UCA1 overexpression is associated with less aggressive subtypes of bladder cancer

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Abstract. Non-coding RNAs (ncRNAs) have been shown to serve important roles in carcinogenesis via complex mechanisms, including transcriptional and post-transcriptional regulation, and chromatin interactions. Urothelial carcinoma-associated 1 (*UCA1*), a long ncRNA, was recently shown to have tumorigenic properties in urothelial bladder cancer (UBC), as demonstrated by enhanced proliferation, migration, invasion and therapy resistance of UBC cell lines *in vitro*. These *in vitro* findings suggested that *UCA1* is associated with aggressive tumor behavior and could have prognostic implications in UBC. The aims of the present study were to therefore to investigate the statistical associations between *UCA1* RNA expression and UBC pathological features, patient prognosis and p53 and Ki-67 expression. Chromogenic *in situ* hybridization and immunohistochemistry were performed on UBC tissue microarrays to characterize *UCA1* RNA, and p53 and Ki-67 expression in 208 UBC cases, including 145 non-muscle-invasive and 63 muscle-invasive cases. *UCA1* was observed in the tumor cells of 166/208 (80%) UBC cases tested. No expression was noted in normal stromal and endothelium cells. Patients with UBC that overexpressed *UCA1* (35%) had a significantly higher survival rate (P=0.006) compared with that in patients with UBC that did not overexpress *UCA1*. This prognostic factor was independent of tumor morphology, concomitant carcinoma *in situ*, tumor grade and tumor stage. In addition, the absence of *UCA1* overexpression was significantly associated with a high Ki-67 proliferative index (P=0.008) and a p53 'mutated' immunoprofile (strong nuclear expression or complete absence of staining; P=0.003). In conclusion, the present results identified *UCA1* as potentially being a novel independent prognostic marker in UBC that was associated with a better patient prognosis and that could serve a pivotal role in bladder cancer carcinogenesis.

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Introduction

Urothelial bladder cancer (UBC) is recorded as the seventh most commonly occurring malignancy in men and the seventeenth most common in women (1). The prevention of risk factors, including smoking and occupational chemicals, has resulted in a decrease of the UBC burden in Western communities; yet, no clinically relevant differences in UBC mortality have been observed over the past 30 years (2).

Standard treatment modalities for UBC have not changed a lot in recent years and encompass intravesical chemotherapy for non-muscle-invasive bladder cancer (NMIBC) and cystectomy with cisplatin-based systemic chemotherapy for MIBC; however, recently, immunotherapy and more precisely, atezolizumab or pembrolizumab, has been approved by the Food and Drug Administration for patients with metastatic disease who progressed during or following cisplatin-based chemotherapy (1,2). Recent insights into the biology of UBC by whole genome, RNA and microRNA sequencing have identified molecular subtypes in MIBC with different putative therapeutic targets and variable sensitivity to currently available chemotherapies (3,4). These studies are part of an ongoing effort to further characterize UBC, using protein and gene expression profiles to identify patients who are at risk for progression or

recurrence and may benefit from a more aggressive therapeutic approach, as well as to avoid over-treatment of tumors with a relatively indolent clinical course (3). Numerous genes have been implicated in the development and progression of UBC, and their altered expression has been attributed to gene mutations and epigenetic changes. In the latter category, non-coding RNAs (ncRNAs) have been demonstrated to serve important roles in carcinogenesis and cancer metastasis via complex mechanisms, including transcriptional and post-transcriptional regulation, and chromatin interactions (5). The ncRNAs are divided into small regulatory RNAs and long ncRNAs (lncRNAs), composed of >200 nucleotides (6). Urothelial carcinoma-associated 1 (*UCA1)* is an lncRNA that was first isolated by Wang *et al* (7) in 2006 and was initially proposed as a urinary biomarker for the detection of UBC, although reports about the efficiency and clinical application of *UCA1* as a diagnostic test have been controversial (8,9). Concurrently, *UCA1* was shown to have tumorigenic properties, as shown *in vitro* by enhanced proliferation, invasion, migration and therapy resistance of UBC cell lines (10,11). A subsequent study identified *UCA1* as a crucial element in cell cycle regulation by positive indirect action on the phosphoinositide 3-kinase (PI3K)-protein kinase B (AKT)-mechanistic target of rapamycin pathway through the p300 coactivator cAMP response element-binding protein (CREB) (12). The majority of these results are provided by *in vitro* cellular assays, and together they suggest that *UCA1* is associated with more aggressive tumor behavior and could therefore have prognostic implications in UBC. Indeed, several studies, mainly conducted in Chinese patients with solid tumors (esophageal, gastric, colorectal, prostate, breast, endometrial, ovarian or non-small cell lung carcinoma) reported that a high level of *UCA1* expression was associated with positive lymph node metastasis, higher clinical stage and poor survival (13,14). To date few and contradictory data are currently available with regard to the expression of *UCA1* [measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)] in series of human UBC and its association with UBC morphology and aggressiveness (7,15). In the present study, chromogenic *in situ* hybridization (CISH) was applied to analyze the expression of *UCA1* in human UBC samples, thus integrating expression levels and *in vivo* morphological context. Additionally, the study aimed to determine the value of *UCA1* as a prognostic marker in patients with UBC and its association with other biological markers associated with UBC aggressiveness, including p53, encoded by key tumor suppressor gene *TP53*, and the proliferation marker Ki-67 (16,17).

