


# In stage pT1 non-muscle-invasive bladder cancer (NMIBC), high KRT20 and low KRT5 mRNA expression identify the luminal subtype and predict recurrence and survival

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**Abstract** Differential expression of cytokeratins (CK) is a characteristic feature of chemoresistant luminal (KRT20) and chemosensitive intrinsic aggressive basal (KRT5) subtypes in muscle-invasive bladder cancer (MIBC). We investigated mRNA expression of KRT5 and KRT20 and its predictive value in stage pT1 bladder cancer. In retrospective analysis of clinical data and formalin-fixed paraffin-embedded tissues (FFPE) of patients with stage pT1 NMIBC who underwent transurethral resection of the bladder, a single-step RT-qPCR was used to measure mRNA expression. Furthermore, immunohistochemical (IHC) staining of CK20, panCK, and MIB1

was performed. Valid measurements were obtained from 231 samples out of a series of 284 patients. Spearman correlation revealed significant associations between mRNA and protein expression of KRT20/CK20 ( $\rho$  0.6096,  $p < 0.0001$ ) and MKI67/MIB1 ( $\rho$  0.5467,  $p < 0.0001$ ). A positive correlation was found between MKI67 and KRT20 expression ( $\rho$  0.3492,  $p < 0.0001$ ), while MKI67 and KRT5 were negatively correlated ( $\rho$  -0.1693,  $p = 0.01$ ). High KRT20 expression ( $\geq 40.26$ ) was significantly associated with worse recurrence free survival (RFS) ( $p = 0.001$ ), progression-free survival (PFS) ( $p = 0.0003$ ), and cancer specific survival (CSS) ( $p = 0.0414$ ). The combination of high KRT20 expression and low KRT5 expression ( $< 36.83$ ) was associated with unfavorable RFS ( $p = 0.0038$ ) and PFS ( $p = 0.0003$ ) and proved to be the only independent predictor for RFS ( $p = 0.0055$ ) and PFS ( $p = 0.0023$ ) in multivariate analysis. KRT20 mRNA determination was superior to CK20 protein estimation with regard to RFS and PFS prediction. KRT20 and KRT5 mRNA quantification can predict recurrence and progression of stage pT1 NMIBC reflecting basal and luminal subtypes of MIBC and is superior to CK20 protein expression determined by IHC.

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## Abbreviations

CSS	Cancer-specific survival
FFPE	Formaline fixed paraffin embedded
GOI	Gene of interest
HR	Hazard ratio
mRNA	Messenger ribonucleic acid
OS	Overall survival

PFS	Progression-free survival
REF	Reference gene
RFS	Recurrence-free survival
RT-qPCR	Reverse transcription quantitative real time polymerase chain reaction

## Introduction

Bladder cancer is the ninth most common malignancy worldwide considering both genders, while 75% of all cases occur in male patients [1]. Approximately 75% of patients are initially diagnosed with non-muscle-invasive bladder cancer (NMIBC) [2]. Depending on stage and grade after transurethral resection, 50–70% of the patients develop disease recurrence and up to 20% of the tumors progress to a more invasive stage [2, 3]. Patients with NMIBC are monitored with cystoscopy over many years, which impose heavy costs to society which is why bladder cancer carries the highest cost among cancers per patient from diagnosis to death [4]. Therefore, molecular markers for stratifying patient treatment and application of novel therapeutic drugs are highly appreciated especially for stage pT1 bladder cancer.

NMIBC is stratified into three risk groups according to clinical (diameter, focality) and pathological (stage, grade, concomitant carcinoma in situ (Cis)) criteria [2]. This is the basis for treatment decision and follow-up strategy. To predict recurrence and progression of NMIBC, the European Organization for Research and Treatment of Cancer (EORTC) developed a score (EORTC score) [5]. To improve this limited clinicopathological risk calculator, various molecular markers have been investigated.

More recently, tumor heterogeneity has been investigated at molecular level, which allowed identification of distinct molecular classes beyond histopathological classification [6–8]. These resemble molecular features of the luminal and basal breast cancer subtypes with similar differences in clinical outcome [6–8]. Muscle-invasive bladder cancer (MIBC) has been shown to be of mainly basal- and luminal-cell origin [6–8].

