BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Multicenter Validation of Cyclin D1, MCM7, TRIM29, and UBE2C as Prognostic Protein Markers in Non-Muscle—Invasive Bladder Cancer

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Transcripts from the four genes encoding cyclin D1, MCM7, TRIM29, and UBE2C have previously been included in gene expression signatures for outcome prediction in stage Ta/T1 urothelial carcinomas. We investigated the prognostic value of the protein expressions in Ta/T1 urothelial carcinomas patients. We used four different tissue microarrays (TMAs) with a total of 859 Ta/T1 urothelial carcinomas from Danish, Swedish, Spanish, and Taiwanese patient cohorts with long-term follow-up. Protein expression was measured by IHC, and antibody specificity was validated by Western blotting. We found the expression of cyclin D1, MCM7, TRIM29, and UBE2C to be significantly associated with progression to muscle-invasive bladder cancer (log-rank test; $P < 0.001$) in the Danish training cohort ($n = 283$). Multivariate Cox regression analysis identified cyclin D1 ($P = 0.003$), TRIM29 ($P = 0.001$), and UBE2C ($P < 0.001$) as independent prognostic markers. The prognostic value of the four proteins was validated in a joint validation cohort from Sweden, Spain, and Taiwan ($n = 576$). Computer-assisted image analysis of the prognostic markers produced results comparable to those obtained by manual scoring. Finally, a four-protein maximum-likelihood classifier was trained on the Danish training cohort and applied to the validation cohort. The four protein markers may help optimize treatment of patients with Ta/T1 bladder cancer. Additional prospective studies are needed for further validation of their clinical relevance. (*Am J Pathol* 2013; 182: 339–349; <http://dx.doi.org/10.1016/j.ajpath.2012.10.017>)

Bladder cancer is the fifth most common cancer in the Western world. The United States alone will experience 73,500 newly diagnosed cases and up to 15,000 deaths due to bladder cancer in 2012.¹ For almost 75% of the patients diagnosed as having bladder cancer, the disease will present as non-muscle—invasive bladder cancer (NMIBC). NMIBC is a prevalent disease, with approximately 60% of patients experiencing one or more recurrences after primary resection.² The non-muscle-invasive tumors of the bladder form a heterogeneous group, spanning from noninvasive papillary tumors with low-grade dysplasia to superficially invasive tumors with high-grade dysplasia and concomitant carcinoma *in situ*. In the latter group, the risk of disease progression to a muscle-invasive stage reaches 60% at

long-term follow-up.^{3,4} The histopathologic parameters primarily used in the clinic today (eg, stage and grade) cannot precisely predict the individual disease course, and no molecular markers for predicting tumor recurrence or later progression are currently in clinical use.⁵ Bladder cancer patients are monitored thoroughly for disease recurrence and progression by urine and cystoscopy examinations. This involves a heavy cost to society, and bladder

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cancer carries the highest cost among cancers per patient from diagnosis to death.⁶

The European Organization for Research and Treatment of Cancer (EORTC) risk tables for estimation of the risk of recurrence and progression have been increasingly used in the clinic in the past years.⁷ These tables are based on several clinical and histopathologic risk factors; however, molecular markers may carry the potential to add further prognostic value to these risk tables and consequently enhance our ability to individualize treatment.⁸ Our group has previously identified and validated a gene expression signature that successfully predicts disease progression to muscle-invasive bladder cancer (MIBC).^{9,10} We have recently validated the prognostic value of cathepsin E, maspin, PLK1, and survivin protein expression. The transcripts encoding these proteins were all included in the gene expression signature.¹¹

In this study, we investigated the prognostic value of the protein expression of four differentially expressed transcripts from the expression signature (ie, CCND1, MCM7, TRIM29, and UBE2C) in a Danish cohort of 283 patients with NMIBC and long-term follow-up. The protein expression was then validated using three cohorts of 576 patients with NMIBC from Sweden, Spain, and Taiwan. Finally, we developed image analysis protocols for computer-assisted scoring of the four proteins, validated the prognostic potential of the markers using this method, and constructed a molecular four-protein classifier for outcome prediction using the computer-assisted scoring.

Materials and Methods

Patient Cohorts and Tumor Material

All Danish patients gave their written informed consent, and the study was approved by the Central Denmark Region Committees on Biomedical Research Ethics (1994/2920). The 283 patients with primary bladder cancer selected for this retrospective study debuted with non-muscle-invasive urothelial bladder tumors between 1979 and 2007. They were treated at Aarhus University Hospital, Aarhus, Denmark. Biopsy specimens from each of the tumors were placed on a TMA with 0.6-mm core biopsy specimens.¹¹

All Swedish patients gave their written informed consent, and the use of the patient samples for this study was approved by the Regional Ethical Review Board of Uppsala (2005:339). Primary tumors from 141 patients were positioned on TMAs with 1-mm core biopsy specimens in duplicates. The patients were treated between 1984 and 2005 at Uppsala University Hospital, Uppsala, Sweden.

