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Cyclin A2 Expression as Predictive Biomarker in Muscle-Invasive Upper Tract Urothelial Carcinoma

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Keywords
Cyclin A2 · Upper tract urothelial carcinoma · Biomarker · Overall survival

Abstract
Introduction: The aim was to evaluate the prognostic value of altered *Cyclin A2 (CCNA2)* gene expression in upper tract urothelial carcinoma (UTUC) and to assess its predictive potential as a prognostic factor for overall survival (OS) and disease-free survival. ***Methods:*** 62 patients who underwent surgical treatment for UTUC were included. Gene expression of *CCNA2*, *MKI67*, and *p53* was analyzed by quantitative reverse transcriptase polymerase chain reaction. Survival analyses were performed using the Kaplan-Meier method and the log-rank test. For Cox regression analyses, uni- and multivariable hazard ratios were calculated. Spearman correlation was used to analyze correlation of *CCNA2* expression with *MKI67* and *p53*. ***Results:*** The median age of the cohort was 73 years, and it consisted of 48 males (77.4%) and 14 females (22.6%). Patients with high *CCNA2* expression levels showed longer OS (HR 0.33; 95% CI: 0.15–0.74; p = 0.0073). Multivariable Cox regression analyses identified *CCNA2* overexpression (HR 0.37; 95% CI: 0.16–0.85; p = 0.0189) and grading G2 (vs. G3) (HR 0.39; 95% CI: 0.17–0.87; p = 0.0168) to be independent predictors for longer OS. *CCNA2* expression correlated positively with *MKI67* expression (Rho = 0.4376, p = 0.0005). ***Conclusion:*** Low *CCNA2* expression is significantly associated with worse OS. Thus, *CCNA2* might serve as a potential biomarker in muscle-invasive UTUC and may be used to characterize a subset of patients having an unfavorable outcome and for future risk assessment scores.

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Introduction

Upper tract urothelial carcinoma (UTUC) is a rather rare malignancy, accounting for 5–10% of all urothelial carcinoma cancer cases with an annual incidence of 1–2/100,000 [1]. At the time of diagnosis, patients often present with a locally advanced or metastatic disease. Compared to urothelial carcinoma of the urinary bladder (UCB), the prognosis of UTUC is relatively poor due to its aggressive nature [2]. Although UTUC is morphologically and histologically similar to UCB, molecular and genetic studies suggest two different tumor entities regarding biological and clinical characteristics [3–5]. Despite definitive surgery as the standard treatment for patients with nonmetastatic UTUC, up to 30% of the patients with UTUC experience disease recurrence and cancer-related death, especially in advanced disease stages [6]. Besides existing and well-established prognostic factors for urothelial carcinoma, such as T stage, tumor grade, or lymph node involvement, the identification of new prognostic molecular factors is mandatory to capture the individual biological variability of the tumor and thus support clinical management and assist in the development of new treatment modalities [7]. Diverse biomarkers are currently under investigation or have already been discovered in UTUC [8, 9]. It could be shown that certain microRNA (miR) types in patient serum, such as miR-141 or miR-151b, are associated with cancer-specific survival and tumor progression and have the potential to predict the prognosis of UTUC [10, 11]. Another well-known biomarker in UTUC is *MKI67*. Krabbe et al. [12] could show that its overexpression was associated with the prediction of recurrence-free survival in patients with UTUC.

Cyclin A2 (CCNA2) is a protein that plays an important role in cell cycle progression [13]. Increased expression of *CCNA2* has been observed in a variety of tumor entities, such as hepatocellular carcinoma, breast cancer, or lung cancer, consistent with its role as a key cell cycle regulator [14–18]. Its altered expression has been reported to have a prognostic value in terms of survival or early disease relapse. However, it is not clear whether the elevation of *CCNA2* in cancer cells is contributing to tumorigenesis or whether it is simply a consequence of increased cell proliferation. Interestingly, a study on drosophila cells implicates that rather a decrease in *CCNA2* expression may contribute to tumorigenesis [19]. Furthermore, there is evidence that *CCNA2* may not only be directly involved in tumorigenesis via deregulating the cell cycle but also by phosphorylating tumor suppressors, such as *p53* [20, 21]. Thus, an increased *CCNA2* expression appears to enhance the activity of *p53* and thereby influence tumorigenesis indirectly. However, the prognostic significance of *CCNA2* expression in patients with UTUC has not been investigated so far. Thus, the purpose of this study was to evaluate whether altered *CCNA2* gene expression can be identified as a prognostic marker in patients with muscle-invasive UTUC.