Recently, a prospective comprehensive transcriptional study investigated gene expression in 460 NMIBC patients [10]. It was shown that NMIBC can be subclassified into three major classes with basal- and luminal-like characteristics and different clinical outcome [10].

Differential cytokeratin (CK) expression is a characteristic feature of muscle-invasive disease [11]. Cytokeratins are crucial for cell integrity and in different epithelia different combinations of the 20 CKs are found. Normal urothelium displays a combination of simple keratins including cytokeratin 20 (KRT20) and cytokeratin 5 (KRT5) [12]. KRT20 is physiologically exclusively expressed in umbrella cells [13], whereas

KRT5 is expressed in basal cells [14]. In MIBC, it could be shown that KRT5 is a feature of the chemosensitive and intrinsic aggressive basal subtype, whereas KRT20 is a feature of the chemoresistant and less aggressive luminal subtype [11]. Sjö Dahl et al. studied bladder cancer of all stages and grades and found that KRT5 is expressed in the “Urobasal B” and “squamous cell carcinoma-like (SCC-like)” subgroups, whereas KRT20 is expressed in a subpopulation of the “Urobasal A” and of the “genomically unstable” groups [9].

To further characterize subtypes of stage pT1 NMIBC and their biological behavior, we investigated expression of “luminal” cytokeratin KRT20 and “basal” cytokeratin KRT5 in this challenging subentity. To avoid the numerous technical limitations of IHC, we developed and used RT-qPCR assessment for objective quantification of KRT20 and KRT5 mRNA expression. Moreover, we did immunostain for CK20 to explore whether approximation of molecular subtyping by standard IHC similarly reflects biological behavior.

## Patients and methods

### Study population

The total study cohort consisted of 284 patients with stage pT1 NMIBC at initial diagnosis who underwent transurethral resection of the bladder (TURB) in a single center between 1989 and 2009. Histopathological parameters of all cases, including grading according to WHO 1973 and WHO 2004 classification, were reassessed by a pathologist specialized in uropathology (A.H.). All patients underwent resection and were treated according to an organ preserving approach.

### Isolation of tumor RNA

For RNA extraction from FFPE tissue, a single 10- $\mu$ m section was processed according to a commercially available bead-based extraction method (XTRACT kit; STRATIFYER Molecular Pathology GmbH, Cologne, Germany). RNA was eluted with 100  $\mu$ l elution buffer and RNA eluates were then stored at  $-80$  °C until use. The section was taken from a paraffin block containing at least 30% tumor cells.

### Gene expression by RT-qPCR

RT-qPCR mRNA expression levels of KRT5, KRT20, and MKI67 and of CALM2 as reference gene (REF) were determined. This involves reverse transcription of RNA and subsequent amplification of cDNA, executed using Taqman Primer/Probes. Each patient sample or control was analyzed

with each assay mix in triplicate. Experiments were run on a Siemens Versant (Siemens, Germany) according to the following protocol: 5 min at 50 °C, 20 s at 95 °C followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. Forty amplification cycles were applied and the cycle quantification threshold (Cq) values of three markers and one reference gene for each sample (S) were estimated as the median of the triplicate measurements. Final values were generated using  $\Delta Cq$  from the total number of cycles, to ensure that normalized gene expression obtained by the test is proportional to the corresponding mRNA expression levels.

### Immunohistochemistry: assessment and evaluation

From the total cohort, a tissue microarray (TMA) was assembled. From formalin-fixed paraffin-embedded tissue blocks, one representative 1.5-mm core was used, proven by histopathologic comparison of the TMA core with full original sections of the entire tumor. Then, 4- $\mu$ m sections were cut and mounted on poly-L-lysine-coated glass slides.