All Swedish patients gave their written informed consent (ethical approval: SAF2009-13035). Primary tumors from 194 patients were positioned on TMAs with 1-mm core biopsy specimens in triplicates. The patients were treated between 1994 and 2008 at Hospital Central de Asturias.

All Taiwanese patients gave their written informed consent (institutional review board certification of approval

V100-C009). Tumors from 241 patients were positioned on TMAs with 2-mm core biopsy specimens. The patients were treated between 1991 and 2005 at Taipei Veterans General Hospital. For further clinical and histopathologic characteristics of the four patient cohorts, see [Table 1](#).

Patient Follow-Up

Patients were followed by control cystoscopy performed according to a routine schedule. Follow-up was censored at the time of the most recent cystoscopy. The follow-up included progression-free survival (PFS), disease-specific survival, and overall survival. Tumor progression was defined as progression to stage T2 to T4, which was verified by pathologic examination and measured from the time of surgery to the time of proven event. Patients were censored due to death or discontinued follow-up after 5 years without disease recurrences.

IHC and TMA Scoring

All antibodies were tested in dilution series on a test TMA, holding 1-mm tumor cores from 60 Ta, T1, and T2 urothelial bladder cancers constructed for the purpose. The dilution retrieving the most specific staining with each antibody was used in the immunohistochemistry (IHC) of the four study cohorts. To control for differences in staining intensities among the different patient cohorts, a slide from the test TMA was stained as a control in all IHC procedures.

After blocking the endogenous peroxidase with hydrogen peroxide for 10 minutes, the cores were demasked with heat-induced epitope retrieval. Nonspecific binding was blocked with 1% bovine serum albumin (Albumin Fraction V, AppliChem, Darmstadt, Germany). The TMA slides were incubated overnight at 4°C with the primary antibody. SignalStain Boost IHC Detection Reagent (8114) (Cell Signaling Technology, Beverly, MA) was used for G1/S-specific cyclin D1 (cyclin D1) antibody. The DakoEnVision visualization system (Dako Denmark A/S, Glostrup, Denmark) was used to visualize the antigen through a chromogen reaction. IHC was performed essentially as described previously,¹² using rabbit polyclonal antibody to cyclin D1 (ab16663; 1:150) (Abcam Inc., Cambridge, UK), mouse monoclonal antibody to mini-chromosome maintenance complex component 7 (MCM7) (ab2360; 1:1200) (Abcam Inc.), goat polyclonal antibody to tripartite motif-containing protein 29 (TRIM29) (sc-1614; 1:1200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and mouse monoclonal antibody to ubiquitin-conjugating enzyme E2C (UBE2C) (H00011065-M0; 1:600) (Abnova, Taipei City, Taiwan). All IHC analyses were performed in the same laboratory (Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark). All TMA slides were scanned using a Hamamatsu Nanozoomer scanner (Hamamatsu Corporation, Hamamatsu City, Japan). The high-resolution images were scored manually using VIS visualization software version 4.3.1.0 (Visiopharm A/S, Hørsholm, Denmark). The IHC stains were scored by two independent observers

Table 1 Clinical and Histopathologic Characteristics of All Cohorts

Characteristic	Danish training cohort	Swedish cohort	Spanish cohort	Taiwanese cohort	Joint validation cohort
No. of patients	283	141	194	241	576
Follow-up time for all patients, median (range), months	103 (2–263)	72 (1–193)	97 (5–216)	78 (4–241)	80 (1–241)
Follow-up time for progressing patients, median (range), months	22 (1–210)	21 (1–96)	18 (3–125)	16 (4–199)	17 (1–199)
Follow-up time for nonprogressing patients, median (range), months	117 (53–250)	80 (60–193)	104 (60–205)	89 (60–241)	95 (60–241)
Age, median (range), years	68 (32–86)	70 (31–96)	69 (28–89)	72 (23–90)	71 (23–96)
Male-female ratio	3.64	3.90	5.69	5.69	5.13
Stage, No. (%)					
Ta	182 (64)	67 (48)	21 (11)	134 (56)	222 (39)
T1	101 (36)	74 (52)	173 (89)	107 (44)	354 (61)
WHO grade 2004					
Low-grade+ PUNLMP	183 (65)	47 (33)	103 (53)	106 (44)	256 (44)
High-grade	100 (35)	94 (67)	91 (47)	135 (56)	320 (56)
Tumor size, No. (%)					
<3 cm	177 (63)	55 (39)	139 (72)	156 (65)	350 (61)
≥3 cm	72 (25)	84 (60)	55 (28)	85 (35)	224 (39)
Unspecified	34 (12)	2 (1)	0	0	2
Growth pattern					
Papillary	248 (88)	114 (81)	177 (91)	225 (93)	516 (90)
Solid	20 (7)	25 (18)	17 (9)	16 (7)	58 (10)
Mixed	14 (5)	2 (1)	0	0	2
Multiplicity					
One	169 (60)	111 (79)	127 (65)	133 (55)	371 (64)
More	87 (31)	30 (21)	64 (33)	108 (45)	202 (35)
Unspecified	27 (9)	0	3 (2)	0	3 (1)
Concomitant CIS					
Yes	95 (34)	NA	26 (13)	11 (5)	37 (6)
No	185 (65)	NA	168 (87)	230 (95)	398 (69)
Unspecified	3 (1)	NA	0	0	141 (25)
Adjuvant therapy					
Yes	70 (25)	43 (30)	118 (61)	185 (77)	346 (60)
No	213 (75)	40 (28)	76 (39)	56 (23)	172 (30)
Unspecified	0	58 (42)	0	0	58 (10)
No. of progression events to stage pT2–4 bladder cancer					
All	113 (40)	38 (27)	48 (25)	59 (24)	145 (25)
Ta	54 (30)	10 (15)	0	13 (10)	23 (10)
T1	59 (58)	28 (38)	48 (28)	46 (43)	122 (34)

