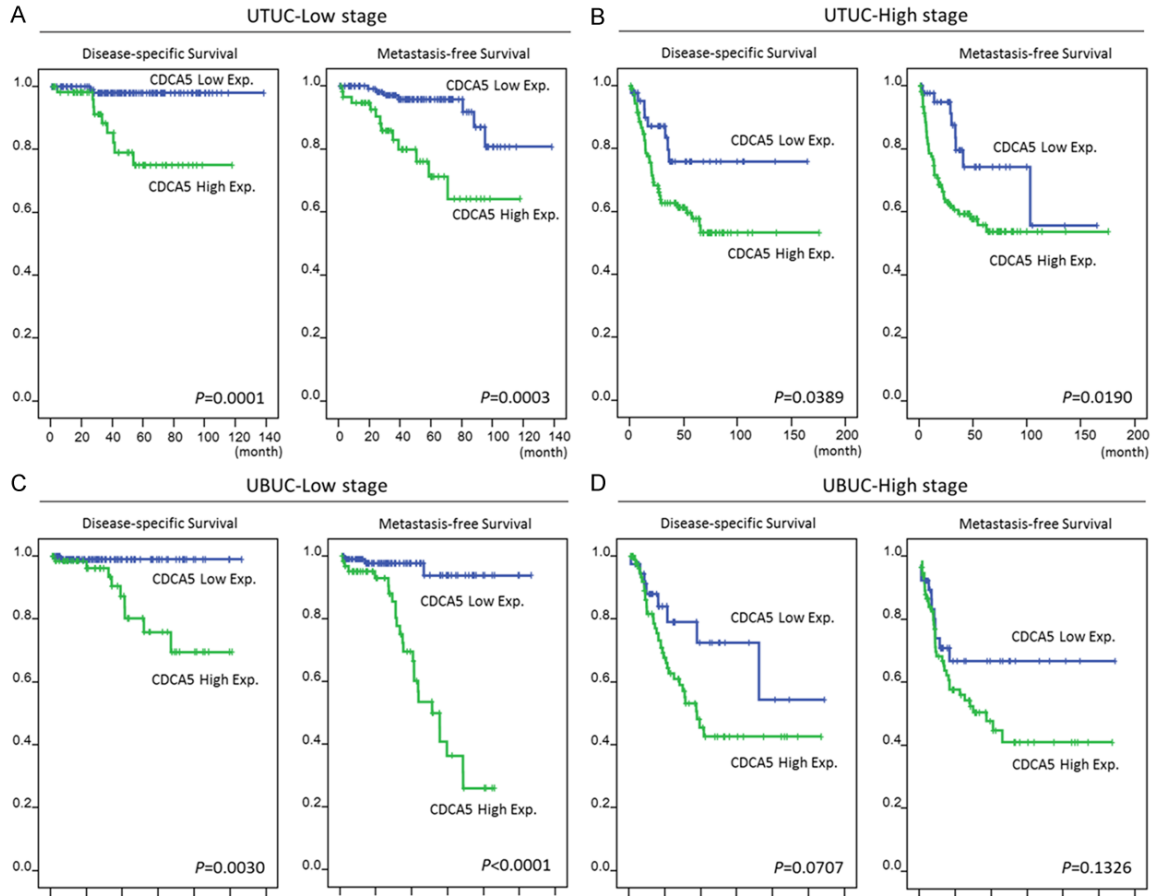
Figure S1. Kaplan-Meier plots reveal that CDCA5 over-expression is associated with poor disease-specific survival (DSS) and metastasis-free survival (MeFS) in the four groups: upper tract urothelial carcinoma (UTUC) of low- and high-stage as well as urinary bladder urothelial carcinoma (UBUC) of low- and high-stage. Notably, in the groups of UTUC-Low stage, UTUC-High stage and UBUC-Low stage, CDCA5 over-expression predicts poor DSS and MeFS with statistical significance (all p < 0.05).