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ORIGINAL ARTICLE

Analysis of the prognostic relevance of sex-steroid hormonal receptor mRNA expression in muscle-invasive urothelial carcinoma of the urinary bladder

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Abstract

Muscle-invasive urothelial carcinoma of the urinary bladder (UCB) often recurs following radical cystectomy (RC). An altered expression of sex-steroid hormone receptors has been associated with oncological outcomes of UCB and may represent therapeutic targets. Here the expression of different hormone receptors was measured on mRNA levels in patients treated by RC and associated with outcomes. Androgen receptor (AR), estrogen receptor 1 (ESR1), and progesterone receptor (PGR) mRNA expression was assessed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) in RC samples of 87 patients with a median age of 66 (39–88) years. Univariate and multivariate analyses were performed to test associations with pathological and clinical characteristics as well as recurrence-free (RFS) and disease-specific survival (DSS). AR mRNA expression was lower in comparison with ESR1 and PGR expression ($p < 0.0001$). In univariate analysis, high expression levels of AR were associated with reduced RFS (HR 2.8, $p = 0.015$) and DSS (HR 2.8, $p = 0.010$). High AR mRNA expression and a positive lymph node status were independent predictors for reduced RFS (HR 2.5, $p = 0.0049$) and DSS (HR 3.4, $p = 0.009$). In patients with low AR mRNA expression, an increased ESR1 and PGR mRNA expression were associated with reduced RFS and DSS. High expression levels of AR are significantly associated with adverse outcome in patients with muscle-invasive UCB following RC. ESR1 and PGR expression status can further stratify patients with low AR expression into subgroups with significantly reduced RFS and DSS. Therapeutic targeting of AR may influence outcomes in patients with UCB.

Keywords Androgen receptor · Bladder cancer · Estrogen receptor · ESR · Progesterone receptor · Urothelial carcinoma

Introduction

Urothelial carcinoma of the urinary bladder (UCB) is the fourth most common malignancy in men with ~ 70,000 new cases and an estimated 16,000 deaths during 2014 in the USA. Local recurrence and the formation of distant metastases are frequent events in many patients who underwent radical cystectomy (RC). The high recurrence rates have barely improved for over two decades, as systemic treatment options remain limited and effective drug targets are lacking [1].

The association of UCB with the hormonal system was postulated based on the fact that the incidence of UCB is

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threefold higher in men than in women, even when adjusted for environmental risk and lifestyle factors [2, 3]. Sex-steroid hormone receptors such as the androgen receptor (AR), estrogen receptor (ESR), and progesterone receptor (PGR) are expressed in the normal bladder urothelium and in UCB at various degrees [4]. Particularly, the role of AR in tumorigenesis and progression of UCB has been assessed in preclinical in vitro and in vivo studies [5]. Several mouse models suggested an association between AR expression and development of urothelial carcinoma [6–9]. For instance, Hsu et al. demonstrated that mice had a lower incidence of UCB and improved survival rates after exposure to carcinogens when AR was ablated in the bladder [7]. A reduced AR expression was observed in high-grade NMIBC (Non-Muscle Invasive Bladder Cancer) and MIBC (Muscle Invasive Bladder Cancer) when compared to low-grade tumors [10–13] and other studies found an increased AR expression in high-grade NMIBC and MIBC patients [14–16]. Conflicting data exist on the prognostic impact of AR expression. Using immunohistochemistry to determine AR protein expression, some investigators found high AR expression ("AR positivity") as a driver of tumor progression [13, 15], whereas others showed an association of AR positivity with lower risk of UCB recurrence [11, 17]. There is also evidence for the involvement of the estrogen and progesterone pathways in UCB tumorigenesis, with conflicting data derived from immunohistochemical studies by our group and others [18–21].

Novel antihormonal agents may influence the treatment of UCB similar to prostate and breast cancer when biological similarities are present in tumor cells [22]. Here, reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was used as a sensitive method with low inter-observer variability [23–25]. Associations of mRNA expression of the hormonal receptors AR, ESR1, and PGR with pathological and clinical data in patients with MIBC treated by RC were assessed.