CIS, carcinoma *in situ*; NA, not applicable; PUNLMP, papillary urothelial neoplasm of low malignant potential; WHO, World Health Organization.

blinded to clinical outcome. In case of interobserver disagreement, a consensus scoring was obtained. For each tumor, the intensity of the stained carcinoma cells (nucleus, cytoplasm, or membrane) was scored as none (0), low (1), or high (2). Furthermore, the percentage of carcinoma cells carrying this particular intensity was also scored. On the basis of this information, we used receiver operating characteristic curves for identifying the optimal cut points.¹³ We defined the low-expression category as only including a vague or diffuse staining, whereas the high-expression category contained the surely positive and high-intensity staining. The definition of the categories was initially determined for each protein by inspecting a large number of tissue cores on the Danish TMA. All cut points

were defined in the Danish patient cohort (training set) and directly applied to the Swedish, Spanish, and Taiwanese patient cohort (validation set). Dichotomization is as follows: cyclin D1: positive nuclear expression (intensity 1 and 2), 20% cut point; MCM7: high nuclear expression (intensity 2), 30% cut point; TRIM29: positive cytoplasmic and/or membranous expression (intensity 1 and 2), 80% cut point; and UBE2C: high nuclear expression (intensity 2), 8% cut point.

Image Analysis Protocols

Image analysis protocols for each of the four proteins for computer-assisted image analysis of the cohorts were developed

using Visiormorph and Tissuemorph software (Visiopharm A/S). First, the tumor tissue region of interest was outlined manually; hence, only the carcinoma cells were subjected to the computer-assisted image analysis ([Supplemental Figure S1](#)). The analysis protocols were tested using the Danish patient cohort (adjusting sensitivity and specificity for each protocol and selecting the optimal cut point for each protein) and then directly applied to the Swedish, Spanish, and Taiwanese patient cohorts for validation.

Western Blotting

The specificity of the antibodies raised against cyclin D1, MCM7, TRIM29, and UBE2C was validated by 4% to 12% SDS-PAGE and Western blotting of extracts from the human cell line Hela S3 and the bladder cancer cell line SW780. Because of a low expression of TRIM29 in the cell lines, extracts from a corresponding fresh frozen bladder cancer sample and HEK 293 cells transfected with a pcDNA3.1/V5-His-TOPO vector with a TRIM29 coding region (Life Technologies Corporation, Carlsbad, CA) were used. Primary antibody dilutions used for Western blotting were as follows: cyclin D1, 1:400; MCM7, 1:500; TRIM29, 1:50; and UBE2C, 1:2000. Secondary antibodies used were as follows: swine anti-rabbit horseradish peroxidase conjugated (P0217), 1:3000; goat anti-mouse horseradish peroxidase conjugated (P0447), 1:3000; and rabbit anti-goat horseradish peroxidase conjugated (P0449), 1:3000 (all Dako Cytomation A/S, Glostrup, Denmark). Loading controls used were as follows: polyclonal antibody to β -actin horseradish peroxidase conjugated (ab49900), 1:30,000 (Abcam Inc.); and monoclonal antibody to α -tubulin (ab7291), 1:5000 (Abcam Inc.). Protein extraction was performed in radioimmunoprecipitation assay buffer added to complete ULTRA Protease Inhibitor (catalog no. 05 892 791 001) and PhosSTOP Phosphatase Inhibitor Cocktail (catalog no. 04 906 845 001) (both Roche Diagnostics, Basel, Switzerland). Protein concentration was determined following a modified Bradford protocol (Bio-Rad Laboratories Inc., Berkeley, CA). Each lane was loaded with 25 μ g of protein except for the TRIM29 overexpressed clone for which 2.5 μ g were loaded. Western blotting was performed essentially as previously described¹⁴ ([Supplemental Figure S2](#)).