Materials and Methods

Cohorts and Patient Samples

A cohort of 125 patients (male: n = 93, 74.4%, median age: 70 years; interquartile range (IQR): 36–91 years; female: n = 32, 25.6%, median age: 76 years; IQR: 59–88) who underwent surgical treatment for UTUC at our tertiary care center between 2002 and 2016 was analyzed. Patients with non-muscle invasive UTUC, a follow-up shorter than 3 months and distant metastases were excluded, which resulted in a final cohort of 62 patients. The exclusion criteria for the individual analyses are shown in Figure 1. The pathological classification was done in accordance to the WHO 1973 and WHO 2004/2016 grading systems.

The study includes data and tissue from human participants in a retrospective study (ethics approval 2015-549N-MA). All patients gave informed consent for participation. All procedures performed in studies involving human participants were in accordance with the Ethical Standards of the Institutional and/or National Research Committee and with the Declaration of Helsinki and its later amendments or comparable ethical standards.

RNA Extraction, cDNA Synthesis, and PCR Analyses of Patient Samples

Representative tumor-bearing slide was selected and reviewed by a board-certified uropathologist. Tumors were marked, macrodissected from subsequent unstained 10 µm cuts, and RNA was extracted using the magnetic-bead-based XTRAKT FFPE kit (Stratifyer, Cologne, Germany). Finally, the RNA was eluted in 100 µL of elution buffer and stored at −80°C.

The cDNA synthesis was performed with sequence-specific reverse PCR primers for the reference gene *CALM2* and target gene *CCNA2*. Furthermore, we analyzed the gene expression of the proliferation marker *MKI67* and the tumor suppressor gene *p53*. The reverse transcriptase Superscript III (Thermo Fisher Scientific, Waltham, MA, USA) was used at 55°C for 120 min, followed by an enzyme inactivation step at 70°C for 15 min. cDNA was used for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) on a Step One Plus qRT-PCR cycler (Applied Biosystems, Waltham, MA, USA) for 40 cycles of amplification with 3 s of 95°C and 30 s of 60°C. The gene expression was normalized to the reference gene *CALM2*, which has been validated in UCB before and determined using the 40-(ΔCt)-method. This method was used to calculate the difference between the Ct value of the target genes (*CCNA2, MKI67* and *p53*) and the Ct of the reference gene (*CALM2*) in each patient [22, 23]. All primers and probes used in this study are shown in Supplementary Table 1 (for all online suppl. material, see https://doi.org/10.1159/000536184).