Patients and methods

Paraffin-embedded tissue samples (FFPE) were obtained from 101 patients with histologically confirmed MIBC (pT2-4 [2 patients with pTa tumors muscle-invasive at TUR]) who were treated with RC as described [26]. Lymphadenectomy was performed in 79 patients (median lymph node yield of 11 [range: 2–28]). One of the patients underwent neo-adjuvant chemotherapy, whereas 13 patients received adjuvant chemotherapy (Cisplatin/Gemcitabine). Fourteen patients were excluded because of pure squamous cell carcinoma (4 patients), missing histology (3 patients), and insufficient RNA yields (7 patients), leaving a total of 87 patients (68 male; 19 female; median age 66, range from 39 to 88) with a median follow-up of 35.1 months (range 0.7–180.8). All patients provided informed consent. The study was approved by the relevant institutional review board (Number 2013-517N-MA/2016-814R-MA).

Hematoxylin-eosin–stained sections were staged according to the 2002 TNM classification of the American Joint Committee on Cancer and graded according to the 1973 WHO classification [27]. Only specimens containing at least 20% tumor cells (median 50%; range 20–100%) were used in this study as suggested to be valid for mRNA-based subtyping in breast cancer studies [28–30].

RNA was extracted from FFPE tissue, according to a commercially available bead-based extraction method (XTRACT kit; STRATIFYER Molecular Pathology GmbH, Cologne, Germany). One-step RT-qPCR was applied for the relative quantification of AR, ESR1, and PGR mRNA expression by using gene-specific TaqMan®-based assays as described [31, 32].

In brief, expression levels of the target genes as well as the reference gene Calmodulin 2 (CALM2) were assessed using the SuperScript III PLATINUM One-Step, quantitative RT-PCR System (Invitrogen, Karlsruhe, Germany) on a Stratagene Mx3005p (Agilent Technologies, Böblingen, Germany) with 30 min at 50 °C, 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C as described [33]. Cycle threshold (Ct) values were normalized by subtracting the Ct value of the housekeeping gene CALM2 from the Ct value of the target genes (ΔCt) [32, 34–36]. RNA results were then reported as 40-ΔCt values which correlate proportionally with the mRNA expression level of the target genes.

The following set of TaqMan TM-based primer/probes (Probes: Fam/Tamra label) was used:

CALM2 Probe TCGCGTCTCGGAAACCGGTAGC
CALM2 Forward Primer GAGCGAGCTGAGTG GTTGTG
CALM2 Reverse Primer AGTCAGTTGGTCAG CCATGCT
ESR1 Probe ATGCCCTTTTGCCGATGCA
ESR1 Forward Primer GCCAAATTGTGTTTGATGGA TTAA
ESR1 Reverse Primer GACAAAACCGAGTC ACATCAGTAATAG
PGR Probe TTGATAGAAACGCTGTGAGCTCGA
PGR Forward Primer AGCTCATCAAGGCAATTGGT TT
PGR Reverse Primer ACAAGATCATGCAAGTTATC AAGAAGTT
AR Probe CAGTCCCACTTGTGTCAAAAGCGAAA TGG
AR Forward Primer CATGGTGAGCAGAGTGCCCTA
AR Reverse Primer GCAGTCTCCAAACGCATGTC

Statistical analyses were performed with Jmp SAS 11 (SAS Institute, Cary, NC, USA) and Graph Pad Prism software

(Version 5, Graph Pad Software Inc., La Jolla, CA, USA). Correlations between variables were assessed using the Spearman's rank correlation coefficient (Rho), scattered plots, and Mann-Whitney *U* test. Cut off definitions were done by Partitioning tests. Univariate analyses were calculated by log-rank tests and displayed as Kaplan-Meier plots. Cox regression analyses were performed to assess the relationship between relevant parameters and DSS (disease specific survival) or RFS (recurrence free survival) in univariate and multivariate analyses. All *p* values were two-sided with p values < 0.05 indicating statistical significance.

Results

Clinical and histopathological characteristics of patients are listed in Table 1. Locally advanced UCB (tumor stage pT3/4) was present in 58 patients (67%). UCB recurrence occurred in 40 patients (46%) at a median time of 8.5 (0.7–135) months after RC. Cancer-specific death was noted in 33 patients (38%) after a median time of 16.4 (0.7–128.1) months after RC.