Statistical Analysis

Stata statistical analysis software version 10.0 (Stata Corporation, College Station, TX) was used for calculation of log-rank tests for equality of survival function, Kaplan-Meier survival plots, and univariate and multivariate Cox regression analysis. Variables with a $P < 0.05$ in the univariate analysis were included in the multivariate analysis to identify variables with independent significance. All reported P values were two-sided. The predictive accuracy of prognostic models was quantified with Harrell's Concordance Index. The assumptions of proportional hazards were verified. The maximum likelihood classifier was trained and tested as previously

described.¹⁵ Data were mean centered and normalized before classifier analysis by GeneCluster version 3.0 (Broad Institute, Cambridge, MA). Java TreeView software version 1.1.3 was used for visualization of the data.¹⁶

Results

The median follow-up was 103 months (range, 2 to 263 months) in the Danish training cohort and 80 months (range, 1 to 241 months) in the joint validation cohort. Progression to MIBC was experienced by 113 patients (40%) in the training cohort and by 145 patients (25%) in the validation cohort. Clinical and histopathologic data for the Danish, Swedish, Spanish, and Taiwanese cohorts are presented in [Table 1](#).

Expression of Cyclin D1, MCM7, TRIM29, and UBE2C

For all four proteins examined, we started by scoring the nuclear, the cytoplasmic, and the membranous staining, if any of these were present. The staining with the cyclin D1 antibody was generally confined to the carcinoma cells. Only a few nuclei stained positive in the adjacent tissue. In the carcinoma cells, the staining with the cyclin D1 antibody was predominantly seen in the nuclei. Occasionally, a little cyclin D1 staining was seen in the cytoplasm. We evaluated the nuclear expression of cyclin D1. We exclusively found the staining with the antibody against MCM7 in the nuclei of the carcinoma cells, and minimal staining was observed in the adjacent tissue. No cytoplasm of the carcinoma cells carried MCM7 staining. We evaluated the nuclear expression of MCM7. The staining with the TRIM29 antibody was exclusively localized to the carcinoma cells. TRIM29 staining was predominantly found in the cytoplasm or membrane of the tumor cells. Nuclear staining was seen only seldomly. We evaluated a combined measure of the cytoplasmic and membranous expression of TRIM29. The staining with the UBE2C antibody was exclusively localized to the carcinoma cells, and the staining was expressed in both the nucleus and the cytoplasm of the carcinoma cells in a positive tumor cell. Therefore, we evaluated a combined measure of the cytoplasmic and nuclear expression of UBE2C ([Supplemental Figure S3](#)).

The manual IHC scoring resulted in the following inter-observer statistics for the Danish, Swedish, Spanish, and Taiwanese cohorts, respectively (free marginal κ): cyclin D1, 0.93, 0.89, 0.81, and 0.84; MCM7, 0.85, 0.72, 0.77, and 0.71; TRIM29, 0.83, 0.73, 0.81, and 0.71; and UBE2C, 0.86, 0.83, 0.71, and 0.84.

Prognostic Value of the Markers in the Danish Training Cohort

The expression of cyclin D1, MCM7, TRIM29, and UBE2C was found to be significantly associated with progression to MIBC (for each marker: log-rank test; $P < 0.001$) ([Figure 1](#)). Stratifying for age, stage, grade, growth pattern, and adjuvant treatment in a multivariate Cox regression analysis, we identified cyclin D1 ($P = 0.003$), TRIM29 ($P = 0.023$), and UBE2C

Figure 1 Correlation of protein expression and outcome in the Danish training cohort. Kaplan-Meier survival curves showing PFS as a function of protein expression of cyclin D1 (A), MCM7 (B), TRIM29 (C), and UBE2C (D). $P < 0.001$ for all panels.

($P < 0.001$) as independent prognostic markers for subsequent progression to MIBC (Table 2). The predictive accuracy (Harrell's Concordance Index) of the clinical risk factors alone was 73.5%, and this number increased to 82.2% when all four

protein markers were included (Table 2). The four examined proteins also had a significant prognostic value when disease-specific survival and overall survival were used as end points (Supplemental Tables S1 and S2). For the full multivariate Cox regression analyses, see Supplemental Table S3.

EORTC Risk Stratification in the Danish Cohort

The EORTC risk table for progression⁷ was applied to the Danish cohort. The risk scores split the cohort into a low-risk group and a high-risk group ($P < 0.001$) (Figure 2A). Evaluating the protein expressions in these low- and high-risk EORTC-based clinical groups, we found a high cyclin D1 expression to be significantly associated with increased PFS in both EORTC low-risk ($P = 0.001$) and high-risk patients ($P = 0.007$) (Figure 2B); high TRIM29 expression was significantly associated with increased PFS in high-risk patients ($P = 0.040$) (Figure 2D), whereas low UBE2C expression was significantly associated with increased PFS in both EORTC low-risk ($P = 0.012$) and high-risk patients ($P = 0.015$) (Figure 2E).