Materials and methods

Ethics statement. The present study was approved by the Ethical Committee of the Erasme University Hospital (Brussels, Belgium; ref., P2015/041). According to Belgian law, no written informed consent was required for archival material in the context of retrospective studies. The ethical committee thus waived the requirement for written informed consent from the participant.

Tissue samples. A total of 11 human UBC tissue microarrays (TMA) and one normal urothelium TMA were manufactured (MiniCore tissue arrayer; Mitogen Ltd., Harpenden, UK) using available archival formalin-fixed and paraffin-embedded (FFPE, ISO15189 standard) samples from cases of 271 UBC and 40 samples of normal urothelium collected between January 1997 and December 2007 in the Erasme Hospital Biobank (Brussels, Belgium; BE_BERA1; Biobanque Hôpital Erasme-ULB (BERA); BE_NBWB1; Biothèque Wallonie Bruxelles (BWB); BBMRI-ERIC). Cases for which archival material was insufficient or not available were excluded from the analysis. The time period was selected in order to obtain a follow-up of at least 5 years for all patients. All the tumors are from patients who were not previously treated for UBC (primary tumor resection), and whose histopathological diagnoses were reviewed and characterized by an uropathologist. Tumor grades and stages were adjusted to comply with the new 2016 WHO classification (18) and the new 2017 TNM Classification of Malignant Tumours (Union for International Cancer Control) (19) (Table I). For each case, six tissues cores of 600 μm in diameter targeting the tumor area (without distinction between the areas of bladder wall) and four tissues cores of 600 μm in diameter targeting the normal urothelium were included in the TMAs. Subsequent to validating each TMA for RNA and tissue fixation quality, 208 out of the 271 preselected UBC cases and 20 samples of normal urothelium were included. The available clinical and pathological features of the patients and their tumors are described in Table I. Patient outcomes were characterized in terms of disease-free survival (DFS) and cancer-specific survival (CSS), i.e., periods from the date of the first tumor resection (the date of the diagnosis) until the date of recurrence or mortality (DFS) or the date of mortality due to tumor progression (CSS).

UCA1 CISH. Detection of *UCA1* RNA was performed by CISH using the RNAscope® Singleplex Target Probe and the RNAscope 2.0 HD Detection kit (Advanced Cell Diagnostics, Newark, CA, USA). Sections (5-μm thick) of all TMAs were baked at 60˚C for 1 h and deparaffinized. Slides were rehydrated and three pre-treat solutions were successively applied according to the manufacturer's recommendations. Following retrieval, *UCA1* anti-sense probe (RNAscope Singlepex Target Probe), positive (RNAscope Positive Control Probe-PPIB; cat. no. 313901) or negative control probe (RNAscope Negative Control Probe-DapB; cat. no. 310043) were dispensed onto slides at 40˚C for 2 h. Following hybridization, slides were incubated with six oligonucleotide probes for signal amplification. The first four amplifiers were hybridized at 40˚C and the last two were incubated at room temperature. Following each hybridization step, slides were washed with a washing buffer two times at room temperature. For chromogenic detection, equal volumes of BROWN-A and BROWN-B DAB substrates from the CISH kit were dropped onto each slide for 10 min at room temperature. Tissue nuclei were then stained using Gill's hematoxylin (Vector Laboratories, Ltd., Peterborough, UK; cat. no. H-3401) for 2 min at room temperature. Only cases for which the positive (PPIB) and the negative (bacterial *DapB* gene, which encodes for the dihydrodipicolinate reductase protein) probes were validated on tissue cores (to ensure the quality of the RNA in the tissue and the absence of false-positive results, respectively) were included in the study. *UCA1* CISH staining was visualized by using Spot Browser V2e

Table I. Patient demographics and baseline features (n=208).

