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ORIGINAL ARTICLE - CANCER RESEARCH

Methylation of *PITX2*, *HOXD3*, *RASSF1* and *TDRD1* predicts biochemical recurrence in high-risk prostate cancer

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Abstract
Purpose To explore differential methylation of *HAAO*, *HOXD3*, *LGALS3*, *PITX2*, *RASSF1* and *TDRD1* as a molecular tool to predict biochemical recurrence (BCR) in patients with high-risk prostate cancer (PCa).
Methods A multiplexed nested methylation-specific PCR was applied to quantify promoter methylation of the selected markers in five cell lines, 42 benign prostatic hyperplasia (BPH) and 71 high-risk PCa tumor samples. Uni- and multivariate Cox regression models were used to assess the importance of the methylation level in predicting BCR.
Results A PCa-specific methylation marker *HAAO* in combination with *HOXD3* and a hypomethylation marker *TDRD1* distinguished PCa samples (>90 % of tumor cells each) from BPH with a sensitivity of 0.99 and a specificity of 0.95. High methylation of *PITX2*, *HOXD3* and *RASSF1*, as well as low methylation of *TDRD1*, appeared to be significantly associated with a higher risk for BCR (HR 3.96, 3.44, 2.80 and 2.85, correspondingly) after correcting for established risk factors. When DNA methylation was treated as a continuous variable, a two-gene model *PITX2* $\times$ 0.020677 + *HOXD3* $\times$ 0.0043132 proved to be the best

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predictor of BCR (HR 4.85) compared with the individual markers. This finding was confirmed in an independent set of 52 high-risk PCa tumor samples (HR 11.89).
Conclusions Differential promoter methylation of *HOXD3*, *PITX2*, *RASSF1* and *TDRD1* emerges as an independent predictor of BCR in high-risk PCa patients. A two-gene continuous DNA methylation model "*PITX2* × 0.020 677 + *HOXD3* × 0.0043132" is a better predictor of BCR compared with individual markers.

Keywords Prostate cancer · Biochemical recurrence · DNA methylation · Prognosis

Introduction

Prostate cancer (PCa) is a disease with substantial phenotypic variability among cases and many uncertainties about its progression at the individual level. Up to 70 % of prostate tumors usually remain slow growing, confined to the prostate gland and never or poorly manifest themselves during the lifetime in the absence of treatment (Ploussard et al. 2011). However, some PCa tumors progress quickly, rapidly invade surrounding tissues and spread to other areas of the body. The critical issue at the stage of diagnosis is to predict accurately which men are at risk of rapid progression, distinguish them from those having a potentially insignificant tumor and identify patients eligible for active surveillance or organ-sparing therapies. Another common challenge is an accurate prediction of the risk of cancer relapse after treatment. Rise in the plasma levels of prostate-specific antigen (PSA), called biochemical recurrence (BCR) or biochemical failure, occurs in up to 50 % of patients by 10 years following radical prostatectomy (RP) (Swanson et al. 2007; Thompson et al. 2009). Other forms of relapse include local recurrence (cancer demonstrated on biopsy of the prostatic bed) and systemic progression (metastases).

Diagnostics of PCa and the choice of treatment are the well-established processes based on several pre- and post-operative clinico-pathological parameters, which are combined in predictive nomograms. However, these predictive models still lack some degree of sensitivity, not always carefully discriminate between clinically insignificant and aggressive tumors, and need to be refined with additional prognostic tools to prevent PCa under staging, or, on the contrary, overdiagnosis and overtreatment (Ploussard et al. 2011; Swanson et al. 2011; Freedland 2011).