Validation in the Swedish, Spanish, and Taiwanese Patient Cohort

The prognostic value of cyclin D1, MCM7, TRIM29, and UBE2C expression was validated in a joint validation cohort consisting of three independent NMIBC cohorts from Sweden, Spain, and Taiwan. For each marker, the exact cut point determined from the training cohort was applied. A univariate Cox regression analysis revealed that the expression of cyclin D1 ($P < 0.001$), MCM7 ($P < 0.001$), TRIM29 ($P = 0.003$), and UBE2C ($P < 0.001$) was significantly associated with progression to MIBC (Table 3). Kaplan-Meier survival curves are shown in Figure 3. Stratifying for age, stage, grade, growth pattern, tumor size, and adjuvant therapy in a multivariate Cox regression analysis, we identified MCM7 ($P = 0.002$) and UBE2C ($P = 0.002$) as independent prognostic markers for subsequent progression to MIBC (Table 3). For the full multivariate Cox regression analyses, see Supplemental Table S4. Because the histopathologic information about concomitant carcinoma *in situ* in the Swedish cohort was unobtainable, it was not possible to subgroup the validation cohort based on the EORTC criteria.

Application of Computer-Assisted Image Analysis

Protocols for computer-assisted image analysis were developed to facilitate a clinically applicable, unbiased scoring of marker expression. The image protocols developed for each of the proteins were applied to the training and validation cohort, and the retrieved results were very similar to those obtained from the manual scoring (Tables 2 and 3 and Supplemental Tables S1 and S2). For the full multivariate Cox regression analyses, see Supplemental Tables S3 and S4. As for the manual scorings, cutoff values were

Table 2 Univariate and Multivariate Cox Regression Analysis of PFS for the Danish Training Cohort

Variable	Univariate Cox regression analysis and prediction accuracy for each variable			Multivariate analysis and prediction accuracy for the PA model (including prognostic markers)		
	HR (95% CI)	P value	PA, %	HR (95% CI)	P value	PA, %
Age (per 5-year increment)	1.18 (1.07–1.31)	0.001	60.3			
Sex (male vs female)	1.03 (0.65–1.61)	0.910	50.1			
Stage (T1 vs Ta)	3.01 (2.07–4.38)	< 0.001	65.6			
Grading WHO 2004						
High vs low+ PUNLMP	3.42 (2.34–4.98)	< 0.001	67.1			
Growth pattern solid/mixed vs papillary	2.35 (1.44–3.83)	0.001	56.0			
Adjuvant treatment (BCG/MMC vs no BCG/MMC)	0.51 (0.29–0.90)	0.019	55.7			
Concomitant CIS (CIS vs no CIS)	1.41 (0.96–2.06)	0.077	53.0			
Size (≥ 3 vs < 3 cm)	1.06 (0.67–1.67)	0.810	51.7			
Multiplicity (more vs one)	1.12 (0.74–1.71)	0.584	51.3			
Cyclin D1	0.36 (0.24–0.54)	< 0.001	61.5	0.51 (0.33–0.79)	0.003	75.9
Cyclin D1 (CAIA)	0.38 (0.25–0.58)	< 0.001	61.2	0.45 (0.29–0.69)	< 0.001	74.8
MCM7	2.29 (1.45–3.61)	< 0.001	58.3	1.52 (0.93–2.49)	0.095	76.1
MCM7 (CAIA)	2.28 (1.50–3.48)	< 0.001	59.4	1.50 (0.95–2.37)	0.082	75.7
TRIM29	0.46 (0.30–0.70)	< 0.001	60.9	0.60 (0.39–0.93)	0.023	75.7
TRIM29 (CAIA)	0.34 (0.22–0.51)	< 0.001	64.2	0.43 (0.28–0.67)	< 0.001	76.0
UBE2C	3.05 (1.94–4.80)	< 0.001	63.4	2.60 (1.60–4.23)	< 0.001	78.3
UBE2C (CAIA)	4.12 (2.60–6.52)	< 0.001	67.6	3.16 (1.89–5.28)	< 0.001	78.8
PA model without markers						73.5
PA model including all markers						82.2
PA model including all markers (CAIA)						82.8

Only risk factors with $P < 0.05$ were included in the multivariate analysis. The molecular markers were included separately and not in combination. PA model included age, sex, stage, grade, growth pattern, adjuvant treatment, concomitant CIS, size, and multiplicity. Bold indicates $P < 0.05$.