Clinical features	NMIBC (n=145)	MIBC (n=63)
Median age (range), years	69.4 (35.4-97)	71.1 (33.9-91.3)
Sex, n (%)		
Male	118 (81.4)	54 (85.7)
Female	27 (18.6)	9 (14.3)
UBC morphology, n (%)		
Papillary lesions	140 (96.6)	46 (73.0)
Flat lesions	5 (3.4)	17 (27.0)
UBC variant histology, n (%)		
Present	11 (7.6)	27 (42.9)
Squamous	3 (2.1)	13 (20.6)
Glandular	2 (1.4)	7 (11.1)
Micropapillary	6 (4.1)	8 (12.7)
Sarcomatoid	1 (0.7)	6 (9.5)
Absent	134 (92.4)	36 (57.1)
Multifocality, n (%)		
Present	40 (27.6)	20 (31.7)
Absent	95 (65.5)	38 (60.3)
Unknown	10 (6.9)	5 (8.0)
Concomitant CIS, n (%)		
Present	19 (13.1)	30 (47.6)
Absent	125 (86.2)	26 (41.3)
Unknown[a]	1 (0.7)	7 (11.1)
Lymphovascular invasion, n (%)		
Present	5 (3.4)	36 (57.1)
Absent	109 (75.2)	18 (28.6)
Unknown[a]	31 (21.4)	9 (14.3)
2016 WHO grading, n (%)		
PUNLMP	31 (21.4)	0 (0.0)
Low grade	64 (44.1)	1 (1.6)
High grade	50 (34.5)	62 (98.4)
Recurrence, n (%)		
Yes	55 (62.1)	31 (49.2)
No	55 (37.9)	32 (50.8)
Mortalities, n (%)		
Yes	4 (2.7)	12 (19)
No	141 (97.3)	51 (81)
Median follow-up (range)		
Months	83.6 (0.1-212)	14.1 (0.0-189.2)
Years	7 (0.0-17.7)	1.2 (0.0-15.8)

Numbers (or percentages) of UBC patients included in the study are displayed and describes their clinical and pathological features; cases are categorized as NMIBC (n=145) and as MIBC (n=63). [a]Patients for whom concomitant CIS and lymphovascular invasion cannot be assessed due to coagulation artifacts or small sample size. UBC, urothelial bladder cancer; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; CIS, carcinoma *in situ*; WHO, World Health Organization; PUNLMP, papillary urothelial neoplasm of low malignant potential.

(Alphelys, Plaisir, France) and scored by two independent observers using a three-tiered scoring system as follows: 0, no staining; 1, a few dots observed in a few tumor cells; and 2, >10 dots homogeneously observed in tumor cells; Fig. 1). For each UBC case, the mean score of the 6 tissue cores was calculated and the case was categorized as '*UCA1*-negative or with low expression' and as '*UCA1*-positive or overexpressed' if the mean score was ≤1 and >1, respectively.

Immunohistochemistry (IHC). Sections (5-μm thick) of all TMAs were subjected to standard IHC on a Ventana Discovery XT (Ventana Medical Systems, Inc., Tucson, AZ, USA) using the DABMap detection system according to the manufacturer's protocols. Briefly, the slides were incubated with the rabbit monoclonal anti-Ki-67 antibody for 24 min at 37˚C (RTU antibody: ~2 μg/ml, clone 30-9; cat. no. 790-4286; Ventana Medical Systems, Inc.) or with the mouse monoclonal anti-p53 antibody for 28 min at 37˚C (RTU antibody: ~2.5 μg/ml, clone Bp53-11; cat. no. 760-2542; Ventana Medical Systems, Inc.). The slides were washed and incubated with the biotinylated secondary antibody (1:200 dilution; cat. no. BA-2001 for anti-mouse antibody; cat. no. BA-1000 for anti-rabbit antibody; both Vector Laboratories, Ltd.) for 24 min at 37˚C and 20 min at 37˚C, respectively, followed by the addition of complex avidin-horseradish peroxidase. Immunostainings were detected by incubation with diaminobenzidine and hydrogen peroxide. All IHC slides were counterstained with Gill's hematoxylin for 2 min at room temperature, dehydrated and mounted. For each staining, an external positive control was included as well as a negative control, which entailed replacing the primary antibody with non-immune serum (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). In addition, anti-vimentin immunostaining (RTU antibody: ~2.5 μg/ml, clone V9; cat. no. 790-2917; Ventana Medical Systems, Inc.) for 24 min at 37˚C was performed on each TMA for the quality control of tissue fixation. Two independent observers performed the semi-quantitative assessment of p53 IHC expression by using a three-tier score as follows: 0, no staining; 1, ≤25% of cells with weak, heterogeneous cytoplasmic and nuclear staining; and 2, >25% of cells with high homogeneous nuclear staining (Fig. 1). For discordant cases, a third pathologist blinded to previous results reclassified the p53 IHC staining. Ki-67 slides were digitalized using a NanoZoomer 2.0 HT (Hamamatsu Photonics K.K., Hamamatsu, Japan) and Ki-67 expression was quantitatively evaluated using Visiomorph DP 5.1 (Visiopharm, Hoersholm, Denmark) in the tumor areas manually selected on the digital slides by a pathologist. The Ki-67 labeling index (Ki-67_LI), corresponding to the ratio between the surface area occupied by the positive nuclei and the total nucleus area, was globally computed for each case (across the 6 cores), as previously detailed (20,21). Based on published data (16), a threshold of 25% in terms of Ki-67_LI was used for statistical analyses.

Statistical analysis. All statistical analyses were performed using Statistica 12 (StatSoft, Inc., Tulsa, OK, USA). Comparisons between two independent groups of numerical data were performed using the non-parametric Mann-Whitney test. The association between two qualitative variables was assessed using Fisher's exact test or the χ^2 test, depending on whether the two variables were binary or not. Univariate survival analyses were performed using a standard Kaplan-Meier analysis and a log-rank test, with the exception of cases of continuous variables, for which univariate Cox regression was used. The analyses were completed using multivariate Cox regression; when analyzing the set of clinical variables, those with univariate results of P<0.05 were selected. The potential contributions of the biological variables were then tested to the final 'clinical' model by adjusting for those for which the univariate results indicated a P-value of <0.1. For each statistical analysis, the cases with missing value(s) in the concerned variable(s) were omitted.