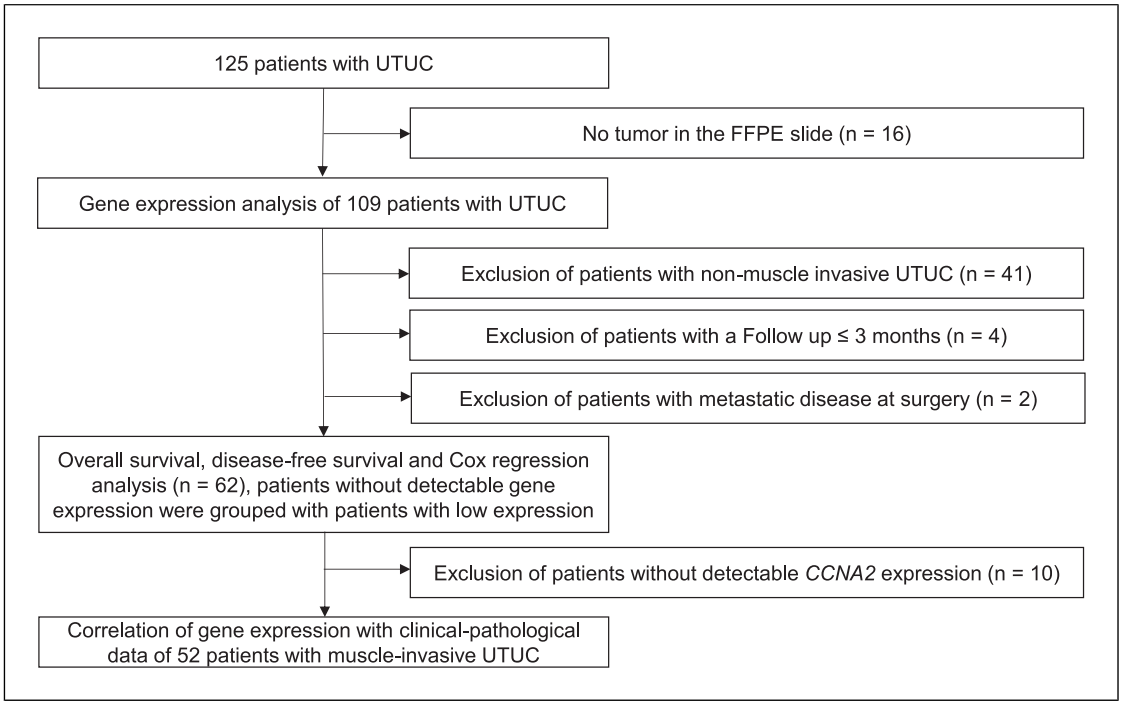

Fig. 1. Flowchart of exclusion criteria, cohort size, and analyses of the cohort.

Statistics

The statistical analyses were performed using JMP® from SAS (version 15; SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Mann-Whitney U-tests were used to compare non-normally distributed continuous data and χ^2 test to calculate categorical data. The cut-off values for high and low gene expression were determined by partition test, with each group representing at least 20% of the total cohort. Survival analyses were performed using the Kaplan-Meier method and the log-rank test. For the Cox regression analyses, uni- and multivariable hazard ratios (HR) were calculated. p values <0.2 from the univariable analyses were included in the multivariable analyses. Spearman analysis was used to correlate the gene expression of the target genes.

Results

The clinicopathologic characteristics of our patient cohort are displayed in Table 1. The median age was 73 years (IQR: 36–91 years) and the cohort consisted of 48 males (77.4%) and 14 females (22.6%). According to the WHO 2004/2016 grading system, two and 57 patients were identified as low and high grades, respectively (no data were available for 3 patients). The median follow-up duration was 55.5 months (IQR: 5–221 months). Disease recurrence, defined as any tumor recurrence, including non-muscle-invasive tumors in the urinary bladder and distant metastasis, was documented in 32 patients (51.6%). Relevant disease recurrence, defined as local recurrence within the pelvic field or at least a T2 stage recurrence in the bladder, lymph node relapse, and distant metastasis, could be detected in 24 of those patients (75%). In 18 patients, distant metastases occurred, for example, in the liver, lung, brain and bones. The median time range until disease recurrence was 4.5 months (IQR: 2–176). Ten patients died of UTUC (no data available for 20 patients).

Measurable gene expression of *CCNA2* was found in 52 patients, whereas *CALM2* expression was detectable in all patients (n = 62). As displayed in Figure 2, none of the selected characteristics, such as gender (male vs. female, p = 0.3338), age (<75 vs. ≥ 75 years, p = 0.3920), pathological T stage (pT2 vs. pT3/T4, p = 0.0691), lymphovascular invasion (LVI) (absent vs. present, p = 0.9316), or clinical and pathological N stage (c/pN0 vs. pN1, p = 0.8096) were significantly associated with the *CCNA2* expression pattern. Similar results were observed for *MKI67* and *p53* (online suppl. Table 2).

Kaplan-Meier curves revealed that patients with a higher *CCNA2* expression level had a longer overall survival (OS) compared to those with lower *CCNA2* expression levels (high vs. low expression 176 vs. 21 months; HR 0.33; 95% CI: 0.15–0.74; p = 0.0073). The 5-year OS rates were 59.9% in patients with high *CCNA2* expression levels and 23.5% in patients with low *CCNA2*