BCG, bacille Calmette-Guérin. CAIA, computer-assisted image analysis; CIS, carcinoma *in situ*; MMC, mitomycin C; PA, prediction accuracy (Harrell's Concordance index); PUNLMP, papillary urothelial neoplasm of low malignant potential; WHO, World Health Organization.

determined from the training cohort and applied directly to the validation cohort. Finally, a maximum-likelihood classifier was trained based on the computer-assisted image analysis of all four proteins in the Danish training cohort, and

the four-protein classifier was applied to the validation cohort (Figure 4). Because of nonevaluable, too little, or missing cancer tissue, we only included patients with three or four valid protein scores in the analysis, thereby including

Figure 2 EORTC risk stratification and marker expression in the Danish training cohort. **A:** Kaplan-Meier survival curves showing PFS as a function of the EORTC progression score. Kaplan-Meier survival curves showing PFS survival in the EORTC low-risk group (progression score, 0 to 6) and in the EORTC high-risk group (progression score, 7 to 23) as a function of the dichotomized expression of cyclin D1 (**B**), MCM7 (**C**), TRIM29 (**D**), and UBE2C (**E**).

Table 3 Univariate and Multivariate Cox Regression Analysis of PFS for the Validation Cohort

Variable	Univariate Cox regression analysis for the Swedish-Spanish-Taiwanese validation cohort			Multivariate Cox regression analysis for the Swedish-Spanish-Taiwanese validation cohort		
	HR (95% CI)	P value	PA, %	HR (95% CI)	P value	PA, %
Age (per 5-year increment)	1.13 (1.04–1.23)	0.006	56.9			
Sex (male vs female)	1.02 (0.66–1.58)	0.925	50.3			
Stage (T1 vs Ta)	3.79 (2.43–5.92)	<0.001	63.0			
WHO grade 2004	5.08 (3.28–7.89)	<0.001	67.3			
High vs low PUNLMP						
Growth pattern solid/mixed vs papillary	2.95 (1.96–4.44)	<0.001	56.7			
Adjuvant treatment (BCG/MMC vs no BCG/MMC)	0.62 (0.44–0.88)	0.007	56.2			
Concomitant CIS (CIS s no CIS)	2.08 (1.20–3.58)	0.009	53.2			
Size (≥ 3 vs < 3 cm)	1.59 (1.15–2.20)	0.006	55.8			
Multiplicity (more vs one)	1.07 (0.76–1.50)	0.711	51.3			
Cyclin D1	0.37 (0.25–0.55)	<0.001	62.1	0.66 (0.42–1.03)	0.065	76.9
Cyclin D1 (CAIA)	0.45 (0.30–0.66)	<0.001	58.8	0.65 (0.42–1.00)	0.048	78.4
MCM7	3.13 (2.11–4.64)	<0.001	60.4	1.98 (1.28–3.08)	0.002	79.3
MCM7(CAIA)	2.61 (1.37–5.00)	0.004	52.6	1.50 (0.74–3.04)	0.263	78.7
TRIM29	0.54 (0.36–0.81)	0.003	57.5	0.79 (0.51–1.25)	0.321	79.2
TRIM29 (CAIA)	0.16 (0.02–1.13)	0.066	52.2	0.30 (0.04–2.18)	0.235	80.0
UBE2C	2.98 (1.88–4.73)	<0.001	61.7	2.34 (1.37–4.00)	0.002	79.9
UBE2C (CAIA)	3.63 (2.19–6.01)	<0.001	62.5	2.47 (1.34–4.56)	0.004	79.7
Risk signature: high vs low	3.88 (2.40–6.26)	<0.001	66.3	2.35 (1.31–4.20)	0.004	82.0
High vs intermediate	1.50 (0.99–2.28)	0.056	56.1	1.13 (0.72–1.76)	0.605	74.8
Intermediate vs low	2.53 (1.58–4.07)	<0.001	61.1	1.88 (1.09–3.26)	0.024	82.8
PA model without markers						78.0
PA model including all markers						78.2
PA model including all markers (CAIA)						80.5

Only risk factors with $P < 0.05$ were included in the multivariate analysis, except CIS due to no clinical annotation for the Swedish cohort. The molecular markers were included separately and not in combination. PA model included age, sex, stage, grade, growth pattern, adjuvant treatment, concomitant CIS, size, and multiplicity. Only variables with information from all three cohorts were included in the analysis. Bold indicates P values < 0.05 .

BCG, bacille Calmette-Guérin; CAIA, computer-assisted image analysis; CIS, carcinoma *in situ*; MMC, mitomycin C; PA, prediction accuracy (Harrell's Concordance index); PUNLMP, papillary urothelial neoplasm of low malignant potential; WHO, World Health Organization.