Results

As illustrated in Fig. 1A-C, the CISH methodology detected UCA1 dot expression in the cytoplasm and in the nucleus of urothelial normal and tumor cells. All the normal urothelium cases showed UCA1 dot positivity (data not shown). No UCA1 dot staining was observed in endothelial, stromal or inflammatory cells (Fig. 1A-C). UCA1 dot positivity was detected in 166 out of the 208 UBC included in the present study (80%). According to the semi-quantitative scoring, UCA1 overexpression (with a positivity mean score of >1) was observed in 72/208 UBC (35%), including 55 (76%) NMIBC [i.e. 32/84 (38%) pTa and 23/61 (38%) pT1] and 17 (24%) MIBC, respectively (Table II). UCA1 overexpression was not statistically associated with tumor morphology (papillary vs. flat lesions), presence of a secondary histological variant (including squamous, sarcomatoid, glandular or micropapillary mixed UBC tumors), tumor multifocality or the presence of concomitant carcinoma *in situ* (CIS) (Table II). However, UBC overexpressing *UCA1* was statistically associated with less lymphovascular invasion compared with UBC that failed to overexpress *UCA1* (P=0.02). No statistical association was found between *UCA1* overexpression and UBC tumor grade (papillary urothelial neoplasm of low malignant potential, low-grade and high-grade tumors) or stage ('pTa, pT1, pT2, pT3, pT4' or 'NMIBC vs. MIBC') (Table II). Regarding p53 expression in UBC, it was noted that the complete absence of p53 expression (i.e., a score of 0; Fig. 1D) and strong and diffuse nuclear p53 staining in >25% of UBC cells (i.e., a score of 2; Fig. 1F) followed similar profiles in terms of UBC aggressiveness as opposed to the weak and heterogeneous p53 expression pattern (i.e., a score of 1) (data not shown); these results were similar to those of previous studies regarding p53 expression in serous ovarian and endometrial carcinoma (22-24). The data obtained from UBC associated with a p53 'score 0' and a p53 'score 2' were grouped (referred to as the 'p53-mutated immunoprofile'), as opposed to the p53 'score 1' (referred to as the 'p53-wild-type immunoprofile'), for use in future statistical analyses. These analyses showed that, compared with the 'p53-wild-type' immunoprofile, the 'p53-mutated' immunoprofile was observed significantly more in association with flat UBC morphology (P=0.01), presence of a secondary histological variant (P=0.00001), concomitant CIS (P=0.0006), lymphovascular invasion (P=0.0002), UBC tumor high-grade (P=0.00001) and UBC tumor high-stage (P=0.00003) (Table II). Similarly, a high Ki-67 proliferative index (>25%) was observed significantly more in association with more aggressive UBC, i.e., UBC with flat urothelial morphology (P=0.04), presence of a secondary histological variant (P=0.003), concomitant CIS (P=0.00003), lymphovascular invasion (P<0.00001), tumor grade (P<0.00001) and tumor stage (P<0.00001; Table II).

To evaluate the prognostic contribution of *UCA1* overexpression in patients with UBC, first, the impact of the clinical factors (listed in Table I) on DFS and CSS (Table III) was analyzed. Univariate survival rate analyses revealed that