The median normalized mRNA expression (40-ΔCt values) for ESR1 and PGR mRNA expression was with 36 (range: 30.1 to 40.3, $p < 0.0001$) and 35.8 (range: 29.9 to 40.5, $p < 0.0001$), higher than median AR mRNA expression with 29.1 (range: 24.4 to 34.7). Spearman's rank correlation showed a high co-expression between PGR and ESR1 mRNA (Rho 0.74, $p < 0.0001$) but neither relevant co-expression between AR and ESR1 nor PGR (Rho 0.075 and 0.11, p = n.s.). Normalized mRNA expression of ESR1, AR, and PGR showed no association with greater age (≥ 75), gender, T-Stage, grading, or Cis. AR mRNA expression was significantly lower in patients with high pathological grade when compared to patients with G2 MIBC ($p = 0.022$, Table 2).

High AR mRNA expression (> 30.95) was significantly associated with reduced DSS (5-year DSS: 17% for high AR; 61% for low AR; $p = 0.0011$, Fig. 1). Higher AR expression was also associated with reduced RFS (5-year RFS: 18% for high AR; 48% for low AR; $p = 0.0050$) in univariate testing. For PGR expression, no significant associations were found between PGR and DSS ($p = 0.091$) or RFS ($p = 0.19$). Similarly, no significant associations were found between

Table 1 Patients characteristics of MIBC study cohort. pTa tumors where muscle invasive at TUR

	n or median	Percentage/range
Age, y	66	39–88
≥ 75	15	17
< 75	72	83
Gender		
Male	68	78
Female	19	22
Tumor stage		
pTa	2	2
pT2	27	31
pT3	41	47
pT4	17	20
Grading		
G2	21	24
G3	66	76
Concomitant carcinoma in situ	23	26
Nodal stage		
pN0	50	58
pN1	10	11
pN2	19	22
n.a. (Nx)	8	9
Lymphovascular invasion		
Positive	40	46
Negative	32	37
n.a.	15	17
Positive margins	6 (2 n.a.)	7
Cancer specific death	33	38
Time to Cancer specific death	16.4	0.7–128.1

Table 2 Association between clinicopathological characteristics and marker expression shows a lower expression of AR in patients with higher grade (gene expression values normalized (40-ΔCT); significant values in bold)

	AR		ESR1		PGR	
	Median	*p*	Median	*p*	Median	*p*
Age, y						
≥ 75	29.06	0.87	36.27	0.94	35.78	0.88
< 75	29.03		35.85		35.79	
Gender						
Male	29.03	0.66	35.85	0.85	35.82	0.61
Female	29.20		36.14		35.68	
Tumor stage						
pTa/2	29.03	0.67	35.06	0.86	35.68	0.41
pT3/4	29.12		36.24		36.10	
Grading						
G2	**30.0**	**0.022**	35.06	0.68	35.83	0.96
G3	**28.63**		36.11		35.78	
No Cis	29.03	0.25	36.1	0.42	35.8	0.259
Concomitant Cis	28.01		35.8		36.3	
Nodal stage						
pN0	29.03	0.97	35.29	0.48	35.76	0.22
pN1/pN2	29.32		36.48		36.46	
pN0	29.03	0.63	35.29	0.37	35.76	0.21
pN2	29.32		36.57		37.08	
LVI−	29.8	0.36	35.06	0.39	35.78	0.27
LVI+	29.32		36.46		36.26	

