

RESEARCH ARTICLE

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Decreased expression of RNA-binding motif protein 3 correlates with tumour progression and poor prognosis in urothelial bladder cancer

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Abstract

Background: Low nuclear expression of the RNA-binding motif protein 3 (RBM3) has previously been found to be associated with poor prognosis in several cancer forms e.g. breast, ovarian, colorectal, prostate cancer and malignant melanoma. The aim of this study was to examine the prognostic impact of RBM3 expression in urinary bladder cancer.

Methods: Immunohistochemical RBM3 expression was examined in tumours from 343 patients with urothelial bladder cancer. Chi-square and Spearman's correlation tests were applied to explore associations between RBM3 expression and clinicopathological characteristics. The impact of RBM3 expression on disease-specific survival (DSS), 5-year overall survival (OS) and progression-free survival (PFS) was assessed by Kaplan-Meier analysis and Cox proportional hazards modelling.

Results: Reduced nuclear RBM3 expression was significantly associated with more advanced tumour (T) stage ($p < 0.001$) and high grade tumours ($p = 0.004$). Negative RBM3 expression was associated with a significantly shorter DSS (HR=2.55; 95% CI 1.68-3.86) and 5-year OS (HR=2.10; 95% CI 1.56-2.82), also in multivariable analysis (HR=1.65; 95% CI 1.07-2.53 for DSS and HR=1.54; 95% CI 1.13-2.10 for 5-year OS). In patients with Ta and T1 tumours expressing reduced RBM3 levels, Kaplan-Meier analysis revealed a significantly shorter PFS ($p = 0.048$) and 5-year OS ($p = 0.006$).

Conclusion: Loss of RBM3 expression is associated with clinically more aggressive tumours and an independent factor of poor prognosis in patients with urothelial bladder cancer and a potentially useful biomarker for treatment stratification and surveillance of disease progression.

Keywords: RBM3, Urothelial bladder cancer, Prognosis

Background

Approximately 20% of patients with urothelial carcinoma of the bladder present with muscle invasive cancer [1]. However, the majority of bladder malignancies do not invade muscle at diagnosis (Tis, Ta, and T1). The clinical problem associated with these tumors is their highly unpredictable potential for recurrence and progression into muscle invasive disease [1]. High-grade bladder tumors with lamina propria invasion (T1) represent those at the greatest risk, rendering the surgical

management of this disease subject to much controversy [2]. Nearly one-third of these patients will require cystectomy as a second-line treatment after failure of Bacillus Calmette-Guerin (BCG) treatment [3]. The challenge is to identify these high-risk cases upfront, to offer them cystectomy as primary treatment. For patients with muscle-invasive bladder cancer, cystectomy with pelvic lymph node dissection remains the mainstay of treatment. Overall, approximately 50% of these patients will develop distant metastases after surgical treatment and die of the disease [4]. The prognosis worsens for patients with tumours involving perivesical fat or adjacent organs (cT3b-4) and those with lymph node involvement. In these patients, cystectomy alone offers a cure rate of

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only 20%–30% [5]. Hence, there is a great need for novel prognostic and treatment predictive biomarkers to improve clinical management of patients with urothelial bladder cancer.

Reduced expression of the RNA-binding motif protein 3 (RBM3) has previously been demonstrated to correlate with an impaired prognosis in several major human cancer forms i.e. breast, ovarian, prostate, colorectal cancer and malignant melanoma [6-10]. While the functional basis for these observations remain to be fully elucidated, the observed association between RBM3 expression and DNA integrity and repair [10,11] may be of importance.

In the present study, we examined the prognostic significance of RBM3 expression in tumours from a large prospective cohort of patients with urothelial bladder cancer.