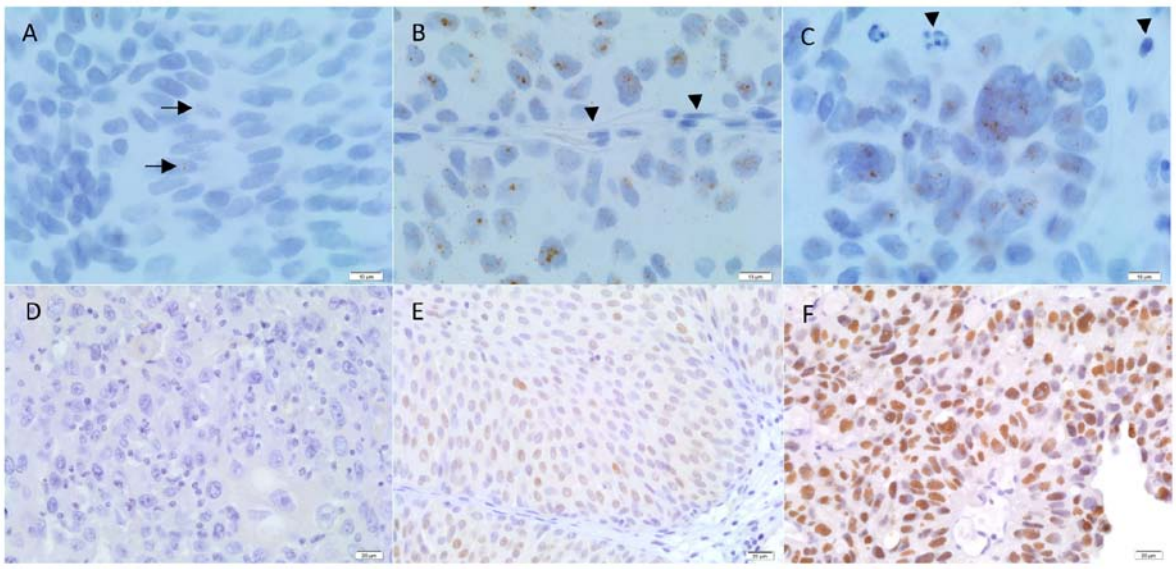

Figure 1. *UCA1* RNA chromogenic *in situ* hybridization and p53 IHC staining in UBC samples. (A-C) *UCA1* expression is located in the cytoplasm and the nucleus of urothelial tumor cells: (A) Few dots were observed in few tumor cells (score 1; arrows) and >10 dots were homogeneously observed in tumor cells (score 2) of (B) low-grade UBC and (C) high-grade UBC cases. (B and C) No *UCA1* dot staining was observed in endothelial, stromal or inflammatory cells (arrows). For each UBC case, the mean score of the 6 tissue cores was calculated and the case was categorized as '*UCA1*-negative or with low expression' and as '*UCA1*-positive or overexpressed' if the mean score was <1 and ≥1, respectively. (D-F) Assessment of p53 IHC expression using a three-tier score: (D) 0 (no staining), (E) 1 (≤25% of cells with weak, heterogeneous cytoplasmic and nuclear staining) and (F) 2 (>25% of cells with high homogeneous nuclear staining). *UCA1*, urothelial carcinoma-associated 1; UBC, urothelial bladder cancer; IHC, immunohistochemistry.

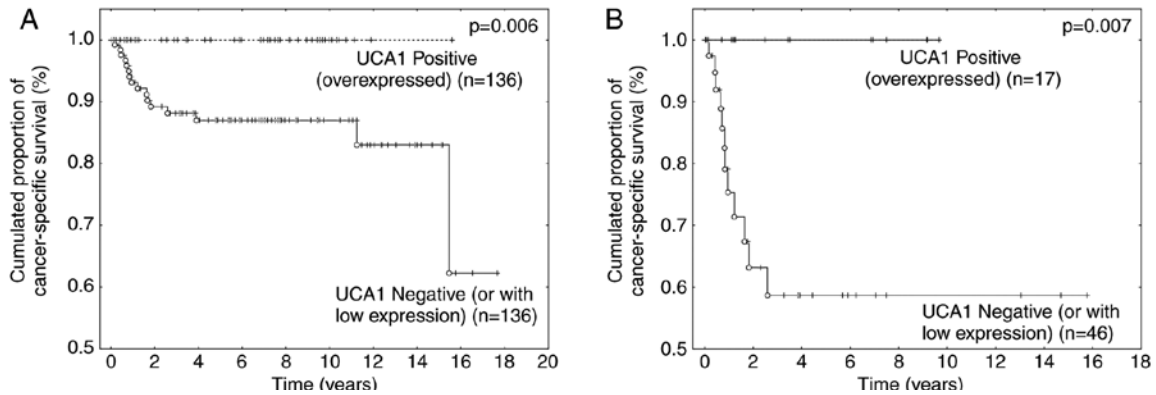

Figure 2. Prognostic value of *UCA1* expression in UBC. Kaplan-Meier plots indicating cancer-specific survival of patients stratified by *UCA1* expression categorized as '*UCA1*-negative or with low expression' and as '*UCA1*-positive or overexpressed' in (A) UBC tumor samples (all cases) and (B) only in muscle-invasive bladder cancer. *UCA1*, urothelial carcinoma-associated 1; UBC, urothelial bladder cancer.

patient age (P=0.0003), multifocality (P=0.03), concomitant CIS (P=0.00005), lymphovascular tumor cell invasion (P<0.00001), UBC tumor high-grade (P=0.0003) and UBC tumor high-stage (P=0.00002) were all significantly associated with reduced DFS rate. With the exception of patient age and multifocality, the same clinical factors were all significantly associated with reduced survival rate (P<0.01 and P<0.001 respectively, Table III). Regarding *UCA1* expression, no significant impact was observed on DFS (Table III). Conversely, *UCA1* overexpression was significantly associated with increased survival rate in UBC patients (P=0.006), and this result was independent of tumor stage (Fig. 2A and B). Indeed, patients with MIBC that overexpressed *UCA1* were associated with a 5-year survival rate of 100%, while the 5-year survival rate was only 58% for patients whose MIBC failed to overexpress the biomarker (P=0.007; Fig. 2B). All patients with UBC that overexpressed *UCA1* in the present series remained alive at the end of this study (Fig. 2A and B). Considering only the *UCA1* group with 'negative or with low expression', multivariate survival analyses combining tumor stage, concomitant CIS and lymphovascular invasion highlighted a high tumor stage (≥pT2) as being the sole independent prognostic factor associated with worse survival rate for UBC patients (P=0.003; data not shown).