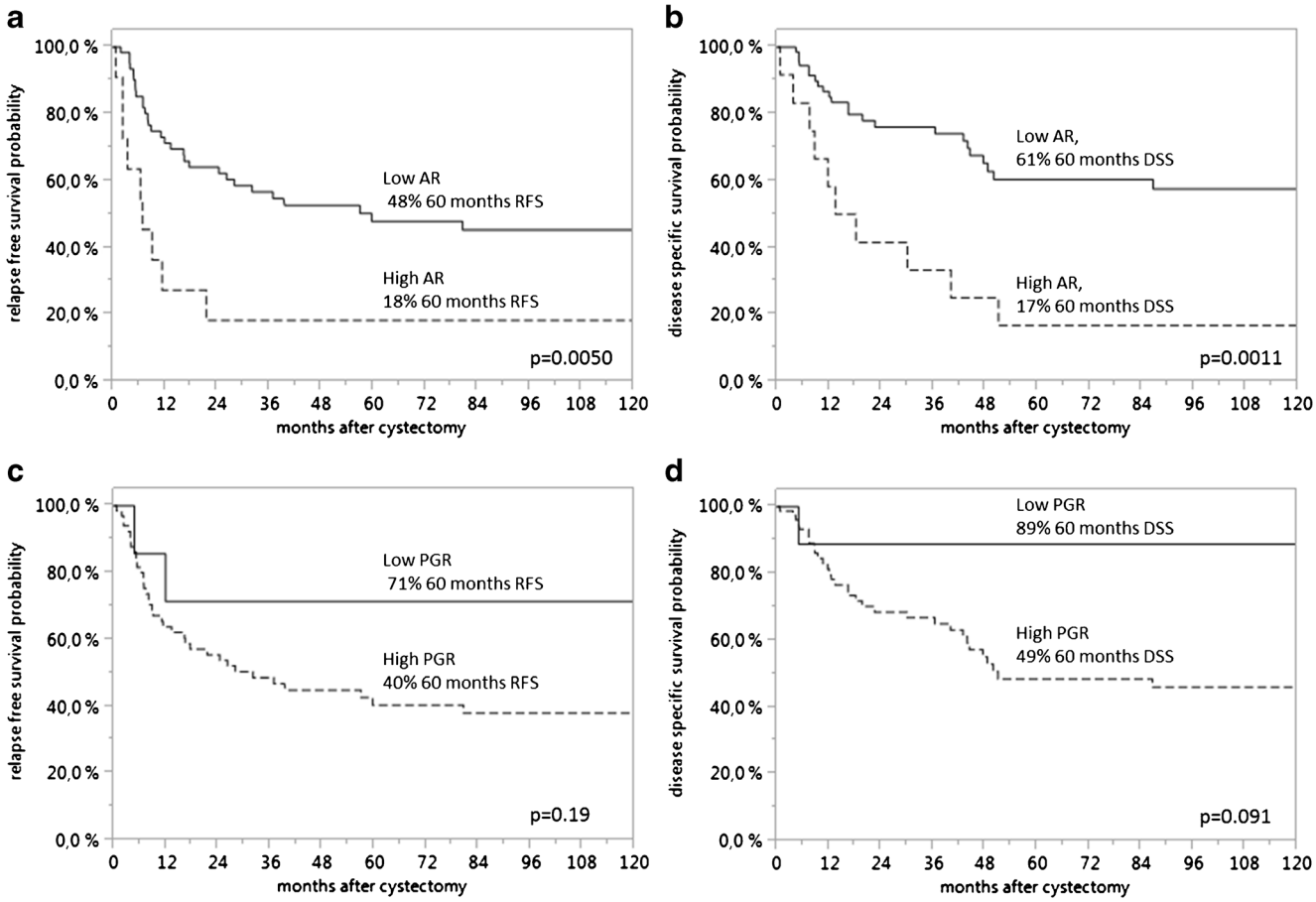

Fig. 1 Kaplan-Meier curves of relapse free survival (RFS) and disease specific survival (DSS) stratified by AR (**a**, **b**) as well as by PGR (**c**, **d**) expression (Cut off level: AR > 30.95, PGR > 32.64). Respective cut-off values for gene expression revealed the presence of 14% patients with high AR, and 9% with high PGR expression

ESR1 mRNA expression and RFS or DSS ($p = 0.81$; $p = 0.43$).

In univariate analysis, high AR expression levels were associated with both reduced RFS (HR 2.8, $p = 0.015$) and DSS (HR 2.8, $p = 0.010$), whereas high PGR expressions were exclusively associated with reduced RFS (HR 4.7, $p = 0.048$). Multivariate Cox-regression analysis adjusting for gender, T-Stage, WHO 1973 grading, age, lymph node status, and LVI revealed prognostic significance of high AR expression for RFS (HR 2.5, $p = 0.049$) and DSS (HR 3.4, $p = 0.009$). The absence of nodal involvement was a favorable variable regarding RFS (HR 3.0, $p = 0.0014$) and DSS (HR 3.2, $p = 0.0027$, Table 3).

Given that only patients with low AR mRNA expression exhibited a significant survival benefit, we further stratified this subgroup by ESR1 and PGR mRNA expression levels. Patients with low AR mRNA expression were divided in a low (AR low, PGR low), intermediate (AR low, PGR high), and high risk (AR high) group with regard to RFS and DSS, depending on their PGR mRNA expressions (Fig. 2). Similarly, patients with low AR mRNA expression showed a significantly different RFS ($p = 0.0049$) and DSS ($p = 0.0011$) when additionally stratified by ESR1 expression levels (low risk (AR low, ESR1 low), intermediate risk (AR low, ESR1 high), high risk (AR high).