Table 1. Clinicopathological characteristics of the cohort

	Total cohort	*CCNA2* low	*CCNA2* high	*p* value	χ^2
Number of patients, *n* (%)	62 (100)	17 (27.4)	45 (72.6)		
Follow-up, months, median (IQR)	55.5 (5–221)				
Age in years, *n* (%)					0.7788
<75 years	38 (61.3)	11 (17.8)	27 (43.5)	<0.0001	
≥75 years	24 (38.7)	6 (9.7)	18 (29.0)	0.0004	
Gender, *n* (%)					0.7389
Male	48 (77.4)	14 (22.6)	34 (54.8)	<0.0001	
Female	14 (22.6)	3 (4.8)	11 (17.8)	0.0126	
Pathological T stage, *n* (%)					0.0652
pT2	19 (30.6)	2 (3.2)	17 (27.4)	n/a[a]	
pT3/T4	43 (69.4)	15 (24.2)	28 (45.2)	<0.0001	
Type of surgical procedure, *n* (%)					nc[a]
Radical nephroureterectomy	59 (95.2)	17 (27.4)	42 (67.8)	<0.0001	
Distal ureterectomy	2 (3.2)	0 (0.0)	2 (3.2)	nc[a]	
Nephrectomy	1 (1.6)	0 (0.0)	1 (1.6)	nc[a]	
N stage, *n* (%)					0.6671
cN0/pN0	53 (85.5)	14 (22.6)	39 (62.9)	<0.0001	
pN1	9 (14.5)	3 (4.8)	6 (9.7)	0.0238	
Lymphovascular invasion, *n* (%)					0.2290
Present	19 (30.6)	8 (14.5)	11 (20.0)	0.0003	
Absent	36 (58.1)	9 (16.4)	27 (49.1)	<0.0001	
Data n/a	7 (11.3)				
Tumor grade[b], *n* (%)					1.0000
Low	2 (3.2)	0 (0.0)	2 (3.4)	nc[a]	
High	57 (91.9)	17 (28.8)	40 (67.8)	<0.0001	
Data n/a	3 (4.9)				
Tumor grade[c], *n* (%)					0.5915
G2	28 (45.2)	9 (15.2)	19 (32.2)	<0.0001	
G3	31 (50.0)	8 (13.6)	23 (39.0)	<0.0001	
Data n/a	3 (4.8)				

CCNA2, Cyclin A2; *n*, number; IQR, interquartile range; nc, not calculable; n/a, not available. [a]Statistical evaluation not possible due to size distribution. [b]According to WHO 2004/2016. [c]According to WHO 1973.

expression levels. Concerning disease-free survival (DFS) including all relapses, DFS including all relevant relapses, as well as metastasis-free survival, no significant differences could be observed between patients with high and low *CCNA2* expression levels ($p = 0.9032$, $p = 0.6330$, and $p = 0.2104$, respectively). Kaplan-Meier curves are displayed in Figure 3. Furthermore, Kaplan-Meier curves for *MKI67* and *p53* were performed. There was no correlation between the gene expression of *MKI67* and *p53* regarding the OS (*MKI67*: $p = 0.1486$ and *p53*: $p = 0.1456$) and DFS (*MKI67*: $p = 0.0998$ and *p53*: $p = 0.1439$, online suppl. Fig. 1). *CCNA2* showed a positive correlation with *MKI67* (Rho = 0.4376, $p = 0.0005$) but not with *p53* (online suppl. Fig. 2).

In addition, we performed univariable and multivariable Cox regression analyses to explore the associations of *CCNA2* expression levels, gender, age, pathological T stage, LVI, and grading with OS and DFS. On univariable analysis, only high *CCNA2* expression (HR 0.41; 95% CI: 0.21–0.81; $p = 0.0137$) and G2 grading (HR 0.34; 95% CI: 0.16–0.71; $p = 0.0027$) could be identified as predictors for a longer OS. This could be confirmed on multivariable analysis: high *CCNA2* expression levels (HR 0.37; 95% CI: 0.16–0.85; $p = 0.0189$) and grading G2 could be identified as independent predictors for a longer OS (HR 0.39; 95% CI: 0.17–0.87; $p = 0.0168$).

LVI was found to be an influencing factor for DFS in the univariable analysis (HR 0.34; 95% CI: 0.15–0.78; $p = 0.0137$) and multivariable analysis (HR 0.37; 95% CI:

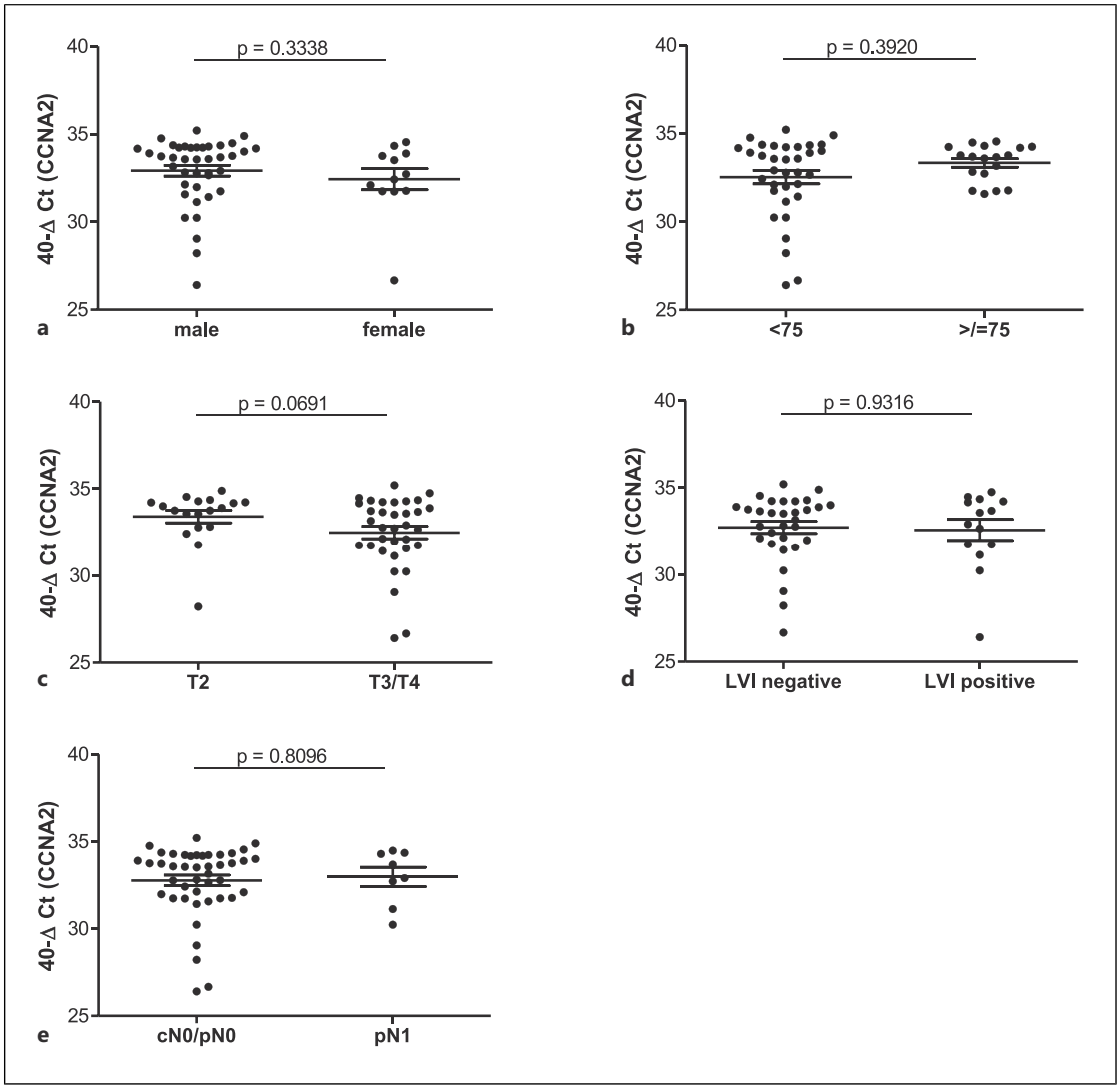

Fig. 2. Gene expression analysis of *Cyclin A2* (*CCNA2*) in tumor samples of patients with muscle-invasive UTUC grouped by gender (**a**), age (**b**), pathological T stage (**c**), lymphovascular invasion (LVI) (**d**), and N stage (**e**) using the 40-ΔCt-method. Higher values correspond to a higher expression and each dot reflects a single patient.

0.14–0.94; $p = 0.0376$) with the presence of LVI reducing DFS significantly. All other variables could not be identified as predictive factors for DFS. The results are displayed in Table 2.

Discussion

UTUC is a tumor entity associated with poor prognosis. Among others, histological grade and pathological stage are commonly used as prognostic factors for risk stratification and treatment decision. However, they may be insufficient to accurately predict the clinical course of UTUC. Thus, the identification of robust molecular markers in patients with UTUC is essential for solidly estimating progression. In this study, the expression levels of the cell cycle regulator *CCNA2* and the association with OS and DFS in muscle-invasive UTUC were examined. Several studies in diverse malignancies revealed that a deregulation of *CCNA2* is involved in human carcinogenesis [24, 25]. However, the link between altered *CCNA2* expression and disease prognosis in muscle-invasive UTUC has not been investigated so far.