IHC staining was carried out in a BenchMark IHC Full System immunostainer (Roche Diagnostics, Mannheim, Germany) using the avidin-biotin peroxidase method with diaminobenzidine as a chromogen according to the manufacturer's instructions. The primary antibodies used were anti-CK20, anti-MIB (each mouse monoclonal, dilution 1:200, incubation time 20 min, DAKO Deutschland, Germany), and anti-panCK (mouse monoclonal, dilution 1:50, incubation time 12 min, Immunotech Laboratories, USA). IHC staining was evaluated by a specialized uro-pathologist (A.H.).

### Statistical methods

Statistical analysis was performed using SPSS version 23 and JMP 9.0.0. Cut-off definitions were done by Partitioning tests and Youden Index analysis. The Spearman product-moment correlation coefficient  $r$  was used as a measure of the strength and direction of the linear relationship between variables. Statistical analyses including Kaplan-Meier survival analysis, multivariate Cox regression, and partitioning testing were performed with JMP SAS (SAS Institute, Cary, NC, USA) and Graph Pad Prism software (Version 5.04; Graph Pad Software Inc., La Jolla, CA, USA).

## Results

### Patient population

The total study cohort consisted of 284 NMIBC tumor samples from 1989 to 2009 from a single institution. All tumors were

staged pT1 in initial histopathological assessment and confirmed by histopathological reassessment in 2015/16. As 53 samples had to be excluded, data from 231 patients (78.4% male, median age 72 years) could be used for final evaluation (Suppl. Fig. 1). Of these, 70 were graded G2 and 161 were graded G3 according to the WHO Grading system 1979 (Table 1). Median follow-up time was 43 months. Median mRNA expression levels were for KRT5 36.86, for KRT20 40.59, and for MKI67 36.35.

### Correlation of cytokeratin mRNA expression and protein expression by IHC

Non-parametric Spearman rank correlation revealed a positive, statistically significant association between mRNA expression and protein expression measured by RT-qPCR