Discussion

The present study investigated mRNA-expression of the hormonal receptors AR, ESR1, and PGR in MIBC in order to evaluate a potential prognostic role in these patients with limited systemic treatment options [1]. Hormonal receptors present very interesting therapeutic targets and were already in the focus of several immunohistochemistry-based studies with controversial results [11–13, 15, 16, 37].

A lower AR mRNA-expression in high-grade tumors was found in the present study, which is in line with several previous findings showing an association with high stage and grade [11, 13]. Boorjian and colleagues suggested that a loss of AR expression leads to invasive UCB as they found reduced AR expression in high stage UCB (AR expression in 53% of urothelial carcinoma and in 86% of the normal urothelium) [12]. Furthermore, an increased AR expression

Table 3 Uni- and multivariate Cox proportional hazard model to test the effect of different parameters on DSS and RFS in MIBC patients treated with RC. Analysis showed an independent prognostic value of AR and nodal status on DSS and on RFS (significant values in bold)

	RFS				DSS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	Hazard RATIO	*p* value	Hazard ratio	*p* value	Hazard ratio	*p* value	Hazard ratio	*p* value
pN	**2.9**	**0.0019**	**3.0**	**0.0014**	**3.3**	**0.0018**	**3.2**	**0.0027**
pT stadium	1.9	0.065			1.86	0.28		
Grade	0.83	0.63			0.87	0.73		
Gender	1.6	0.19			1.3	0.52		
Age (< 75)	1.87	0.14			2.3	0.058		
LVI	1.7	0.094			1.6	0.19		
AR	**2.8**	**0.015**	**2.5**	**0.049**	**2.8**	**0.010**	**3.385**	**0.0091**
ESR1	1.1	0.82			1.3	0.43		
PGR	2.5	0.15			**4.7**	**0.048**		

in patients with increased T-stage was shown by Mir et al. (9.0% of NMIBC expressed AR versus 15.1% of MIBC) [16].

High AR expression has previously been associated with UCB progression [15]. High AR expression levels were significantly associated with reduced DSS and RFS in the present study. Mashadi et al. demonstrated that patients with positive AR expression had a worse prognosis and a higher rate of metastases than their AR negative counterparts. Zheng