The DNA methylation is a perspective biomarker powerful enough to improve currently existing predictive models (Nelson et al. 2009; Park 2010). A generally accepted concept of cancer-related DNA methylation aberrations states that hypermethylation is associated with inappropriate gene silencing, while DNA hypomethylation causes activation of oncogenes and genetic instability (Schulz 2005; De Smet et al. 2013). Although a causative role of aberrant DNA methylation in PCa initiation now seems rather questionable (Pellacani et al. 2014), these aberrations persistently accumulate with cancer progression (Phé et al. 2010). Still, the estimates of the prognostic potential of the DNA methylation markers are highly variable, which possibly stems from population differences, the heterogeneity of the studied patient groups and the use of distinct methylation detection methodologies (Chao et al. 2013). Standardization of the procedure, careful selection of the study groups and the combining of several promising markers into one multiplexed assay are required to set up a reliable methylation-based clinical test for PCa management.

Here, we combined several PCa-associated markers in one quantitative assay with a purpose to find a methylation signature with the enhanced prognostic capability. The most intensively studied of these markers is *Ras association* (*RalGDS/AF-6*) *domain family member one* (*RASSF1*), aberrantly hypermethylated in more than 40 types of sporadic human cancers, including PCa (Donninger et al. 2007). A recent systemic review denoted *RASSF1* as a potential methylation biomarker in PCa diagnosis associated with high Gleason score (Pan et al. 2013). *Paired-like homeodomain 2* (*PITX2*) was initially identified as a marker associated with metastasis-free survival in breast cancer patients (Maier et al. 2007; Harbeck et al. 2008). In a study of 605 RP-treated PCa patients, those having greater than median *PITX2* methylation were four times more likely to experience BCR within 8 years after surgery than patients with low *PITX2* methylation (Weiss et al. 2009). Hypermethylation of the *homeobox D3* (*HOXD3*) gene is regarded as an independent predictor of BCR in a combination with a pathological stage (Kron et al. 2010). *HOXD3* in a combination with *APC* and *TGFb2* outperformed single markers for the prediction of BCR (Liu et al. 2011). *3-Hydroxyanthranilate 3,4-dioxygenase* (*HAAO*) was identified as a gene frequently hypermethylated in PCa (Mahapatra et al. 2012). The promoter of *lectin, galactoside-binding, soluble, 3* (*LGALS3*), unmethylated in normal prostate and BPH samples becomes heavily methylated in early PCa, but is only lightly methylated in more advanced stages (Ahmed et al. 2009). The unusual methylation pattern of *LGALS3* in the various stages of PCa may indicate the possible involvement of hypomethylation in its reactivation during PCa progression.

Up to date, the role of DNA hypomethylation in carcinogenesis is disclosed to a much lesser extent compared with that of CpG-island hypermethylation. Most of the data about promoter-specific demethylation in cancer is related to germline-specific genes expressed exclusively in gametogenic cells, but usually silenced in somatic tissues by DNA methylation. The autosomal spermatogenesis-specific

Table 1 Clinico-pathological characteristics of the groups of patients

Clinical variable	Cohort		
	BPH	PCa1	PCa2
Number of patients	42	71	52
Median age (range) (y)	71 (48–94)	66 (46–76)	66 (51–73)
Median preoperative PSA (range) (ng/ml)	4.68 (0.31–92.05)	19.90 (2.70–141.00)	9.31 (1.49–46.75)
Positive surgical margins [*n* (%)]		28 (39)	N/A
Pathological T stage [n (%)]			
pT2		19 (27)	26 (50)
pT3a		29 (41)	14 (27)
pT3b		19 (27)	11 (21)
pT4		4 (5)	1 (2)
Gleason score [n (%)]			
2–6		21 (30)	0 (0)
7		33 (46)	32 (62)
8–10		17 (24)	20 (38)
Number of BCR (%)		34 (48)	16 (31)
Median follow-up (range) (y)		11.50 (1.42–18.83)	1.58 (0.11–4.28)

BCR biochemical recurrence, *N/A* not available, *PCa1-2* cohorts 1 and 2 of high-risk PCa patients, *PSA* prostate-specific antigen and *y* year

gene *Tudor domain containing 1* (*TDRD1*) is reported to be frequently overexpressed in prostate and breast tumors, which is associated with complete demethylation of the promoter region (Loriot et al. 2003). A prognostic significance of this hypomethylation marker still needs clarification.