Our results revealed an overexpression of *CCNA2* to be an independent and predictive factor for longer OS in this

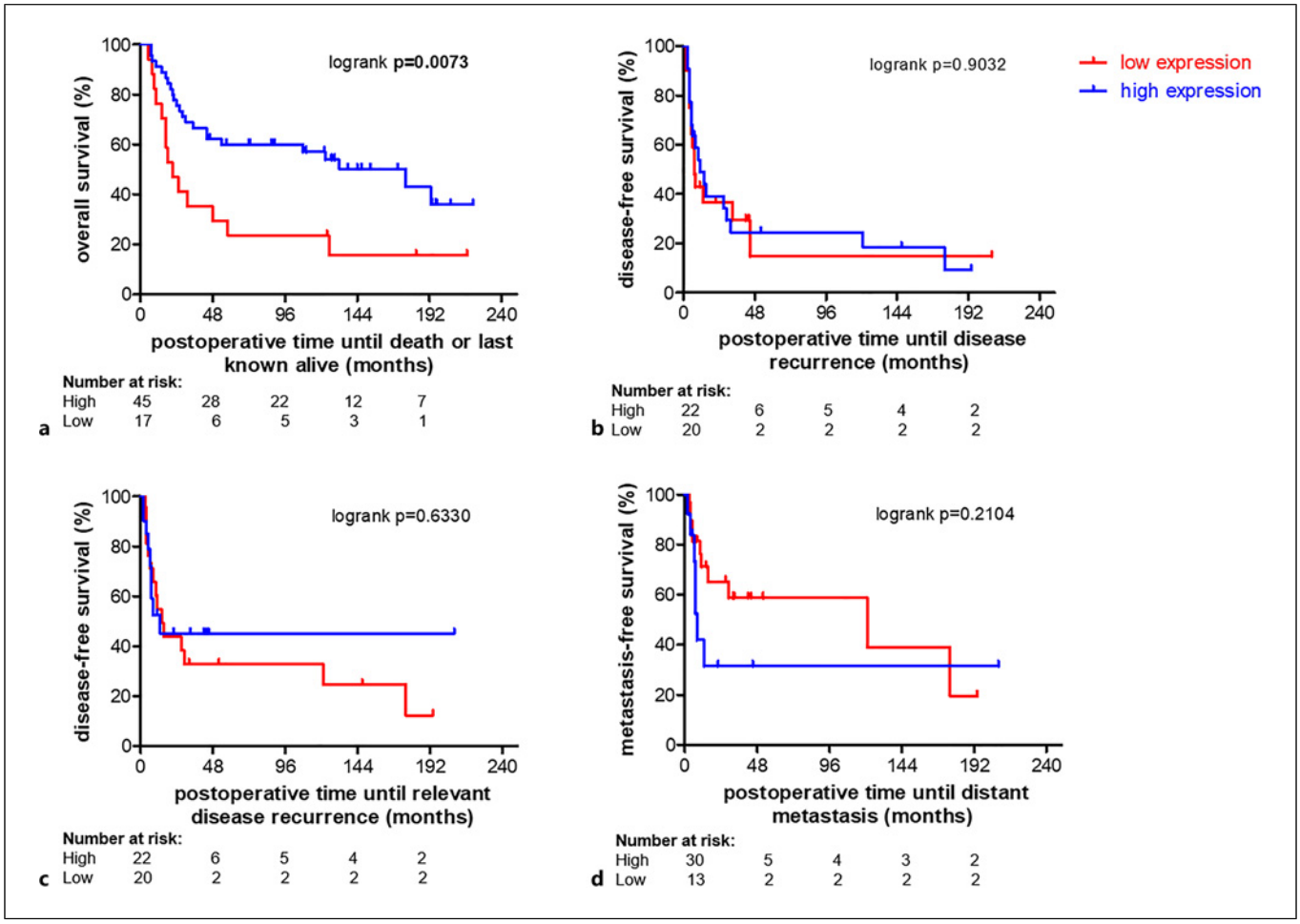

Fig. 3. Kaplan-Meier plots displaying overall survival (**a**), disease-free survival with local relapses and metastasis (**b**), disease-free survival with relevant relapses (**c**), and metastasis-free survival (**d**) with regard to *CCNA2* expression.

cohort, whereas other factors, such as gender, age, pathological T stage, or LVI, did not show a relevant influence on OS in the multivariable analysis. Histological grading G2 (vs. G3) was another predictor for longer OS in the multivariable analysis. In regards to DFS, we could not observe an influence of altered *CCNA2* expression levels, but the presence of LVI showed to be an independent predictor for a reduced DFS.