Finally, the study investigated whether the expression of *UCA1*, p53 and Ki-67 was interrelated. As noted in Table IV, *UCA1* overexpression was more frequently observed in association with weak and heterogeneous expression of p53 (i.e., a 'p53-wildtype immunoprofile'; P=0.003) and with a low Ki-67 proliferative index (<25%; P=0.008), compared with the absence of *UCA1* overexpression.

Table II. Biomarker expression and associations with pathological features of UBC.

	UCA1 RNA (CISH) (n=208)			p53 nuclear staining (IHC) (n=199)			Ki-67 nuclear staining (%) (n=205)		
Feature	Negative or low expression (n=136), n (%)	Positive (overexpression) (n=72), n (%)	P-value	Weak and heterogeneous (n=125), n (%)	Negative or high (n=74), n (%)	P-value	<25% (n=149) n (%)	≥25% (n=56) n (%)	P-value
UBC morphology									
Papillary lesions	121 (89)	65 (90)	ns	118 (94)	62 (84)	0.01	137 (92)	46 (82)	0.04
Flat lesions	15 (11)	7 (10)		7 (6)	12 (16)		12 (8)	10 (18)	
UBC variant histology[a]									
Absent	107 (79)	63 (87.5)	ns	114 (91)	48 (65)	0.00001	129 (87)	38 (68)	0.003
Present	29 (21)	9 (12.5)		11 (9)	26 (35)		20 (13)	18 (32)	
Multifocality									
Absent	92/129 (71)	41/64 (64)	ns	84/114 (74)	45/71 (63)	ns	97/139 (70)	34/51 (67)	ns
Present	37/129 (29)	23/64 (36)		30/114 (26)	26/71 (37)		42/139 (30)	17/51 (33)	
Unknown	7	8		11	3		10	5	
Concomitant CIS									
Absent	95/130 (73)	56/70 (80)	ns	101/120 (84)	44/71 (62)	0.0006	122/147 (83)	26/50 (52)	0.00003
Present	35/130 (27)	14/70 (20)		19/120 (16)	27/71 (38)		25/147 (17)	24/50 (48)	
Unknown[b]	6	2		5	3		2	6	
Lymphovascular invasion									
Absent	83/117 (71)	44/51 (86)	0.02	83/97 (86)	38/64 (59)	0.0002	103/119 (87)	21/46 (46)	<0.00001
Present	34/117 (29)	7/51 (14)		14/97 (14)	26/64 (41)		16/119 (13)	25/46 (54)	
Unknown[b]	19	21		28	10		30	10	
2016 WHO grading									
PUNLMP	20 (15)	11 (15)		23 (18)	8 (11)		31 (21)	0	
Low grade	41 (30)	24 (33)	ns	52 (42)	12 (16)	0.00001	61 (41)	3 (5)	<0.00001
High grade	75 (55)	37 (51)		50 (40)	54 (73)		57 (38)	53 (95)	
TNM staging (UICC 2017; 8th ed.)									
NMIBC (<pT2)	90 (66)	55 (76)	ns	101 (81)	39 (53)	0.00003	121 (81)	21 (37)	<0.00001
MIBC (≥pT2)	46 (34)	17 (24)		24 (19)	35 (47)		28 (19)	35 (63)	

Statistical associations between *UCA1* RNA (CISH), p53 (IHC) and Ki-67 (IHC) expression and the pathological features of the UBC cases included are shown. Semi-quantitative scoring was used for *UCA1* and p53; i.e., 'Negative or with low expression' vs. 'positive or overexpressed' for *UCA1* and 0 (no staining), 1 (≤25% of cells with weak, heterogeneous cytoplasmic and nuclear staining) and 2 (>25% of cells with high homogeneous nuclear staining) for p53. Ki-67 expression was quantitatively evaluated using Visiomorph DP 5.1 (Visiopharm) in the tumor areas and expressed in terms of Ki-67 labeling index with a threshold of 25% for statistical analyses. [a]Including squamous differentiation, sarcomatoid, glandular and micropapillary variants. [b]Pathological features that could not be accurately specified on UBC samples. UBC, urothelial bladder cancer; NMIBC, non-muscle invasive bladder cancer; CIS, carcinoma *in situ*; WHO, World Health Organization; PUNLMP, papillary urothelial neoplasm of low malignant potential; CISH, chromogenic *in situ* hybridization; *UCA1*, urothelial carcinoma-associated 1; ns, not significant.

Table III. Univariate survival analyses for patients with UBC.