Table 1 Distribution of patient and tumour characteristics in the evaluated cohort (n=343)

Age	
Mean	71.85
Median	73.00
(Range)	31-96
Sex	
Female	83 (24.2)
Male	260 (75.8)
Smoking	
Smoker	130 (79.8)
Non-smoker	33 (20.2)
Unknown	180
T-stage	
pTa	115 (33.5)
pT1	116 (33.8)
pT2	90 (26.2)
pT3	17 (1.5)
pT4	5 (5.0)
Grade	
Low	82 (23.9)
High	261(76.1)
N-stage	
0	43 (78.2)
1	12 (21.8)
unknown	288
M-stage	
0	92 (70.2)
1	39 (29.8)
unknown	212

Methods

Patients

All patients with newly diagnosed urothelial bladder cancer at Uppsala University Hospital have been registered prospectively since 1984. This study included patients diagnosed up until 2005 for whom histological specimens were available. Since the majority of tumours

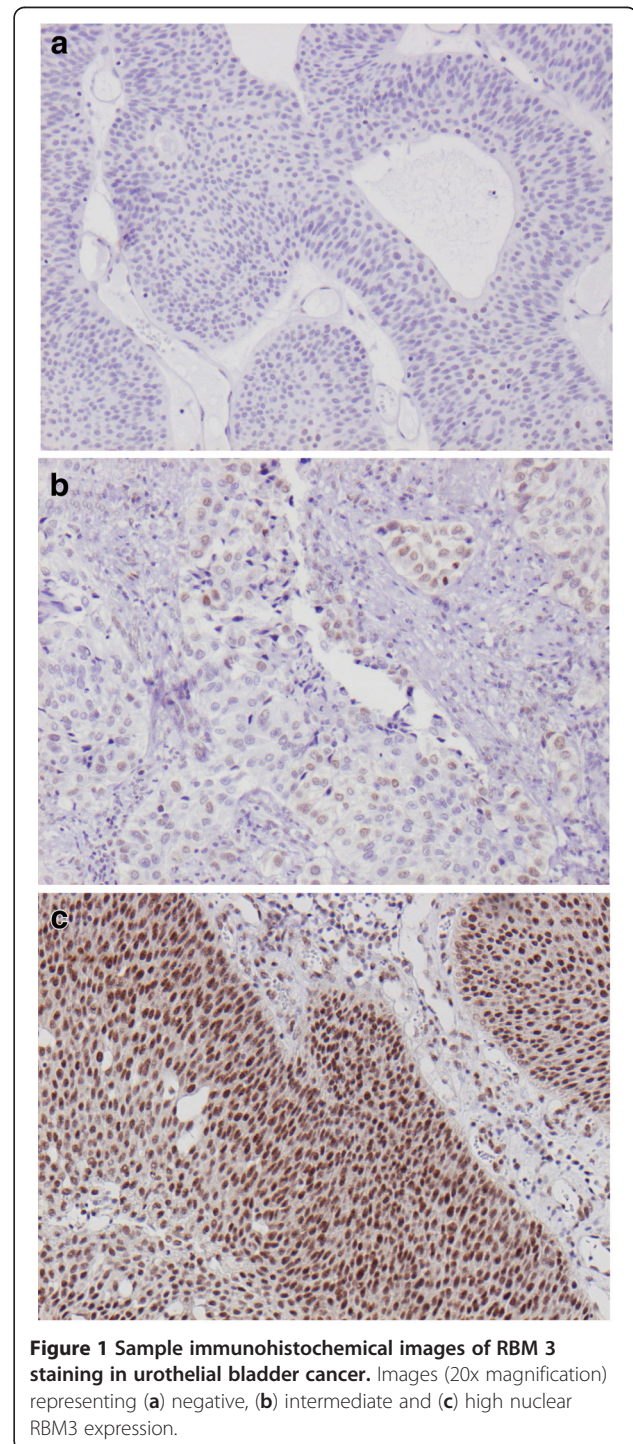


Table 2 Associations between RBM3 expression and clinicopathological characteristics.