CCNA2 is a molecular marker that is present during multiple phases of the cell cycle and a key component of the S-phase and the G2/M-phase [26–28]. Evidence on CCNA2 as a predictive and prognostic marker is heterogeneous. On one side, earlier studies in other tumor entities examining *CCNA2* expression levels mostly indicated that high *CCNA2* expression levels were associated with a poorer prognosis compared to lower *CCNA2* expression levels. This could also be observed in other proteins of the Cyclin family, such as Cyclin E1 [29]. On the other side, there are also studies reporting *CCNA2* deficiency to be a risk factor for a poor clinical outcome [14, 19]. Kanakkanthara et al. [30] showed that *CCNA2* is critically involved in an efficient DNA repair, which is important to maintain genomic stability in cells. The results of their study in mice suggested that a decreased *CCNA2* expression may promote malignant transformation to lung and skin cancer. Similar findings were reported by Su et al. [19] in drosophila cells. Contrarily, Dong et al. [31] observed that *CCNA2* overexpression in patients with pancreatic ductal adenocarcinoma (PDAC) was associated with advanced tumor stage and showed prognostic impact for PDAC outcome. Patients with increased *CCNA2* levels were more likely to experience disease recurrence and progression and had a worse OS and DFS. It was suggested

Table 2. Uni- and multivariable analyses of factors regarding favorable OS and DFS

	Univariable analysis			Multivariable analysis		
	HR	95% CI	*p* value	HR	95% CI	*p* value
Overall survival						
Gender (male vs. female)	0.64	0.30–1.36	0.2658	–	–	–
Age (<75 vs. ≥ 75)	0.57	0.30–1.09	0.0949	0.59	0.27–1.28	0.1830
Pathological T stage (T2 vs. T3/T4)	0.68	0.33–1.41	0.2860	–	–	–
LVI (absent vs. present)	0.58	0.29–1.17	0.1347	0.72	0.32–1.60	0.4206
N stage (cN0/pN0 vs. pN1)	0.64	0.28–1.45	0.3066	–	–	–
Grading (G2 vs. G3)	0.34	0.16–0.71	0.0027	0.39	0.17–0.87	**0.0168**
CCNA2 expression (high vs. low)	0.41	0.21–0.81	0.0137	0.37	0.16–0.85	**0.0189**
MKI67 expression (high vs. low)	0.62	0.32–1.20	0.1521	0.73	0.35–1.55	0.4125
p53 expression (low vs. high)	0.62	0.32–1.19	0.1560	0.88	0.38–2.05	0.7728
Disease-free survival						
Gender (male vs. female)	0.82	0.33–2.01	0.6712	–	–	–
Age (<75 vs. ≥ 75)	0.83	0.41–1.68	0.6135	–	–	–
Pathological T stage (T2 vs. T3/T4)	0.48	0.22–1.06	0.0568	0.49	0.19–1.27	0.1222
N stage (cN0/pN0 vs. pN1)	0.51	0.19–1.37	0.2120	–	–	–
LVI (absent vs. present)	0.34	0.15–0.78	0.0137	0.37	0.14–0.94	**0.0376**
Grading (G2 vs. G3)	0.56	0.25–1.27	0.1540	1.00	0.39–2.53	0.9942
CCNA2 expression (high vs. low)	0.96	0.47–1.94	0.9057	–	–	–
MKI67 expression (high vs. low)	0.55	0.26–1.16	0.1242	0.71	0.32–1.60	0.4164
p53 expression (low vs. high)	0.60	0.30–1.22	0.1627	0.53	0.24–1.19	0.1276

OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; LVI, lymphovascular invasion; *CCNA2*, *Cyclin A2*.

that *CCNA2* should be considered a novel biomarker in PDAC based on its negative influence on cell cycle genes and thus its worse outcome when overexpressed.