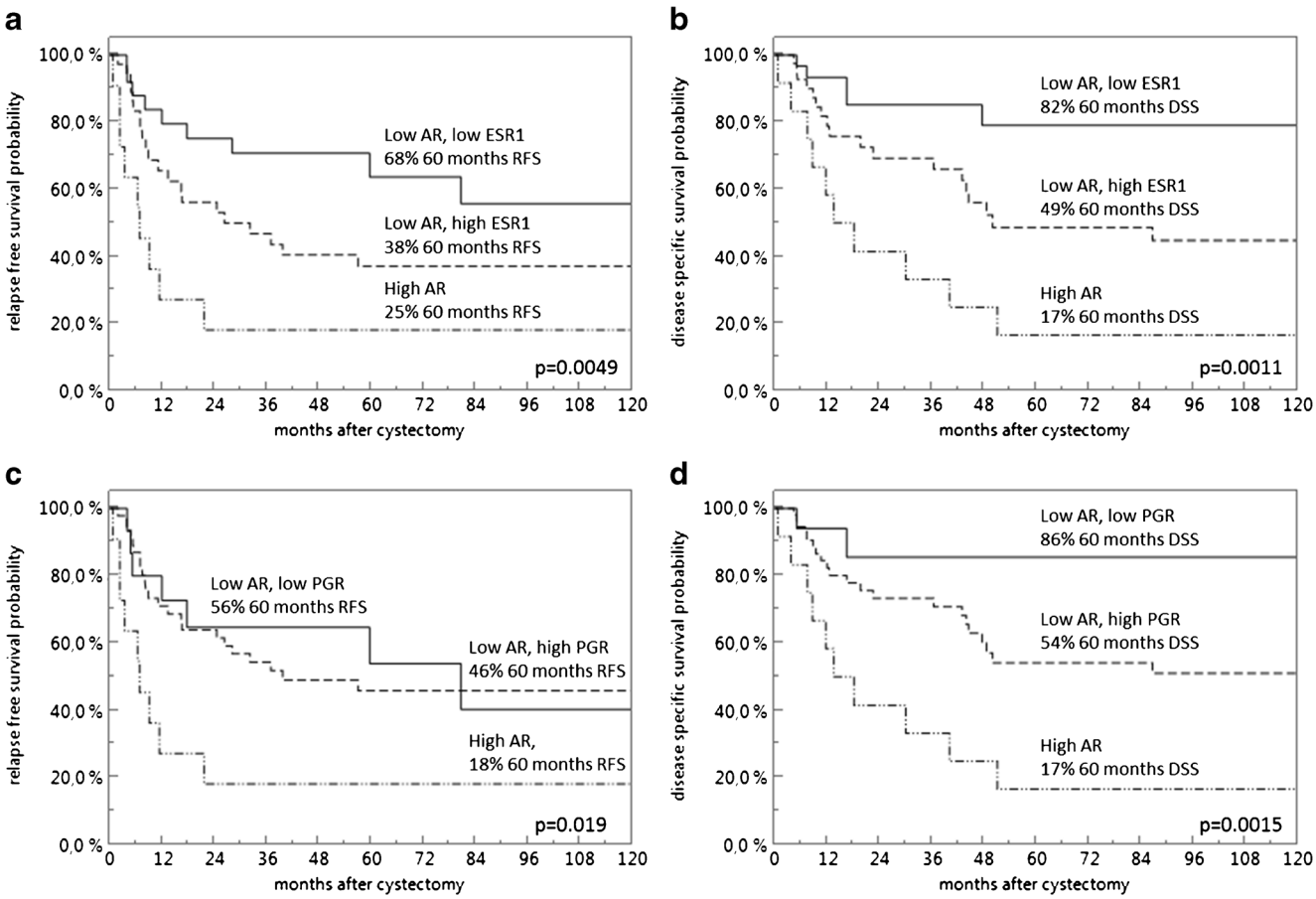

Fig. 2 Kaplan-Meier curves of relapse free survival (RFS) and disease specific survival (DSS) stratified by AR as well as by ESR1 (**a**, **b**; AR > 30.95, ESR1 > 35.29) or PGR expression (**c**, **d**; AR > 30.95, PGR > 34.53)

et al. found an association between AR positivity and progression of UCB as well [14]. A small pilot study using a RT-qPCR approach found more frequent recurrence rates in patients with high AR expression [8]. In an immunohistochemical study, however, no significant association of AR expression and recurrence or survival was found, although a tendency towards higher progression rates in AR positive tumors after RC was described [13]. A study by Mir et al. failed to show a significant difference for DSS in patients with stage T2 UCB when stratified by AR expression [16]. The reason for these conflicting results might be attributed to immunohistochemistry which was mainly used for detecting AR protein expression in most of the previously cited studies. Immunohistochemistry is prone to high inter-observer variability and different results, depending on the applied antibody and scoring system [38]. Furthermore, immunohistochemistry was previously shown to detect only 5.7% of nuclear AR expression and 26.3% of combined nuclear and cytoplasmatic AR expression in UCB, potentially masking prognostically meaningful results [39]. For example, even in highly AR-expressing prostate cancer, the correlation between AR protein staining intensity and AR mRNA expression was shown to be limited [40], which contributes to the assumption that AR expression in UCB may be too low to be reliably detected using immunohistochemistry.

In the present study, mRNA expression of AR, ESR1, and PGR was measured using RT-qPCR by analogy with previous studies to avoid technical limitations of immunohistochemistry [31, 32, 41]. We chose to analyze ESR1 instead of ESR2 mRNA expression based on the data accumulated by the Cancer Genome Atlas (TCGA) Research Network which showed a higher expression and broader dynamic range for ESR1 mRNA expression when compared to ESR2 in MIBC [42]. Unlike AR, we found no significant association between the mRNA expression of ESR1 and PGR with tumor stage, grade, and survival. This is in concordance with our previous findings [18]. Using immunohistochemistry, we previously found ESR1 protein to be expressed at a very low rate in UCB (9%) and not to be related with DSS, while PGR was not expressed in UCB at all [18]. Our results are comparable with the findings of Shen et al. [43]. Using immunohistochemistry to analyze the tissue microarrays of 224 patients with UCB, the authors suggested that ESR2 is the predominant estrogen receptor (ESR) in UCB, as ESR2 was positive in 63% of the patients with an increasing expression in advanced tumor stages [43].