472 of the 576 patients (82%) in the analysis. We determined a low-risk, intermediate-risk, and high-risk progression signature using a 33% cutoff in both directions from the classifier mean value. We found the high-risk signature to be significantly associated with progression to MIBC when compared with the low-risk signature in both univariate [hazard ratio (HR) = 3.88, $P < 0.001$] and multivariate (HR = 2.35, $P = 0.004$) Cox regression analyses of PFS (Table 3). The intermediate-risk signature could not be statistically significantly separated from the high-risk signature in univariate and multivariate analysis, but the intermediate-risk signature was significantly associated with progression to MIBC when compared with the low-risk signature in both univariate (HR = 2.53, $P < 0.001$) and multivariate (HR = 1.88, $P = 0.024$) Cox regression analyses (Table 3). For the full multivariate Cox regression analyses, see Supplemental Table S5.

Finally, the classifier was further developed to also include information on stage and grade. The classification results were highly significant in univariate Cox regression analysis of PFS when comparing the high-risk signature with the low-risk signature [HR = 7.55; 95% confidence

interval (CI) = 4.31 to 13.23; $P < 0.001$], the high-risk signature with the intermediate-risk signature (HR = 1.61; 95% CI = 1.07 to 2.45; $P = 0.024$), and the intermediate-risk signature with the low-risk signature (HR = 4.35; 95% CI = 2.39 to 7.91; $P < 0.001$). Using this combined approach, we defined a large fraction of the patients to have a very good prognosis (1-year progression risk, 0.5%; 5-year progression risk, 6.8%) (Supplemental Figure S4).

Discussion

The present work identified and validated four protein markers with prognostic value in NMIBC. Cyclin D1, MCM7, TRIM29, and UBE2C were demonstrated to be independent prognostic protein markers in a training cohort consisting of 283 patients with long-term follow-up, and these results were validated in a large multicenter validation cohort consisting of 576 patients with long-term follow-up. The present study thus lends further evidence to previous studies that have reported that the transcripts encoding these

Figure 3 Correlation of protein expression and outcome in the Swedish-Spanish-Taiwanese validation cohort. Kaplan-Meier survival curves showing PFS as a function of dichotomized protein expression of cyclin D1 (**A**), MCM7 (**B**), TRIM29 (**C**), and UBE2C (**D**). The protein expression cut points were defined by the Danish training cohort and directly applied to the validation cohort. $P < 0.001$ in **A**, **B**, and **D**, and $P = 0.001$ in **C**.

proteins are significantly associated with subsequent disease progression.^{9,10} In addition, we developed and validated image protocols for computer-assisted image analysis of the four proteins. The prognostic value of these image protocols nearly matched that of the manual scoring; the present study also offers promising perspectives for the future use of computer-assisted evaluation of prognostic markers in NMIBC management. However, a large prospective validation study needs to be performed before the developed image protocols can be used in the clinic. The final main finding of this study is that it presents a four-protein classifier based on the computer-assisted scoring of the protein expressions and demonstrates how this progression signature contains significant prognostic value in the validation cohort, thereby proposing a possible beneficial effect of combining molecular markers in NMIBC prognostication. When compared with the prognostic gene signature previously published by our group,¹⁰ the four-protein progression signature is based on formalin-fixed, paraffin-embedded tissue instead of fresh frozen tissue, which consequently may make the clinical implementation of the test easier, owing to tissue availability. Both signatures were independent of other clinical and histopathologic variables in multivariate Cox regression analysis.

Adding prognostic molecular markers to a risk table of clinical and pathologic parameters like the EORTC risk table may have great potential in future clinical practice. A more precise risk evaluation may potentially improve patient survival, and the use of computer-assisted image analysis of IHC stainings may be a possible way to standardize protein expression evaluation and reduce the evaluation time for the pathologist.¹⁷ The image analysis presented holds promise for future clinical use. An optimization of already existing risk tables may also be necessary because it has recently been argued that the EORTC risk table may overestimate the actual risk of progression to MIBC,¹⁸ at least in patients treated with bacillus Calmette-Guerin. Combining IHC markers and stage and grade in a computer-assisted scoring analysis identified a large fraction of patients with a very low risk of progression. This finding is potentially of clinical interest because these patients may have a reduced cystoscopy frequency, reducing patient burden and cost.

Several biases can be associated with the patient cohorts included in this study. In general, the patients included were treated at different hospitals for many years using different standard treatment protocols. This may consequently weaken the uniformity in the staining intensity of the antibody against the proteins, thereby making it difficult to validate certain staining patterns across countries. The Danish cohort has a relative overrepresentation of Ta tumors and low-grade disease compared with the Swedish, Spanish, and Taiwanese cohorts. This may complicate a comparison of the training cohort with the validation cohort. Furthermore, the progression rate in the Danish cohort surpasses the progression rate in the validation cohort and in NMIBC in general, separating the

Figure 4 Four-protein classifier results based on the computer-assisted analysis of the proteins. Heat map of the four-protein signature for progression monitored in 472 patients with NMIBC. Columns denote tumor samples and rows denote proteins. Up-regulation (yellow) and down-regulation (blue) compared with the median expression of the protein (black). The samples are ordered according to classification values (difference between the two distances to the classifier group mean values), with the samples classified with a progression signature to the right. **A:** The dotted vertical red line indicates the classifier mean values, and the dotted vertical green lines indicate the 33% cutoff limits when changing from the low-risk signature to the intermediate-risk signature and again from the intermediate-risk signature to the high-risk signature. **B:** Kaplan-Meier curves showing PFS as a function of the three risk signatures.