We have developed a two-step quantitative multiplexed nested MSP procedure to evaluate the prognostic potential of *HAAO*, *HOXD3*, *LGALS3*, *PITX2*, *RASSF1* and *TDRD1* in a set of RP tumor samples obtained from high-risk PCa patients. These patients represent an excellent cohort to study candidate PCa biomarkers because of the relatively high rate of clinical events as compared to the low- or intermediate-risk patient groups. Our results identify a two-gene continuous DNA methylation model, which can be used as a molecular tool to predict BCR after RP.

Materials and methods

Patients and sample collection

Formalin-fixed, paraffin-embedded tissue samples were obtained at the University Hospital Leuven (Belgium) from patients with benign prostatic hyperplasia (BPH, $n = 42$; types of surgery: transcapsular adenomectomy or transurethral resection of the prostate) and two independent cohorts of RP-treated high-risk PCa patients (PCa1, $n = 71$; PCa2 and $n = 52$). High-risk PCa patients were selected according to the criteria adopted by the European Association of Urologists (EAU), i.e., a clinical stage ≥T3a, a biopsy Gleason score of 8–10 and/or a PSA level >20 ng/ml (Heidenreich et al. 2014). None of the patients were treated with neoadjuvant hormonal, radiation or chemotherapy. Preoperative staging included a digital rectal examination, an abdominopelvic-computed tomography (CT) scan and a bone scan. Prostate specimens (whole mount sections, 4 mm intervals) were staged and graded according to the 2002 TNM classification and the Gleason grading system. Follow-up was performed every 3 months for the first 2 years after surgery, every 6 months in the following 3 years and annually thereafter. BCR following RP was defined as an initial serum PSA level greater than or equal to 0.2 ng/ml, with a second confirmatory level of PSA greater than 0.2 ng/ml, in agreement with the EAU guidelines (Aus et al. 2005). Table 1 shows a summary of the clinico-pathological characteristics of all cohorts.

Additionally, the prostate cell lines LNCaP, DU 145, PC-3, PZ-HPV-7, BPH-1 (American Type Culture Collection, Rockville, MD, USA) and whole blood human genomic (HG) DNA (Clontech Laboratories, Inc., Mountain View, CA, USA) were used in the experiments. The study was approved by the UH Leuven Medical Ethics Commission.

DNA extraction and bisulfite conversion

In samples from PCa1 cohort, the areas with at least 90 % tumor cell content were marked on the paraffin sections (all done by the same pathologist), macro-dissected, and genomic DNA was extracted following a standard phenol–chloroform procedure. For BPH and PCa2 cohorts, genomic DNA was extracted from whole paraffin sections (containing a mixture of tumor and benign adjacent cells in PCa2 samples) using the WaxFreeTM DNA kit (TrimGen, Sparks, MD, USA). About 500 ng of genomic DNA from each sample was