**Table 1** Patient characteristic of the stage pT1 bladder cancer study cohort

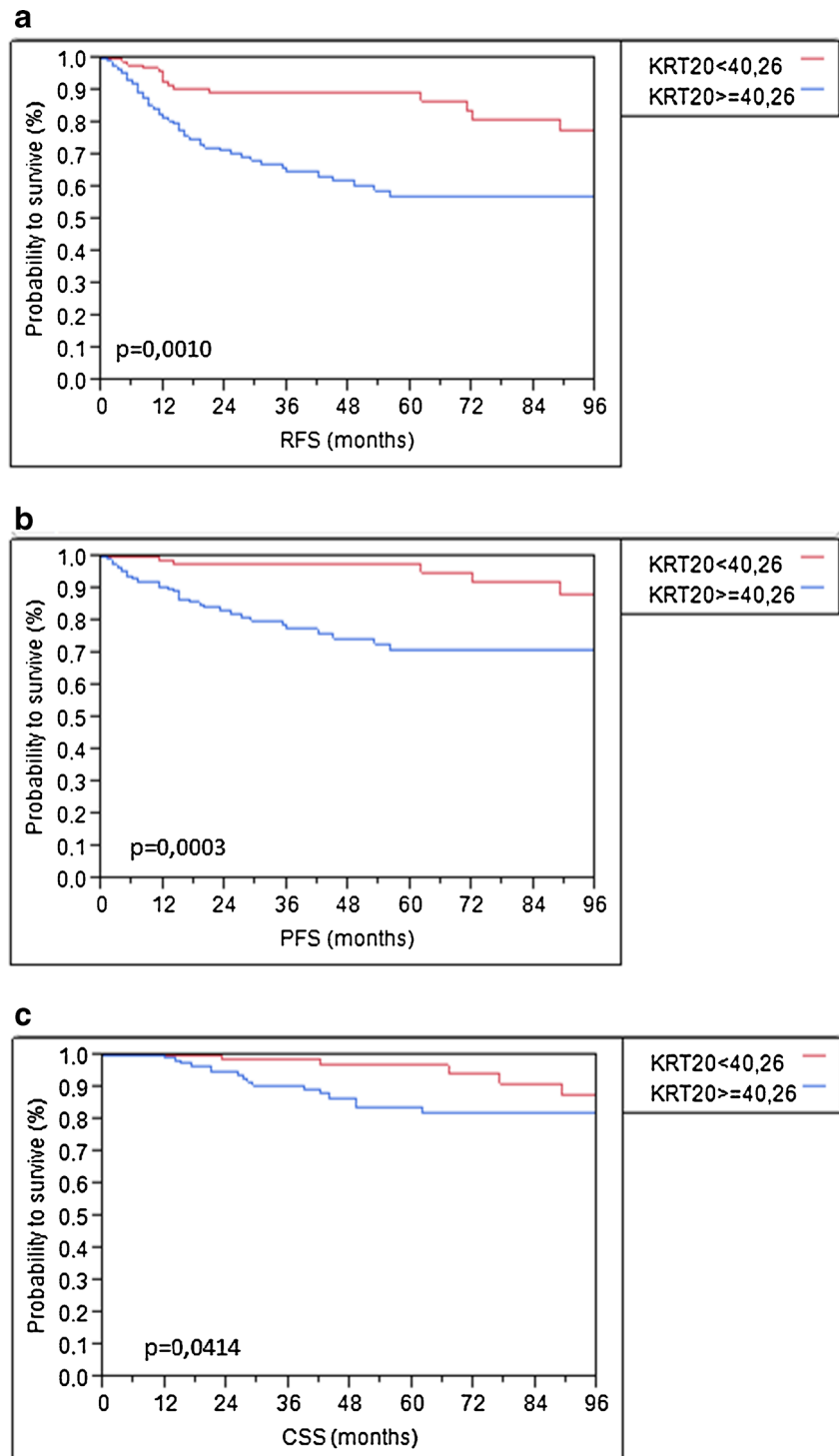
Patient data	<i>n</i> (%)
Total stage pT1 patients 1989–2009	231 (100.0)
Female patients	50 (21.6)
Male patients	181 (78.4)
Median age (years)	72
IQ range (years)	65–79
WHO grading classifications	
WHO classification 1973	
pT1G2	70 (30.3)
pT1G3	161 (69.7)
WHO classification 2004	
Low grade	4 (1.7)
High grade	227 (98.3)
Additional histopathological factors	
Solid tumors	24 (10.4)
Multifocal tumors	42 (18.2)
Tumor size $\geq 3$ cm	129 (55.8)
Associated Cis	56 (24.2)
Treatment of UBC and follow-up results	
Instillation therapy approved	117 (50.6)
Need for secondary Cx	29 (12.6)
Median follow-up (months)	42
IQ range (months)	26–80
Maximum follow-up (months)	231
Recurrence ( $\leq$ pT1)	67 (29.0)
Progression ( $\geq$ pT2)	38 (16.5)
Overall survival	134 (58.0)
Death of disease	21 (9.1)

and IHC between KRT20/CK20 ( $\rho$  0.6096,  $p < 0.0001$ ) and MKI67/MIB1 ( $\rho$  0.5467,  $p < 0.0001$ ) (Suppl. Fig. 2). MKI67 and KRT20 showed a positive correlation ( $\rho$  0.3492,  $p < 0.0001$ ), whereas both MKI67 and KRT5 ( $\rho$   $-0.1693$ ,  $p = 0.010$ ) and KRT20 and KRT5 ( $\rho$   $-0.1804$ ,  $p = 0.006$ ) were negatively correlated (Suppl. Fig. 2).

### Predictive value of cytokeratin expression

Kaplan-Meier analysis revealed a statistically significant association between high KRT20 mRNA expression ( $\geq 40.26$ ) and poor recurrence-free (RFS) ( $p = 0.001$ ), progression-free (PFS) ( $p = 0.0003$ ), and carcinoma-specific survival (CSS) ( $p = 0.0003$ ), and carcinoma-specific survival (CSS) ( $p = 0.0003$ ), and carcinoma-specific survival (CSS) ( $p = 0.0003$ ).

**Fig. 1** Kaplan-Meier analysis for RFS (a), PFS (b), and CSS (c) based on KRT 20 mRNA expression in stage T1 bladder cancer



( $p = 0.0414$ ) (Fig. 1). High protein expression of CK20 in IHC with a cut-off of  $\geq 80\%$  of cells positive for CK20 was only associated with PFS ( $p = 0.0047$ ) (Suppl. Fig. 3).