RBM3 expression	Negative (NS=0)	Intermediate (NS=1-6)	High(NS=9)	<i>p</i> -value
n(%)	77 (22.4)	213 (62.1)	53 (15.5)	
Age				
≤ average	24 (31.2)	107 (50.2)	27 (50.9)	0.013
>average	53 (68.8)	106 (59.8)	26 (49.1)	
Gender				
Female	26 (33.8)	45 (21.1)	12 (22.6)	0.092
Male	51 (66.2)	168 (78.9)	41 (77.4)	
T-stage				
Ta	18 (23.4)	74 (34.7)	23 (43.4)	<0.001
T1	15 (19.5)	77 (36.2)	24 (45.3)	
T2-4	44 (57.1)	62 (29.1)	6 (11.3)	
Grade				
Low	8 (10.4%)	58 (27.2)	16 (30.2)	0.004
High	69 (89.6%)	155 (72.8)	37 (69.8)	

was made up of Ta tumours, this group was reduced to include 115 tumours. Patient and tumour characteristics are summarised in Table 1. Progression-free survival (PFS), overall survival (OS) and disease-specific survival (DSS) were calculated from the date of surgery to date of event or last follow-up. At follow up, patients with non-muscle invasive tumours were categorized as having none, few or frequent recurrences. Definition of few recurrences was less than three recurrent tumours within 18 months, whereas frequent recurrences were three or more recurrences within the same time period. Progression was defined as shift of the tumor into a higher stage. Median time to progression for patients with non-muscle invasive disease was 18.0 months (range 2.0-55.0). Follow-up time for non-recurrent and non-progressing cases were ≥ 4 and ≥ 5 -years, respectively.

Tissue microarray construction

The use of these patient samples for protein profiling was approved by the regional ethical review board of Uppsala (reference number 2005:339). All tumours were histopathologically re-evaluated and classified according to the WHO grading system of 2004 [12] by a board certified pathologist. Tissue microarrays (TMAs) were constructed using a semi-automated arraying device (TMArrayer, Pathology Devices, Westminster, MD, USA). All tumour samples were represented in duplicate tissue cores (1mm).

Immunohistochemistry and staining evaluation

For immunohistochemical analysis, 4 μ m TMA-sections were automatically pre-treated using the PT Link system and then stained in an Autostainer Plus (DAKO; Glostrup, Copenhagen, Denmark) with the mouse monoclonal anti-

RBM3 antibody AAb030038 (Atlas Antibodies AB, Stockholm, Sweden) diluted 1:10000. The specificity of the antibody has been validated previously [6,10], also in the human bladder cancer cell line RT-4, in which RBM3 was demonstrated to be highly expressed [6]. RBM3 staining was evaluated by two independent observers (KB and KJ) who were blinded to clinical and outcome data. RBM3 was mainly expressed in the nuclei and the fraction of cells with nuclear positivity (NF) denoted as 0 (0-1%), 1 (2-25%), 2 (26-75%), 3 (>75%), and the intensity of staining (NI) as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The fraction of positively staining cells was estimated across both sampled cores, and the dominating staining intensity denoted. A combined nuclear score (NS), e.g. multiplier of NF \times NI, was then constructed as previously described [9,10].

Statistics

Spearman's rho and Chi-square tests were applied for analysis of the correlation between RBM3 expression and clinicopathological characteristics. Kaplan-Meier analysis and log rank test were used to illustrate differences in progression free survival (PFS), disease-specific survival (DSS), overall survival (OS) and 5-year OS in strata according to RBM3 expression. For survival analyses, RBM3 expression was trichotomized into negative (NS=0), intermediate (NS=1-6) and high (NS=9), or dichotomised into negative (NS=0) vs positive (NS ≥ 1) or negative-intermediate (NS 0-6) versus high (NS=9). Cox regression proportional hazards modelling was used to estimate the impact of negative vs positive RBM3 expression on DSS, 5-year OS, PFS in both univariable and multivariable analysis, adjusted for age, sex, T-stage and grade. All tests were two sided. A *p*-value of 0.05 was

considered significant. All statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Associations between RBM3 expression, patient and tumour characteristics

Following antibody optimisation and staining, RBM3 expression could be evaluated in tumours from 343/344 (99.7%) cases. There was no obvious heterogeneity in RBM3 expression between duplicate TMA cores. Negative RBM3 staining (NS=0) was denoted in 77 (22.4%) cases, weak-strong intensity in <75% of the cells (NS 1–6) in 213 (62.1%) cases and strong staining in > 75% of the cells (NS=9) was denoted in 53 (15.5%) cases. Heterogeneity regarding the nuclear staining fraction between duplicate cores was only observed in some cases with intermediate RBM3 expression, and never exceeding 1 category. Images representing different patterns of RBM expression are shown in Figure 1a-c.