However, this is in contrast to previous reports using immunohistochemistry showing a lower positivity rate of ESR1 in high grade tumors and in MIBC [13]. The discrepancies might be attributed to the specific characteristics of our patient cohort as well as analysis methods which showed higher positivity when using RT-qPCR in comparison with immunohistochemistry for ESR1 [13].

Based on existing observations in breast cancer describing opposed expression of AR and ESR as well as different prognostic groups based on AR and ESR expression [44], we subdivided patients with low AR expression according to PGR and ESR1 mRNA expression. Thus, we were able to define risk groups for recurrence and disease-specific death based on altered expression of a hormonal receptor expression panel. The highest risk group included all patients with high AR mRNA expression. Patients with low AR mRNA expression, but high ESR1 or PGR mRNA expression showed intermediate risk for disease recurrence and cancer-specific death. Patients with overall low hormonal receptor expression had the lowest risk. The reason for this might be that there is some crosstalk between AR and ESR as they also share some coactivators [44], meaning that in AR-negative patients the negative effects usually mediated through the AR pathway may still be active to some extent in the presence of ESR and PGR. It has previously been shown in breast cancer that the ESR negative and AR positive subtype has a similar gene expression pattern as ESR-positive breast cancers [44, 45]. Chromatin immunoprecipitation sequencing studies in breast cancer cell lines demonstrated that AR-binding events are similar to those of ESR1, indicating that AR may be able to substitute for ESR1 in the absence of ESR1 [44, 46]. The same might be possible vice versa in AR negative and ESR1 positive UCB.

With the prognostic association of hormonal receptor status in the present study, our results also implicate a benefit of an antihormonal targeted treatment for patients with UCB expressing hormonal receptors, which has been described. Izumi et al. demonstrated in a retrospective analysis that men with primary UCB and concomitant prostate cancer experienced prolonged RFS for UCB under androgen deprivation therapy for prostate cancer [47]. Furthermore, lower recurrence rates in patients with NMIBC under androgen deprivation therapy were described [48]. Currently, novel AR inhibitors such as enzalutamide are under evaluation for UCB in phase I/II trials (NCT02605863; NCT02300610) which can further support the use of antiandrogen therapy in UCB. Furthermore, an enhanced cytotoxicity of gemcitabine in combination with tamoxifene was demonstrated in UCB cell lines [49], while response to the combination of tamoxifen and chemotherapy was comparable to chemotherapy alone in a previous pilot study [50]. However, administration of tamoxifen in patients with UCB might offer better results depending on the expression of hormonal receptors. Moreover, antihormonal therapy in UCB in the light of basal and luminal subtypes of UCB [51, 52] can further be beneficial because it was presented that hormonal receptors are mainly expressed in the luminal-like subtype of UCB [53] which suggests that patients with luminal tumors may benefit from antihormonal therapy.

Our study has some limitations. First, the expression analyses were performed in a retrospective cohort of non-

consecutive patients. Moreover, no immunohistochemical assessment was made for validation of AR, ESR1, and PGR on a protein level. Furthermore, ESR2 mRNA expression was not analyzed. Given the relatively small sample size of our cohort, additional larger validation studies are necessary. While the presented method of sensitive and quantitative assessment of mRNA expression levels may overcome some inherent limitations of any immunohistochemical method, one possible limitation of mRNA expression analysis is the potential contamination with non-neoplastic urothelium. While we used only samples containing more than 20% tumor cells by analogy with studies in breast cancer [28–30], a contamination cannot be entirely excluded.

In conclusion, we found that high AR mRNA expression is significantly associated with worse outcome in patients with muscle-invasive bladder cancer undergoing RC. ESR1 and PGR mRNA expression status can further stratify patients with low AR mRNA expression into subgroups with different RFS and DSS. These data offer a rationale for AR targeting in appropriately selected patients and background for further prospective studies on the role of hormonal receptors and antihormonal treatments in UCB.

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Compliance with ethical standards

Informed consent Informed consent was obtained from all the individual participants included in the study. The study was conducted after approval of the local ethics committee (board (Number 2013-517 N-MA/ 2016-814R-MA).

Conflict of interest Ralph M. Wirtz is a founder of STRATIFYER Molecular Pathology GmbH. All other authors declare that they have no conflict of interest.

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