Table 2 Primers for the assay

Primer ID	Sequence 5′–3′	Fragment length (bp)
HAAO-MI F	TGTTTTTAAGACGTTTAAGGAGTTTAGTA	210
HAAO-MI R	CAAAATAAACCCCAAACCTACTAC	
HAAO-MSP F	TTGAGTTCGGGTTTCGTAGTTC	88
HAAO-MSP R	CCAAACCTACTACGTACGACCG	
HAAO-USP F	TGTTTTGAGTTTGGGTTTTGTAGTTT	94
HAAO-USP R	CCCCAAACCTACTACATACAACCA	
HOXD3-MI F	AGGGAGAGAAGTTGGTGTTT	172
HOXD3-MI R	CCCAACAACCCTACAAAAA	
HOXD3-MSP F	TAACGTGAGTTAGGAGTAGCGTTTC	110
HOXD3-MSP R	GAACGCACAAACCGACG	
HOXD3-USP F	GTGTTTAATGTGAGTTAGGAGTAGTGTTTT	121
HOXD3-USP R	AACAACAAACACACAAACCAACA	
LGALS3-MI F	AATTTTTTATTTAGGTGATTTTGGAGA	151
LGALS3-MI R	CAAAAACGACCAAAAAACTCC	
LGALS3-MSP F	AGTTTAGGTTTCGGGAGCGTTAC	61
LGALS3-MSP R	ACTAAAAAACGCGACCTCCG	
LGALS3-USP F	GTTGTAGTTTAGGTTTTGGGAGTGTTAT	71
LGALS3-USP R	CAAACACTAAAAAACACAACCTCCA	
PITX2-MI F	TTTTTGGTTTTAAGATGTTAGGTTAATA	89
PITX2-MI R	CGCAACTCAACTCCAAACAC	
PITX2-MSP F	GTTAATAGGGAAGCGCGGAGTC	59
PITX2-MSP R	AAACACCCAAACGAACGACG	
PITX2-USP F	ATGTTAGGTTAATAGGGAAGTGTGGAGTT	63
PITX2-USP R	CCAAACACCCAAACAAACAACA	
RASSF1-MI F	GTCGTTTAGTTTGGATTTTGG	131
RASSF1-MI R	CTCAAACTCCCCCGACATAA	
RASSF1-MSP F	GGTTCGTTTTGTGGTTTCGTTC	72
RASSF1-MSP R	CCCGATTAAACCCGTACTTCG	
RASSF1-USP F	GGGTTTGTTTTGTGGTTTTGTTT	78
RASSF1-USP R	CATAACCCAATTAAACCCATACTTCA	
TDRD1-MI F	GGAATACGTGGGTATATTGAGTTGT	139
TDRD1-MI R	GACTACCGATACTAAAAACCCTACC	
TDRD1-MSP F	GGTATATTGAGTTGTACGTGGACGC	57
TDRD1-MSP R	CCTCCTAACCTCAACGCACG	
TDRD1-USP F	GTGGGTATATTGAGTTGTATGTGGATGT	63
TDRD1-USP R	CACCCTCCTAACCTCAACACACA	

F forward, *MI* methylation-independent, *MSP* methylation-specific PCR, *R* reverse and *USP* unmethylation-specific PCR

bisulfite converted using the EZ DNA methylation kit (Zymo Research Corp., Orange, CA, USA), according to the manufacturer's protocol, and eluted in 25-µl H_2O. LNCaP (100 % methylation of *HAAO*, *LGALS3*, *RASSF1* and *PITX2*), PC-3 (100 % methylation of *HOXD3*) and HG DNA (0 % methylation of *HAAO*, *LGALS3*, *RASSF1*, *HOXD3* and *PITX2*) served as a control for correct bisulfite conversion.

Quantification of DNA methylation

A two-step nested approach was applied to quantify the promoter methylation state. In step 1, the 100–200 base-pair (bp) fragments around the transcription start sites of the selected genes were co-amplified with methylation-independent (MI) primers containing maximally one CpG site close to the 5′ end (Table 2). PCR was performed in a total volume of 25 µl of buffer A containing 67 mM Tris–HCl at pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM of each dNTP (Fermentas GmbH, St. Leon-Rot, Germany), 0.5 U DNA polymerase (IMMOLASE™, Bioline USA Inc., Boston, MA, USA, 0.2 µM of the appropriate MI forward (F) and MI reverse (R) primers (Sigma-Aldrich N.V. Bornem, Belgium) and bisulfite-converted DNA template (25–50 ng). Reactions were carried out in triplicate in

a T-Personal thermocycler (Biometra GmbH, Goettingen, Germany) under the following conditions: denaturation for 10 min at 95 °C, 30 PCR cycles (30 s at 95 °C, 30 s at 57 °C and 30 s at 69 °C) and a final extension step for 3 min at 69 °C. A negative control (water only) was included. The final multiplex PCR product from each of the three repeats was diluted 500-fold in sterile distilled water.