We examined the relationship between three categories of RBM3 expression; negative (NS=0), intermediate (NS=1-6) and high (NS=9), and established clinicopathological parameters (Table 2). This revealed a significant positive association between RBM3 expression and a younger age at diagnosis ($p=0.013$), lower T-stage ($p<0.001$) and high grade tumours ($p=0.004$).

Association between RBM3 expression and survival in the full cohort

Kaplan-Meier analysis and log-rank test revealed a step-wise reduced DSS (Figure 2a) and 5-year OS (Figure 2b) with decreasing RBM3 expression, with the shortest DSS ($p<0.001$) and 5-year OS ($p<0.001$) for patients with tumours lacking RBM3 expression. These associations were confirmed in Cox univariable analysis (HR=2.55; 95% CI 1.68-3.86 for DSS and HR=2.10; 95% CI 1.56-2.82 for 5-year OS) and remained significant in multivariable analysis adjusted for age, gender, T-stage and grade (HR=1.65; 95% CI 1.07-2.53 for DSS and HR=1.54; 95% CI 1.13-2.10 for 5-year OS) (Table 3). Separate analysis of categories of nuclear fraction and staining intensity yielded similar results for DSS and 5-year OS (Additional file 1).

Association between RBM3 expression, disease progression and survival in patients with Ta and T1 tumours

Next, we examined the association between RBM3 expression, disease progression within 24 months and 5-year OS in patients with Ta and T1 tumours. Kaplan-Meier analysis using three categories of RBM3 expression revealed a significantly reduced PFS for patients with RBM3 negative tumours compared to tumours with

high RBM3 expression ($p=0.030$). A similarly reduced, borderline significant, PFS was demonstrated for tumours with intermediate RBM3 expression (Figure 3a), and when a dichotomised variable of high versus negative/intermediate RBM3 expression was applied, a significantly reduced PFS was seen for the latter category ($p=0.048$, Figure 3b). Analysis of 5-year OS using the trichotomised variable of RBM3 expression (Figure 3c)