Taken together, high expression of KRT20 ( $\geq 40.26$ ) and low expression of KRT5 ( $< 36.83$ ) showed a statistically significant association with poor RFS ( $p = 0.0038$ ) and PFS ( $p = 0.0003$ ) without an effect on CSS (Fig. 2). Expression levels of KRT5 were associated with PFS, but not with RFS and CSS.

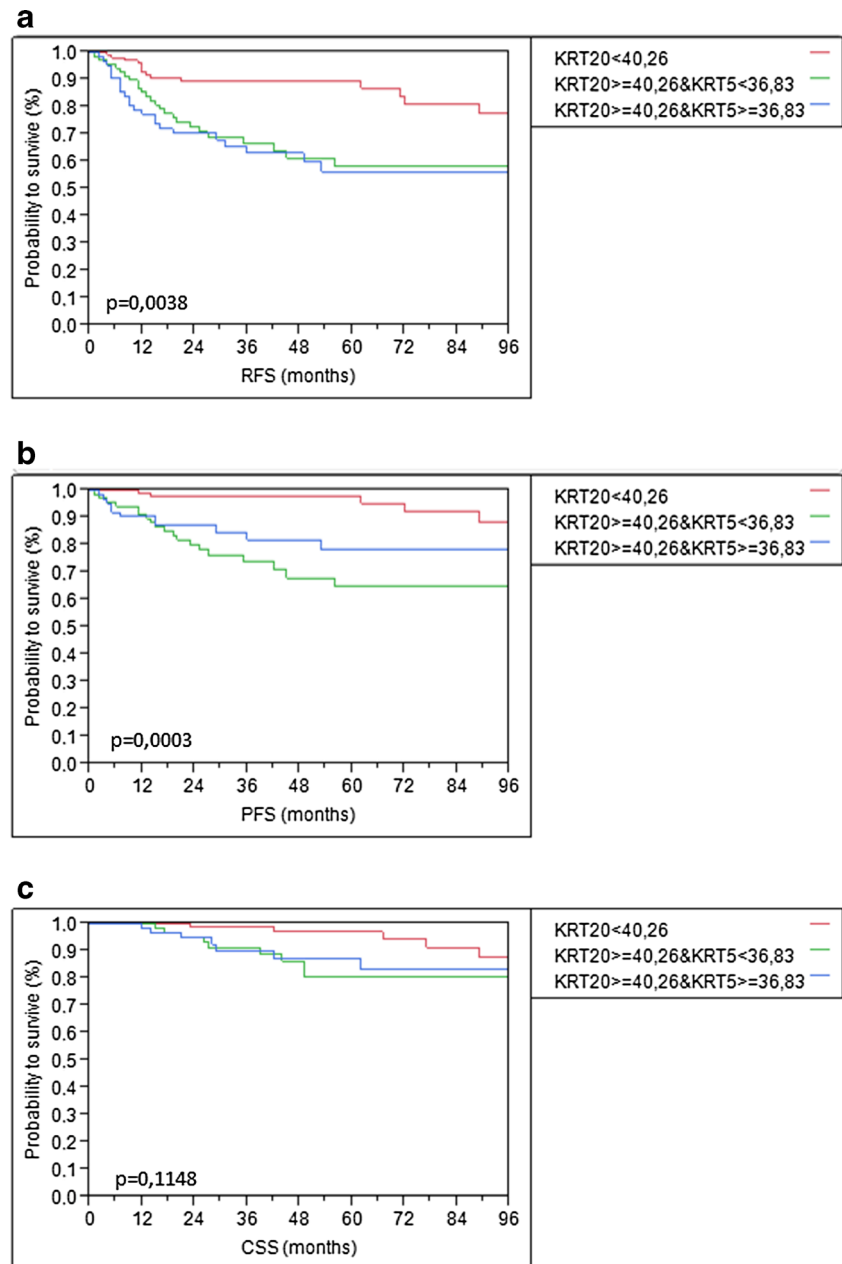
Multivariate Cox regression analysis adjusted for gender, associated Cis, tumor size, focality, and WHO 1973 grading revealed subtyping according to the KRT mRNA expression (KRT20  $\geq 40.26$  and KRT5  $< 36.83$ ) as the only statistically