In step 2, gene fragments corresponding to methylated and unmethylated DNA sequences (fragments M and U) after bisulfite conversion and preamplified in step 1 were separately quantified, using M- and U-specific primer pairs (MSP- and USP-primers, respectively, Table 2). Independent quantitative PCR (qMSP) and qUSP reactions for each of the three repeats were carried out in a Rotor-Gene TM 6000 (Corbett Life Science Pty Ltd., Mortlake, NSW, Australia) in a total volume of 15 µl containing buffer A, 1 µM EvaGreen® dye (Biotium Inc., Hayward, CA, USA), 0.4 µM of MSP or USP primer pairs and DNA template (5 µl of the diluted amplification product from step 1). The thermocycling conditions were as follows: denaturation for 10 min at 95 °C followed by 35 PCR cycles (20 s at 95 °C, 15 s at 61 °C and 15 s at 69 °C). Separate standard curves were generated for qMSP and qUSP, using four serial dilutions ($3 \times 10^7 - 3 \times 10^3$ copies per reaction in triplicate, Fig. S1a and d) of plasmid vectors (pGEM®-T Easy Vector System, Promega Corporation, Madison, WI, USA) with inserted M or U fragments of each marker gene (plasmid vectors pM and pU). All qMSP and qUSP reactions had a correlation coefficient of the standard curve ≥0.99 and a slope of approximately −3.3, indicating twofold increases in PCR product per cycle in the linear phase of the real-time PCRs (Figure S1b and e). The appearance of at least one of the M or U signals from each sample within the area of amplification of serially diluted standards (pM1-4 or pU1-4, i.e., >3,000 gene copies, Fig. 1a, d) at the stage of quantification was used as a criterion of successful pre-amplification of each gene. Melting curve analysis step following each qM/USP was included to estimate whether the melting profile of the amplified fragments corresponded to that of the control (Fig. S1c and f). No-template control and erroneous templates (pU for qMSP and pM for qUSP) were included in each qM/USP to exclude the possibility of contamination and mispriming (Fig. S1a and d). The percentage of methylation (%M; average of three repeats) was defined as $[M/(U + M)] \times 100$. A mixture (1:1 ratio) of pM and pU standards at four serial dilutions was added to each qM/USP reaction to control the adequacy of the quantification procedure.

Statistical analysis

The sensitivity and specificity were determined using MedCalc for Windows, version 12.5 (MedCalc Software, Ostend, Belgium). For the comparison of two median values, the Mann–Whitney *U* test was applied. A Cox proportional hazard model was used for exploration of the relationship between DNA methylation and the outcome (BCR). A linear trend was compared with quadratic and cubic splines-based functions. Evidence of nonlinearity was tested by the likelihood ratio test (Harrell 2002). In case of a linear relationship, DNA methylation was dichotomized (low/high methylation), whereas a trichotomization (low/medium/high methylation) was applied in case of a nonlinear function. Cox models were used for selecting cutoff values to categorize DNA methylation by considering all possible dichotomizations. Cutoff values were selected based on a model fit (likelihood) as well as on clinical arguments.

Log-rank test and univariate Cox models were used to analyze the difference between DNA methylation categories with respect to risk of BCR. Results are presented by means of hazard ratios (HR) and their 95 % confidence intervals (CI). The *p* values refer to a Wald test. Graphical representation of the results is given by plotting the Kaplan–Meier estimates. Multivariate Cox models were used to correct for possible confounders. To explore the association between DNA methylation treated as a continuous variable and BCR, regularized linear regression modeling was used to create multivariate models (Friedman et al. 2010).