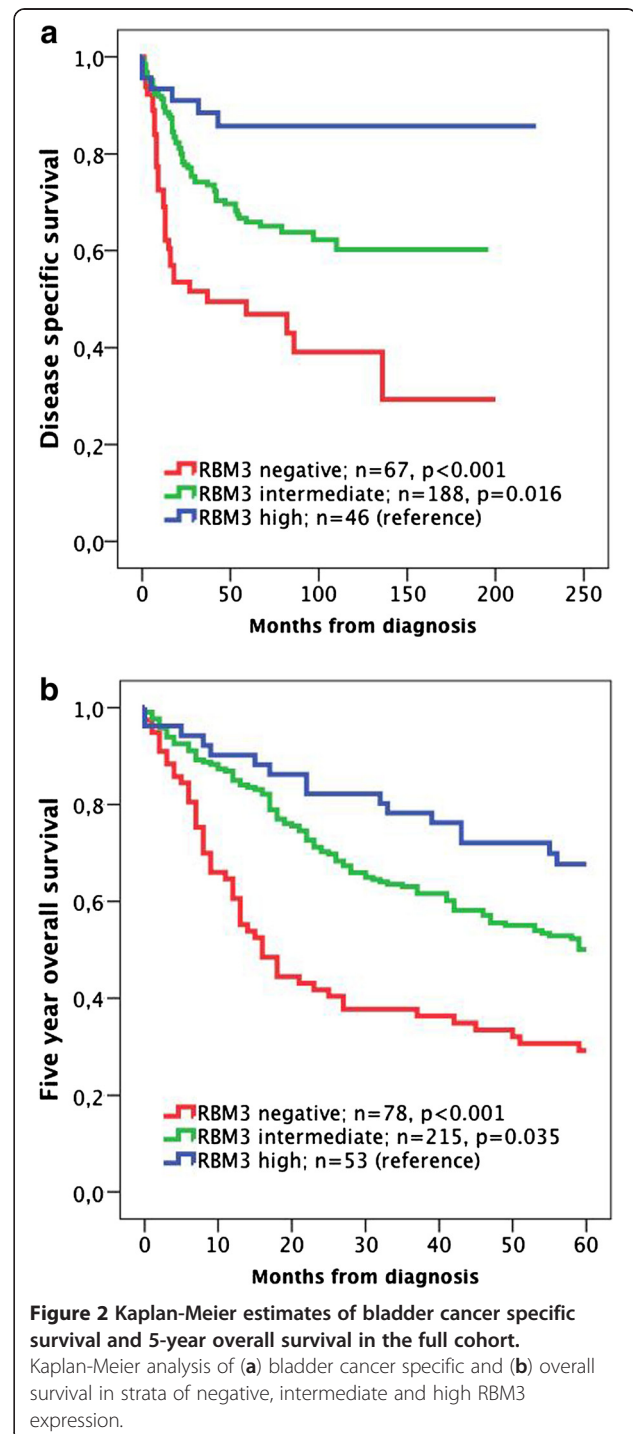


Table 3 Relative risks of death from disease and overall death within 5 years according to clinicopathological factors and RBM3 expression in all patients (n=343)

	n (events)	Risk of death from disease		n (events)	Risk of death within 5 years	
		Univariable HR (95% CI)	Multivariable HR (95% CI)		Univariable HR (95% CI)	Multivariable HR (95% CI)
Age						
Continuous	299 (100)	1.05 (1.03-1.07)	1.04 (1.02-1.07)	343 (123)	1.07 (1.05-1.08)	1.07 (1.05-1.08)
Gender						
Female	72 (28)	1.00	1.00	83 (56)	1.00	1.00
Male	227 (72)	0.79 (0.51-1.23)	1.07 (0.68-1.67)	260 (167)	0.95 (0.70-1.29)	1.24 (0.91-1.70)
Stage						
Ta	104 (13)	1.00	1.00	115 (61)	1.00	1.00
T1	97 (25)	2.20 (1.13-4.31)	1.87 (0.85-4.08)	116 (71)	1.37 (0.97-1.94)	1.44 (0.96-2.15)
T2-4	99 (62)	8.86 (4.86-16.16)	6.77 (3.21-14.26)	112 (91)	2.79 (2.01-3.87)	2.90 (1.92-4.40)
Grade						
Low	75 (7)	1.00	1.00	82 (41)	1.00	1.00
High	224 (93)	5.75 (2.66-12.40)	1.52 (1.58-3.96)	261 (182)	2.04 (1.45-2.87)	0.90 (0.58-1.39)
RBM3 expression						
Positive	232 (66)	1.00	1.00	266 (160)	1.00	1.00
Negative	67 (34)	2.55 (1.68-3.86)	1.65 (1.07-2.53)	77 (63)	2.10 (1.56-2.82)	1.54 (1.13—2.10)

revealed a significantly reduced survival for patients with RBM3 negative tumours compared to those with high expression ($p=0.005$) while survival was similar for the intermediate category for RBM3 expression, as also demonstrated using a dichotomised variable comparing negative RBM3 expression with any RBM3 expression ($p=0.006$, Figure 3d).

Cox regression analysis demonstrated that neither RBM3 expression nor any of the established clinicopathological factors were significantly associated with PFS, but that grade and RBM3 expression (negative-intermediate vs high) were the best predictors of disease progression within 24 months in both univariable and multivariable analysis (Table 4). However, a significantly shorter 5-year OS was seen for RBM3 negative compared to RBM3 positive tumours in univariable analysis ($n=231$; HR=2.00 95% CI 1.21-3.33) and borderline significant in multivariable analysis (HR=1.64 95% CI 0.96-2.78) (Table 4). There was a borderline significant association between RBM3 expression and DSS in both univariable analysis ($n=201$; HR=3.06 95% CI 0.95-10.00) and multivariable analysis ($n=201$; HR=3.24 95% CI 1.00-10.53). There was no significant association between RBM3 expression and the rate of recurrence (none, few or frequent) (data not shown).