Nonetheless, how *CCNA2* deregulation influences malignant growth is unknown. It is not clear whether a *CCNA2* overexpression is the reason for tumorigenesis, poor prognosis, and early relapse or rather the simple consequence of increased cell proliferation. *CCNA2* is typically coexpressed with other proliferation markers, such as *MKI67* or proliferative cell nuclear antigen. Thus, increased *CCNA2* levels are not unexpected in highly proliferative tissue, such as tumor tissue. Hence, one could assume that high *CCNA2* expression may simply reflect cell proliferation after development of a tumor. In this study, the positive correlation between *CCNA2* and *MKI67* could be confirmed. Another interesting aspect and potential explanation for the association between clinical outcome and *CCNA2* overexpression could be the fact that *CCNA2* is a protein phosphorylating other oncoproteins and tumor suppressors and hereby can influence their activity. For instance, this could potentially result in an enhancement of the activity of the tumor suppressor *p53* and consequently influence the regulation of expression of genes involved in cell cycle control, DNA repair, and induction of apoptosis [20, 21]. Consistent with the explanation of an activation of the tumor suppressor *p53* leading to a better outcome when *CCNA2* overexpression is detected, there is evidence that cells lacking *CCNA2* are associated with an increased number of lagging chromosomes in drosophila cells [19]. Thus, decreased *CCNA2* expression levels may have a negative influence on its function as a DNA damage checkpoint and thus be associated with deficient DNA repair and tumorigenesis. In our cohort, no correlation between *p53* and *CCNA2* could be observed, for which the cohort size may be the reason. Overall, both high and low expression levels could be observed being associated with positive and negative outcomes in diverse malignancies. However, there is no consistent definition of high, low, and normal gene expression levels in those studies. A discrepancy between studies regarding the expression levels of cell cycle regulators and their prognostic value was also reported in the cell cycle control protein *p16* in patients with UCB. It could be shown that both a low and a high expression level of *p16* could be predictors for a worse outcome after radical cystectomy [32, 33]. These results mirror the complex interactions of cell cycle regulator proteins in the tumor cell cycle machinery and the

heterogeneous biology among diverse tumor entities and molecular subtypes within one entity.

Several limitations of this study should be considered. First, the retrospective study design might have introduced statistical bias and thereby limited the strength of the study. Another limitation is the rather small sample size due to the rarity of the disease. At the same time, taking into account that this is a monocentric study, the number of patients with muscle-invasive UTUC we could include is notable. The fact that comorbidities were not recorded could be another limitation. At the same time, this could strengthen our results and make them more robust as *CCNA2* expression showed a significant association with OS in this real-life cohort, without stratification for comorbidities. This could make it an even more pragmatic biomarker.

Conclusion

Although various molecular markers mirroring cell proliferation in UTUC have already been examined, it is not known which of these markers are clinically most useful. To our knowledge, we report the first evaluation of *CCNA2* expression levels and the association with OS and DFS in patients with muscle-invasive UTUC who underwent surgical treatment for UTUC. We could show that high *CCNA2* expression may provide important prognostic information concerning OS in terms of being a predictive factor for a longer OS. In addition to other prognostic factors, *CCNA2* might be used to characterize a subset of patients having an unfavorable outcome. Thus, *CCNA2* might serve as a potential biomarker in muscle-invasive UTUC, and its clinical value should be confirmed in larger and prospective patient cohorts.

Statement of Ethics

The protocol for this research project has been approved by a suitably constituted Ethics Committee of the institution, and it conforms to the provisions of the Declaration of Helsinki. University of Heidelberg's Ethics Committee II, Medical Faculty Mannheim, reference number 2015-549N-MA. All written informed consent was obtained from the participants to participate in the study and for publication of the details of their medical case.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author Contributions

M.T. Walach: protocol/project development, data analysis, writing – original draft, and writing – review and editing; K. Nitschke: protocol/project development, data analysis, methodology, visualization, writing – original draft, and writing – review and editing; M. Groß-Weege, J. Jarczyk, F. Wessels, M. Neuberger, K.-F. Kowalewski, M.C. Kriegmair, Z. Popovic, and T. Gaiser: performance of work and writing – review and editing; J. Großhans, L. Wildner, and L. Pause: performance of work, data curation, writing – review and editing; T.S. Worst: protocol/project development, scientific input, and writing – review and editing; and P. Nuhn: protocol/project development, supervision, scientific input, and writing – review and editing.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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