Danish training cohort from a direct comparison with the validation cohort and NMIBC in general. On the other hand, the progression rate of the validation cohort mimics that of NMIBC in general, making the results from the validation cohort comparable with NMIBC. The training cohort does not represent a consecutive cohort of patients and is biased toward inclusion of a large number of patients with disease progression. This may complicate a direct comparison of the results from the training cohort with NMIBC in general. The validation cohort received more adjuvant treatment than the Danish cohort, thereby complicating a direct comparison; this difference has been controlled for in the multivariate analyses. The increase in predictive accuracy when adding molecular markers to the available clinical variables was less for the validation cohort when compared with the Danish training cohort. This may partly be due to the direct application of the optimal cut point from the training cohort, instead of using the optimal cut point for each of the protein markers from the validation cohort. The optimal cut point may differ due to, for example, differences in tissue fixation between the cohorts. We have examined the four cohorts for possible differences between patients experiencing early (<1 year) and late (>1 year) disease progression. We found that patients with early progressing tumors had predominantly stage T1, high-grade solid tumors, and they did not receive adjuvant therapy. This finding underlines that the patient cohorts used are characterized by well-established clinical and histopathologic risk factors.

However, despite these biases, the markers examined in this study showed prognostic potential in both EORTC low- and high-risk bladder cancer and in NMIBC in general. Furthermore, the data may be affected by possible differences in ischemia time, different tissue fixation, and different tissue handling of samples in the four patient cohorts. For example, in the present study, the expression of TRIM29 and MCM7 was found to be markedly lower in the validation cohort than in the training cohort, which may have weakened the validation. Furthermore, it can be seen as a strength that the study includes two proteins correlated with progression and two proteins inversely correlated with progression. This may

represent complementary changes involved in the development of more aggressive disease. Also, use of inversely correlated markers for a clinical test may probably increase test usability and eliminate the need for additional normalizers or controls. A multicenter study under standardized conditions with numerous prospectively collected specimens is therefore needed before possible clinical use.¹⁹

All protein markers reported here have been previously investigated. Cyclin D1 has been extensively examined in cancer development and is seen as an important regulator of the G1- to S-phase transition in the cell cycle.²⁰ However, it has recently been shown that cyclin D1 also mediates DNA repair.²¹ In the field of bladder cancer, the search for a prognostic value of cyclin D1 expression has produced different results. Cyclin D1 protein expression has been correlated with both poor prognosis^{22,23} and good prognosis.²⁴ MCM7 is part of the MCM complex that ensures that DNA undergoes a single round of replication per cell cycle.²⁵ It has also been found to unwind double-stranded DNA at the replication forks.²⁶ The *MCM7* gene has been found to be up-regulated in a number of cancers compared with normal tissue. Of special interest is that the gene has been found to be overexpressed in prostate cancer compared with the normal prostate, and this overexpression has been associated with prostate cancer progression.²⁷ Small-interfering RNA-mediated knock-down of the *MCM7* gene in bladder cancer cell lines has been reported to diminish proliferation.²⁸ These findings underline the aggressive potential associated with high MCM7 protein expression uncovered in the present study. TRIM29 is a member of the family of tripartite motif-containing proteins.²⁹ TRIM29 has been found to bind p53³⁰ and Tip60 and to antagonize p53.³¹ The *TRIM29* gene has been reported to be overexpressed in different cancer types in a range of studies,³² including bladder cancer,³³ and up-regulation has been correlated with an improved prognosis.¹⁰ UBE2C participates in mitotic spindle checkpoint control and is important for the destruction of mitotic cyclins, and UBE2C consequently assists the regulation of cell cycle progression through the M phase.³⁴ UBE2C is also important for

maintaining the euploidy status of the cells.³⁵ In agreement with our findings, several studies have reported a high expression of the *UBE2C* gene to be prognostic for poor survival.³⁶ *UBE2C* has previously been reported to be up-regulated in primary bladder cancer samples compared with corresponding normal tissue³⁷ and overexpressed in grade 3 compared with grade 2 tumors.³⁸

We conclude that cyclin D1, MCM7, TRIM29, and *UBE2C* may be clinically relevant for better guidance of treatment in patients with NMIBC. Furthermore, a combination of prognostic markers and the use of computer-assisted scoring analysis may be advantageous in the management of NMIBC. Addition of molecular markers to the existing EORTC risk table holds great promise and may be a step toward further individualized treatment. Multi-center prospective studies are needed for further validation of the clinical relevance of the four protein markers.

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Supplemental Data

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