Similar findings were seen in subgroup analysis of patients with T1 tumours, where none of the patients with tumours expressing high levels of RBM3 ($n=14$) had disease progression during 24 months follow-up, in contrast to tumours with intermediate and negative expression ($n=52$, 12 events) (data not shown). In patients with T1 tumours, a

significant correlation was seen between negative RBM3 expression and a reduced 5-year OS in both univariable analysis ($n=116$; HR=2.44 95% CI 1.25-4.75) and multivariable analysis (HR=1.98 95% CI 1.01-3.90).

Discussion

The results from this study show that reduced RBM3 expression is significantly associated with more aggressive tumours and an independent predictor of reduced survival in patients with urothelial bladder cancer. Moreover, in patients with Ta and T1 tumours, reduced RBM3 expression correlated with a significantly shorter time to disease progression, despite the comparatively low number of events, and negative RBM3 expression was an independent predictor of a reduced 5-year overall survival. The association between RBM3 expression and a favorable clinical outcome has been demonstrated in several other cancer forms [6-10] but this is, to our knowledge, the first report of the prognostic impact of RBM3 expression in urothelial bladder cancer.

In this comparatively large study of retrospectively collected tumours from a prospective cohort of patients with urothelial bladder cancer, the most evident impact of RBM3 expression was seen for disease specific and overall survival, both in the full cohort and in subgroup analysis of patients with Ta and T1 tumours. The fact that the impact on progression-free survival in patients with Ta and T1 tumours was not as significant may be due to the lower number of events, and therefore, the association between RBM3 expression and tumour progression merits