All statistical tests were two sided. The *p* values <0.05 were considered statistically significant. Analyses were performed using SAS (version 9.2, SAS Institute Inc., Cary, NC, USA), SPSS (version 17.0, SPSS Inc., Chicago, IL, USA), Mathlab 7.13 (The MathWorks, Inc, Natick, MA, USA) and the survival R package (v 2.36-10) of the R statistical software.

Results

Methylation of the marker genes in cell lines, BPH and PCa tissues

In order to validate the developed quantification procedure, we used the PCa cell lines LNCaP, PC-3 and DU 145, non-cancerous cells BPH-1 and PZ-HPV-7, and whole blood HG DNA (as a "no-methylation" control) from a cancer-free person to quantify promoter methylation of *HAAO*, *RASSF1*, *HOXD3*, *LGALS3*, *RASSF1* and of the *TDRD1* hypomethylation marker. The five hypermethylation markers were highly (>70 %) methylated in at least one PCa cell line (Fig. 1a–e), with *HAAO* and *LGALS3* demonstrating cancer-specific methylation pattern (Fig. 1a, b). *PITX2*, *RASSF1* and *HOXD3* were also methylated in one of non-cancerous genotypes (BPH-1 or PZ-HPV-7), although to a lower extent compared with the PCa cell lines (Fig. 1c–e).