Feature	Disease-free survival, P-value	Cancer-specific survival, P-value
Age, years[a]	0.0003	ns
UBC morphology		
Papillary lesions		
Flat lesions	ns	ns
UBC variant histology[b,c]		
Absent		
Present	ns	ns
Multifocality[b]		
Absent		
Present	0.03	ns
Concomitant CIS[b]		
Absent		
Present	0.00005	<0.00001
Lymphovascular invasion[b]		
Absent		
Present	<0.00001	0.0006
2016 WHO grading[b]		
Low grade		
High grade	0.0003	0.002
TNM staging (UICC 2017; 8th ed.)[b]		
NMIBC (<pT2)		
MIBC (≥pT2)	0.00002	<0.00001
UCA1 overexpression[b]		
Negative or low expression		
Positive (overexpression)	ns	0.006

The table describes the univariate statistical survival analyses of well-known prognostic features for patients with UBC in our series. Each variable is considered as either a continuous variable in [a]univariate Cox regression or a [b]two-class factor analyzed by the log-rank test. [c]Including squamous differentiation, sarcomatoid, glandular and micropapillary variants. UBC, urothelial bladder cancer; NMIBC, non-muscle invasive bladder cancer; CIS, carcinoma *in situ*; WHO, World Health Organization; ns, not significant.

Discussion

Advances in the sequencing of the human genome led to the determination that protein-coding genes compose <3% of the human genome. Yet >80% of genes are actively transcribed to RNA without protein-coding potential, referred to as ncRNAs (5). Accumulating evidence has shown that lncRNAs are often altered in urological cancer types, notably in the prostate and kidneys, and in UBC (25,26). Among these lncRNAs, *UCA1* was originally reported to be involved in UBC carcinogenesis, promoting *in vitro* tumorigenicity and invasive behavior (10-12) Moreover, recent meta-analyses investigating the association between the expression levels of *UCA1* and prognosis (using RT-qPCR methodology approaches) noted that *UCA1* was implicated in the biology of other solid tumors, including gastric, colorectal, lung, breast and ovarian carcinoma (13,14). Data from those meta-analyses (mainly conducted in Chinese patients) concluded a global positive association between a high expression level of *UCA1* RNA and tumor stage, lymph node metastasis and poor survival (13,14). Taking into account the previous *in vitro* results regarding *UCA1* involvement in the carcinogenesis of UBC cell lines, it was notable and unexpected that these meta-analyses did not included studies on patients with UBC (13,14). In 2017, Droop *et al* (15) reported the RT-qPCR expression levels of several lncRNAs (including *UCA1*) in a series of 106 UBC cases in order to assess the correlation with clinicopathological parameters, including tumor grade, tumor stage and patient survival; the data of the publicly available bladder urothelial carcinoma dataset from The Cancer Genome Atlas (TCGA) was also analyzed (15). In the series, it was found that patients with high *UCA1* expression experienced considerably better overall survival rate compared with that of patients with low levels of *UCA1* expression, but this result was not confirmed in the TCGA dataset (15). Notably, the present study confirmed this result in a larger series of UBC cases and using another methodology (CISH instead of RT-qPCR). Indeed, it was shown that patients whose UBC overexpressed *UCA1* were associated with improved overall survival rate compared with that of patients whose UBC failed to overexpress this biomarker, and this result was significantly maintained in the aggressive group of MIBC. In the present series, no patient with MIBC that overexpressed *UCA1* succumbed during the follow-up (>5 years), as opposed to a 5-year survival rate of 58% in the group with UBC that did not overexpress *UCA1*. Notably [and similarly to Droop *et al* (15)], no statistical association was found between *UCA1* overexpression and known pathological prognostic factors in UBC, including tumor architecture (papillary vs. flat UBC), presence of a histological secondary variant, tumor multifocality, concomitant CIS, tumor grade and tumor stage. By consequence, *UCA1* overexpression appears to be a potential novel independent molecular biomarker associated with an improved CSS in patients with UBC in general, and with MIBC, in particular. This result opposes data provided by Wang *et al* (7) in 2006, which positively associated the expression of *UCA1* in 46 UBC cases with tumor stage, grade and multicentricity. However, precise data regarding UBC sample characterization and patient follow-up were not available in the study manuscript or supplementary data. The present results were also strengthened by the negative association between *UCA1* expression and two biomarkers (Ki-67 and p53) associated with UBC aggressiveness. Indeed, UBC that overexpressed *UCA1* more often presented a low Ki-67 proliferative index and a p53 'wild-type' immunoprofile and thus behaved less aggressively as compared with UBC that failed to overexpress *UCA1*.

In the present study, the CISH methodology was selected instead of RT-qPCR as CISH enables the gathering of genetic information in the context of tissue morphology, i.e., the tumor cells and their microenvironment. This methodology is currently used in pathology labs to improve patient management (27). *UCA1* signal dots was detected in 80% of all the UBC samples, but clear *UCA1* overexpression was only

Table IV. Interrelation between expression of *UCA1*, p53 and Ki-67 in urothelial bladder cancer.