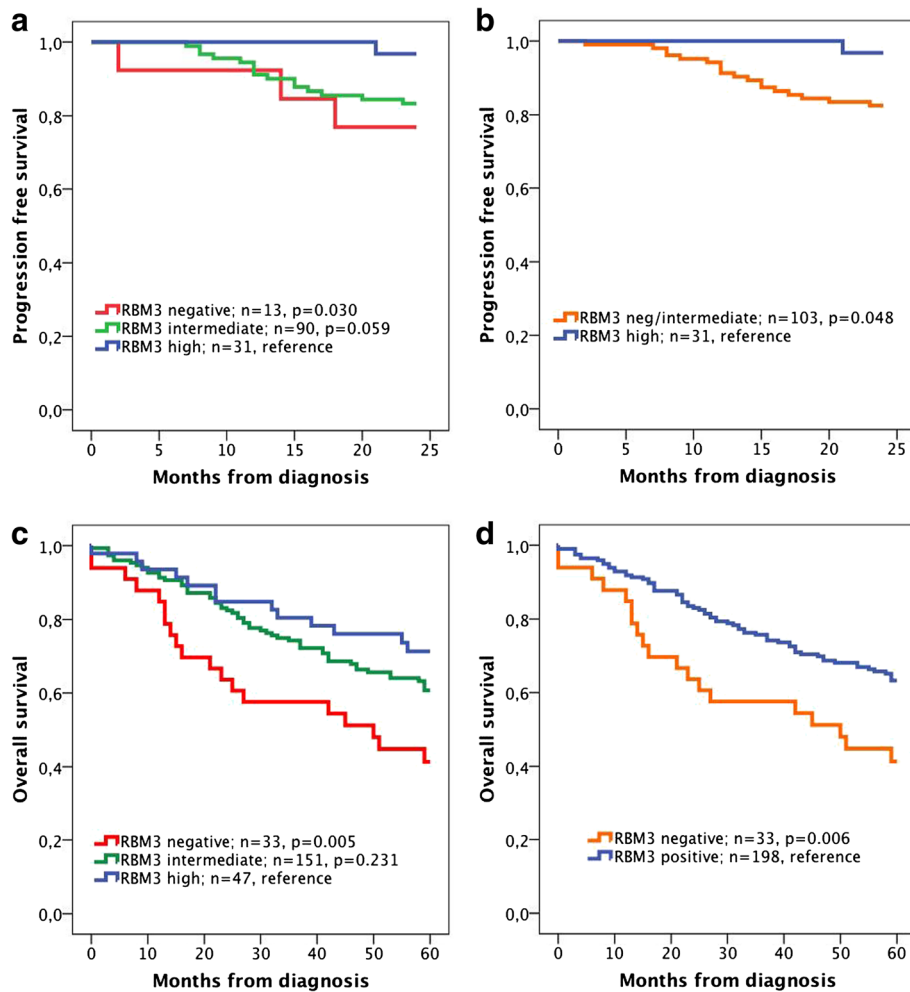


Figure 3 Kaplan-Meier estimates of progression free survival and 5-year overall survival in patients with Ta and T1 tumours. Kaplan-Meier analysis of progression free survival in strata according to (a) negative, intermediate and high RBM3 expression and (b) negative-intermediate versus high expression, and 5-year overall survival in (c) negative, intermediate and high RBM3 expression and (d) negative versus positive expression.

further validation in other cohorts. Notably, RBM3 expression was not associated with neither occurrence nor frequency of local recurrence. These findings may indeed indicate different, or even opposing, tumour biological roles for RBM3 in tumour initiation and progression of urothelial bladder cancer. Previous studies, e.g. in malignant melanoma, have demonstrated that the correlation between reduced RBM3 expression and overall survival was more evident than the correlation to recurrent disease [8]. Speculatively, these findings could indicate that loss of RBM3 expression results in a tumour phenotype more prone to metastatic spread than local aggressiveness. RBM3 has previously been shown to be upregulated in neoplastic as compared to normal tissue [7,9], which also seems to be true for urothelial bladder epithelium versus urothelial neoplasms (www.proteinatlas.org). While urothelial bladder cancer is in its nature a recurrent

disease [1], a recurrence does not per se affect survival from the disease. It is therefore plausible to assume that tumours with high RBM3 expression may well recur, while loss of RBM3 expression will generate a disease more likely to muscle invasion and distant spread.

The precise functional mechanisms behind loss of RBM3 and a more aggressive tumour behaviour remain to be elucidated. However, in vitro models need to be designed and interpreted with the impact of RBM3 expression on clinical outcome in mind, and the previously demonstrated pro-tumourigenic properties for RBM3 in vitro, e.g. that siRNA mediated silencing of the gene renders cancer cells less proliferative [10,13,14], should not be interpreted as being contradictory to its association with a prolonged survival when expressed in human tumours.

In a translational context, further probing of the association between RBM3 and DNA integrity and repair,

Table 4 Relative risks of progression within 24 months and overall death within 5 years according to clinicopathological factors and RBM3 expression in patients with Ta and T1 tumours

	n(events)	Risk of progression within 24 months		n(events)	Risk of death within 5 years	
		Univariable HR(95%CI)	Multivariable HR(95%CI)		Univariable HR(95%CI)	Multivariable HR(95%CI)
Age						
Continuous	134(19)	1.02(0.98-1.07)	1.01(0.97-1.06)	231(88)	1.06(1.04-1.09)	1.06(1.04-1.08)
Gender						
Female	29(2)	1.00	1.00	43(12)	1.00	1.00
Male	105(17)	2.48(0.57-10.74)	1.98(0.45-8.64)	188(76)	0.62(0.34-1.13)	1.67(0.90-3.10)
Grade						
Low	48(3)	1.00	1.00	81(19)	1.00	1.00
High	86(16)	3.24(0.94-11.12)	3.32(0.97-11.40)	150(69)	2.22(1.33-3.68)	1.31(0.73-2.35)
Stage						
Ta	68(7)	1.00	1.00	115(35)	1.00	1.00
T1	66(12)	1.90(0.75-4.83)	1.24(0.44-3.46)	116(53)	0.61(0.40-0.93)	1.61(1.04-2.49)
RBM3 expression*						
High	31(1)	1.00	1.00	Positive	198(69)	1.00
Negative/Intermediate	103(18)	5.92(0.79-44.39)	6.10(0.81-45.69)	Negative	33(19)	2.00(1.21-3.33)

For PFS a dichotomised variable comparing patients with negative or intermediate RBM3 expression to those with high expression was applied. For 5 year OS, a dichotomised variable of negative versus any RBM3 expression was applied.