significant risk factor for RFS (L-R Chi2 10.40,  $p = 0.0055$ ) and PFS (L-R Chi2 12.13,  $p = 0.0023$ ) (Table 2). For CSS, tumor size (L-R Chi2 5.17,  $p = 0.0229$ ) and grade 3 after WHO classification of 1973 (L-R Chi2 5.29,  $p = 0.0215$ ) were the only statistically significant risk factors (Table 2).

### KRT20 mRNA expression is predictive for grade 3 tumors

High KRT20 mRNA expression ( $\geq 40.26$ ) subdivided high risk pT1G3 tumors into a high and a low risk group with 65% PFS versus 95% PFS after a 5-year follow-up, the latter with the same risk as pT1G2 tumors ( $p < 0.0001$ ) (Fig. 3b). This was also observed for RFS ( $p = 0.0121$ ) (Fig. 3a). Low

**Fig. 2** Kaplan-Meier analysis in the total cohort for RFS (a), PFS (b), and CSS (c) based on KRT5 and KRT20 mRNA expression in stage T1 bladder cancer



**Table 2** Cox regression analysis for RFS (a), PFS (b), and CSS (c) regarding KRT5, KRT20 mRNA expression, and clinicopathological features in stage T1 bladder cancer

	L-R Chi <sup>2</sup>	<i>p</i> value
a)		
KRT mRNA expression	10.3976163	<i>0.0055</i>
Gender male vs. female	0.04227714	0.8371
Concomitant Cis yes vs. no	2.26029231	0.1327
Tumor size ≥3 cm vs <3 cm	3.8397026	0.0501
Grading WHO 1973 G3 vs.G2	1.1565218	0.2822
Focality multifocal vs. unifocal	0.57778089	0.4472
b)		
KRT mRNA expression	12.1324562	<i>0.0023</i>
Gender male vs. female	0.06368442	0.8008
Concomitant Cis yes vs. no	2.22695179	0.1356
Tumor size ≥3 cm vs <3 cm	3.06027035	0.0802
Grading WHO 1973 G3 vs.G2	3.34072917	0.0676
Focality multifocal vs. unifocal	0.86329775	0.3528
c)		
KRT mRNA expression	3.61959305	0.1637
Gender male vs. female	0.11610398	0.7333
Concomitant Cis yes vs. no	0.5498462	0.4584
Tumor size ≥3 cm vs <3 cm	5.17484078	<i>0.0229</i>
Grading WHO 1973 G3 vs.G2	5.28635345	<i>0.0215</i>
Focality multifocal vs. unifocal	1.38346461	0.2395

Significant results indicated in italic

KRT20-expression in a pT1G3 tumor was associated with intermediate CSS comparable to that of a pT1G2 tumor, while a pT1G3 tumor with high KRT20 expression carried the highest risk ( $p = 0.038$ ) (Fig. 3c).