	UCA1 RNA (CISH) (n=208)			p53 nuclear staining (IHC) (n=199)[a]			Ki-67 nuclear staining (%) (n=205)[a]		
Factor	Negative or low expression (n=136), n (%)	Positive (overexpression) (n=72), n (%)	P-value	Weak and heterogeneous (n=125), n (%)	Negative or high (n=74), n (%)	P-value	<25% (n=149) n (%)	≥25% (n=56) n (%)	P-value
UCA1 RNA (CISH)									
Negative or low expression	na	na		73/125 (58)	58/74 (78)	0.003	89/149 (60)	44/56 (79)	0.008
Positive (overexpression)	na	na		52/125 (42)	16/74 (22)		60/149 (40)	12/56 (21)	
p53 nuclear staining (IHC)									
Weak and heterogeneous	73/131 (56)	52/68 (76)	0.003	na	na		107/145 (74)	18/54 (33)	<0.00001
Negative or high	58/131 (44)	16/68 (24)		na	na		38/145 (26)	36/54 (67)	
Unknown[a]	5	4					4	2	
Ki-67 nuclear staining (IHC, %)									
<25%	89/133 (67)	60/72 (83)	0.008	107/125 (86)	38/74 (51)	<0.00001	na	na	
≥25%	44/133 (33)	12/72 (16)		18/125 (14)	36/74 (49)		na	na	
Unknown[a]	3	0							

Statistical associations between *UCA1* RNA (CISH) expression and the expression of two biological markers associated with UBC aggressiveness, p53 and the proliferation marker Ki-67, are shown. Semi-quantitative (*UCA1* and p53) or quantitative (Ki-67) scorings were used as aforementioned (see Materials and methods section). [a]Missing data in terms of p53 (9/208) or Ki-67 (3/208) IHC staining compared with *UCA1* data due to TMA quality. na, not applicable; ns, not significant; CISH, chromogenic *in situ* hybridization; *UCA1*, urothelial carcinoma-associated 1; IHC, immunohistochemistry.

observed in 35% of them. As illustrated in Fig. 1B-C, *UCA1* overexpression was only considered when numerous *UCA1* dots were observed in urothelial tumor cells, and these results were concordantly obtained by two different pathologists. Consequently, the positivity threshold for CISH used in the present study should be easily applied by pathologists in daily practice for the management of patients with UBC.

Genes and pathways that are key drivers of UBC have been identified by previous genome-wide expression and sequencing studies, and a complex landscape with numerous molecular subclasses that travel across conventional tumor grade and stage have been revealed (3,28). UBC are genomically heterogeneous, with frequent alterations in genes regulating receptor kinase signaling, cell cycle control and chromatin state, resulting in distinct clinical outcomes (29). Using next-generation sequencing, Kim *et al* (29) identified *PI3K/AKT* pathway alterations in 35% of UBC cases, and noted that in UBC patients treated with radical cystectomy, *PIK3CA* mutation or *PI3K/AKT* pathway alterations are associated with a significant favorable prognosis, whereas *TP53* and *CDKN2A* alterations are associated with poor outcomes. Notably, two recent publications linked *UCA1* to PI3K/AKT pathway activation (12), but also to *CDKN2A-p16INK* mRNA stabilization (30). Yang *et al* (12) showed that *UCA1* stimulated cell cycle progression by increasing CREB expression via activation of the PI3K/AKT pathway *in vitro* in the human bladder cancer BLZ-211 cell line, with *UCA1* expression being positively correlated with AKT1 expression and AKT phosphorylation. In 2014, Kumar *et al* (30) demonstrated that *UCA1* may act as a tumor suppressor gene, as its overexpression of *UCA1* was able to induce cellular senescence at least partially by the disruption of *p16INK* mRNA and hnRNPA1 interactions, resulting in increased *CDKN2A-p16INK* mRNA stability (30). In accord with this study, the present study showed that the overexpression of *UCA1* was more frequently observed in association with a low Ki-67 proliferative index. Taken together, these previous studies appear to be in line with the present results suggesting that *UCA1* overexpression should be associated with less aggressive bladder tumors.

In conclusion, the present study provides novel evidence regarding *UCA1* expression in urothelial tumor cells and its involvement in UBC carcinogenesis. The results highlight the independent contribution of *UCA1* overexpression towards improved outcomes for patients with UBC. The findings confirm the large heterogeneity that composes the MIBC group and may open novel avenues in order to better stratify patients with regards to management and treatment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LL and DM: Constitution of the clinical series, validation of quality controls and biomarkers quantification, and assistance in writing the manuscript. MLM: Implementation of the methodology for setting up *UCA1* detection by CISH and assiatnce in writing the manuscript. JA: TMA manufacturing, IHC and CISH experiments, quality control validation, and assistance in writing the manuscript. YRVE: Slide digitalization and computer-assisted quantitative analyses of KI-67 labeling index using Visiomorph DP 5.1. TR: Assistance with clinical case selection. CD: Statistical analyses and writing of the manuscript. IS: Data analyses and assistance in writing the manuscript. SR: Case reviews and classifications, biomarker quantification, data analyses and manuscript writing.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the Erasme University Hospital (Brussels, Belgium; ref., P2015/041).

Patient consent for publication

According to Belgian law, no written informed consent was required for archival material in the context of retrospective studies. The ethical committee thus waived the requirement for written informed consent from the participant.

Competing interests

The authors declare that they have no competing interests.

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