that has been demonstrated in epithelial ovarian cancer in vivo and in vitro [11], may give more insight into the mechanistic basis for the favourable prognostic impact of RBM3 expression, once a tumour has been established. The link between RBM3 expression and an attenuated DNA damage response not only fits with its previously demonstrated association with cisplatin sensitivity, but also its association with good prognosis, since a deficient DNA repair system may well decrease the capability of invasion and metastatic spread [15,16]. Along this line, it would also be of interest to investigate the impact of RBM3 as a predictor of response to platinum-based chemotherapy, both in the neoadjuvant, adjuvant and palliative setting, cisplatin being one of the cornerstones in the medical treatment of bladder cancer [17-20]. Few molecular markers have proven to be efficient predictors of treatment response, and none have been incorporated into clinical protocols. The question of the role of RBM3 as a marker for platinum sensitivity could not be addressed in the present study, as treatment data was lacking for the majority of the patients.

The clinical management of urothelial bladder cancer holds room for improvement. The monitoring is not only troublesome for the patients, but is also costly for the health care system. In fact, due to its high rate of recurrence and invasive monitoring requirements, urothelial bladder cancer has the highest lifetime treatment cost per patient of all cancers in the United States [21]. Hence, the benefit of finding tools to better identify patients more likely to have, or being at risk of developing, muscle

invasive and metastatic disease is evident. Here, we have demonstrated a stepwise reduced survival with decreasing levels of nuclear RBM3 expression. While the greatest impact on DSS and OS was seen for patients with tumours lacking RBM3 expression, an increased risk of disease progression was also observed in the patient group with intermediate expression. This observation, together with the strong correlation between loss of RBM3 and muscle invasive disease, indicates that loss of RBM3 expression may indeed be a marker of disease progression. Therefore, immunohistochemical assessment of RBM3 expression could prove to be a valuable tool to more accurately predict muscle invasion, even in suboptimal samples from transurethral resections of the bladder. Moreover, it would be of interest to evaluate whether immunocytochemical RBM3 expression could be a useful diagnostic tool for urine cytology samples, to distinguish between different low-grade urothelial neoplastic lesions, e.g. papillary neoplasms of low malignant potential and low-grade papillary urothelial carcinomas, entities with considerable cytologic-histologic discrepancies [22]. While the findings in the present study are based on analyses of transitional cell carcinoma, it would also be of interest to examine RBM3 expression and its possible prognostic implications in other histological subtypes of bladder cancer, e.g. squamous cell carcinoma or adenocarcinoma.

Conclusions

The results from this study suggest that immunohistochemical evaluation of RBM3 expression in urothelial

bladder cancer specimens may be a valuable diagnostic and prognostic biomarker, and a useful tool for improved treatment stratification of patients suffering from the disease.

Additional file

Additional file 1: Prognostic value of categories of nuclear fraction and intensity of RBM3 staining. Kaplan-Meier analysis of (A, B) bladder cancer specific and (C, D) overall survival according to the nuclear fraction and intensity of RBM3 staining, respectively (logrank p over strata <0.001 for all).

Abbreviations

RBM3: RNA-binding motif protein 3; DSS: Disease-specific survival; OS: Overall survival; PFS: Progression-free survival, NS, Nuclear score; TMA: Tissue microarray; HR: Hazard ratio; CI: Confidence interval.

Competing interests

Jakob Eberhard and Karin Jirstrom hold pending intellectual property in relation to RBM3 as a prognostic biomarker in the treatment of bladder cancer. The remaining authors declare that they have no competing interests.

Authors' contributions

KB evaluated the immunohistochemical stainings, performed the statistical analyses and drafted the manuscript, US collected clinical data, assisted with the statistical analysis and helped draft the manuscript, GA assisted with the statistical analysis and helped draft the manuscript, JE contributed with the conception and design of the study, MU contributed with antibody validation, KJ conceived of the study, evaluated the immunohistochemistry, and helped draft the manuscript, PUM is responsible for the patient cohort and clinical database, and helped draft the manuscript. All authors read and approved the final manuscript.

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