## Discussion

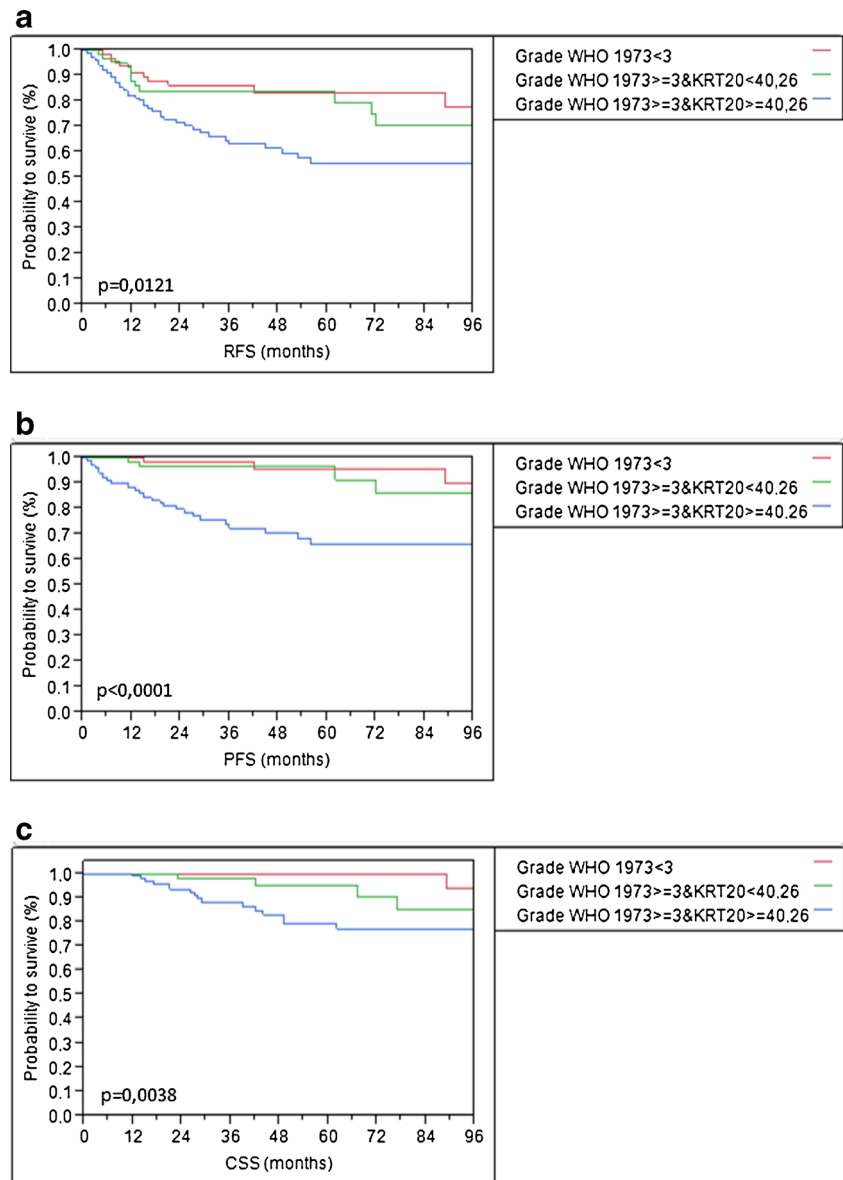
Recently, different studies reported that MIBC can be subdivided into luminal and basal subtypes with different biological behavior [6–10]. The basal subtype presents with advanced stage and metastatic disease is intrinsically aggressive but chemosensitive [11]. The luminal subtype has less aggressive potential but often emerges as chemoresistant [11]. To distinguish between these subtypes, CK expression seems to be a potentially useful marker [11]. KRT5, which marks stem or progenitor cells and is expressed in the basal compartment of urothelium, characterizes the basal subtype [6, 14–16]. KRT20, which is expressed in superficial umbrella cells, is a marker for luminal subtypes [11, 13, 16]. As these findings have been reported for MIBC, we investigated whether differential CK expression has a predictive role in stage pT1 NMIBC. Conventional clinical and pathological risk factors have limited potential for risk stratification of this

subentity, even though reliable and reproducible risk stratification is needed for these patients.

We show that mRNA expression of KRT20 is significantly associated with IHC expression of CK20. As IHC analysis is limited by high inter-observer variability which is also a problem in histopathological staging and grading [2], we used KRT20 mRNA expression as an objective and reproducible method for CK20 expression. Survival analysis revealed a statistically significant worse RFS, PFS, and CSS for NMIBC with high KRT20 expression. Furthermore, we show that KRT20 and MKI67 expression are positively correlated. This allows us to conclude that stage pT1 tumors with luminal characteristics, as reflected in high KRT20 expression, have a higher proliferative activity and are more aggressive. This is in contrast to previous reports in which luminal phenotype of MIBC was associated with less aggressive behavior after chemotherapy [11]. However, a recent prospective comprehensive transcriptional study of a large series of NMIBC showed that a subgroup of BC with luminal differentiation has a higher progression rate than basal-like cancers [10]. Our findings are also in line with previous IHC studies indicating that IHC expression of CK20 in NMIBC increases with stage and grade [17]. Alsheik et al. showed that CK20 is expressed in low grade NMIBC but without any correlation between CK20 IHC expression and recurrence [18]. In stage pT1 NMIBC, high CK20 expression by IHC is associated with unfavorable RFS and CSS [19, 20]. Various studies investigated KRT20 mRNA expression in different stages and grades of urothelial carcinoma of the bladder and in other tissues. In one study comparing normal urothelium with bladder cancer tissue, increased KRT20 mRNA expression was found to be associated with higher grade tumor tissue [21]. Two other studies reported an association between KRT20 mRNA expression in peripheral blood and higher stage and worse prognosis [22, 23]. Ribal et al. found KRT20 mRNA expression in lymph nodes of patients after radical cystectomy (RC) to be associated with higher stage and suggested that this might be used as a biomarker [24]. Subsequently, Gazquez et al. showed that KRT20 mRNA expression in lymph nodes of patients with MIBC is a more sensitive marker for the presence of micrometastasis than histologic evaluation, with predictive value for survival [25]. Furthermore, patients with high KRT20 expression in bone marrow prior to RC were found to have worse outcome [26]. Sjødahl et al. found that KRT20 is expressed in genomically unstable high grade tumors [9]. This subgroup shows intermediate disease-specific survival when all stages and grades are taken together [9].

We found in contrast that KRT5 mRNA expression is negatively correlated with MKI67 and KRT20 mRNA expression. The combination of high KRT20 and low KRT5 expression proved to be a statistically significant predictor for worse RFS and PFS. This also contrasts with previous findings in MIBC, of an association of high expression of KRT5 as basal

**Fig. 3** Kaplan-Meier analysis in the total cohort for RFS (a), PFS (b), and CSS (c) based on KRT20 mRNA expression regarding grade 3 subentity in stage T1 bladder cancer



marker with worse outcome after chemotherapy. Sjöhdahl et al. identified a subgroup of patients, with a “Urobasal B” or “SCC-like” tumor with high expression of KRT5 in IHC, that showed the worst disease-specific survival when all stages and grades were taken together [9]. A consensus conference defined the “basal-squamous-like” (BASQ) subtype, which is consistently associated with worse outcome and characterized by the expression of KRT5 [27]. Volkmer et al. postulated three different subtypes that stratify overall survival into a worse (KRT14+, KRT5+, KRT20–), intermediate (KRT14–, KRT5+, KRT20–), and differentiated (KRT14–, KRT5–, KRT20+) risk group [28]. Most studies focus on MIBC and there is the need for more detailed analysis of NMIBC as was stipulated in a recent consensus conference [27].

In our cohort, high expression of KRT20 divided stage pT1G3 tumors into two risk groups in defining a group of

patients with high risk for progression and worse CSS who might benefit from early cystectomy. Earlier studies showed that early cystectomy is associated with improved long-term CSS in the subgroup of high risk T1G3 tumors [29]. As radical cystectomy has a 90-day mortality rate of up to 8%, a bladder preserving approach with BCG maintenance instillation therapy should be kept in mind [2]. To date, several IHC parameters have been evaluated to support risk stratification of this challenging entity, with limited potential [30]. We show that the single assessment of KRT20 mRNA expression is a valuable and simple approach towards reliable stratification of these patients.

The major weakness of the present study is its retrospective nature with data from a single center. These results need to be validated in a prospective multi-center study. We conclude that expression of KRT20 and KRT5 is predictive for recurrence

and progression of stage pT1 NMIBC, reflecting basal and luminal subtypes of MIBC. This information might be used in risk stratification of this difficult entity, i.e., in identifying patients who might benefit from early cystectomy.

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**Compliance with ethical standards** Informed consent was obtained from all the individual participants included in the study. The study was conducted after approval of the local ethics committee.

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**Competing interests** RMW and SE are founders of STRATIFYER Molecular Pathology GmbH. RMW is an employee of STRATIFYER Molecular Pathology GmbH.

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