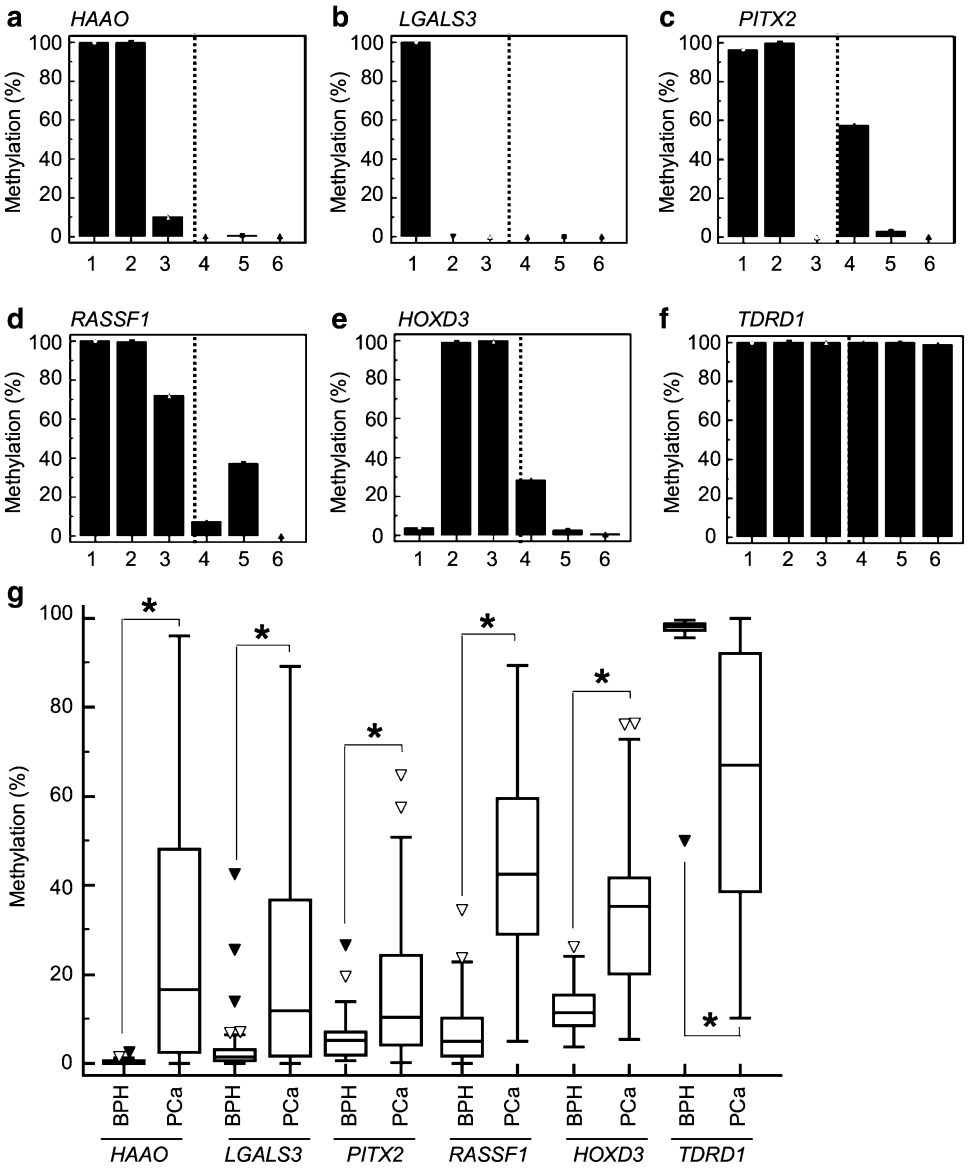

Fig. 1 Comparison of DNA methylation in cell lines, BPH and high-risk PCa. Methylation of *HAAO* (**a**), *LGALS3* (**b**), *PITX2* (**c**), *RASSF1* (**d**), *HOXD3* (**e**) and *TDRD1* (**f**) was quantified in LNCaP (*1*), PC-3 (*2*), DU 145 (*3*), BPH-1 (*4*), PZ-HPV-7 (*5*) cells and in whole blood human genomic DNA (*6*), as well as in radical prostatectomy samples from 42 patients with BPH (BPH) and 71 patients with high-risk PCa (PCa1) (**g**). *Vertical-dotted lines* separate the PCa cell lines (*1–3*) from nonmalignant (*4–6*) genotypes (**a–f**). The figure shows *box-whisker graphs*, with 25–75th percentiles (*boxes*), median values (*horizontal lines*) and minimal and maximal values (*whiskers*) for methylation of the marker genes (**g**). Median methylation of five genes in PCa1 cohort was significantly higher than in BPH group, as determined by the Mann–Whitney *U* test $p < 0.001$ (*asterisk*). The reverse trend was observed for *TDRD1*. *Empty triangles* denote "outside" values (larger than the upper quartile plus 1.5 times the interquartile range). *Filled triangles* denote "far out" values (smaller than the lower quartile minus three times the interquartile range or larger than the upper quartile plus three times the interquartile range)

LNCaP was the most hypermethylated genotype with four hypermethylation markers showing ~100 % methylation values, while none of the five markers were methylated in HG DNA. For this reason, LNCaP and HG DNA were used in further analysis as a control for efficient bisulfite conversion and multiplex preamplification. In contrast to alternative methylation of the five markers in cancerous and benign genotypes, *TDRD1* was ~100 % hypermethylated in all genotypes analyzed (Fig. 1f).

Next, we quantified promoter methylation in DNA samples from 42 BPH and 71 high-risk PCa patients (PCa1 cohort, Table 1). Median DNA methylation values of all genes except *TDRD1* were significantly higher in PCa samples compared with BPH (Fig. 1g, Mann–Whitney *U* test *p* values <0.001), although absolute methylation values never reached those observed in the PCa cell lines, apparently due to a high heterogeneity of the PCa tumor samples. Methylation of *HAAO* was the most PCa-specific marker detected in 80 % of PCa cases with the specificity of 0.98 at the lowest cutoff methylation value discriminating between BPH and PCa (2 %, Table 3). Methylation values of the four other hypermethylation markers were higher in BPH compared with *HAAO*, ranging from 0 to 7 % (*LGALS3*), 14 % (*PITX2*) and 23 % (*RASSF1*), and from 3 to 20 % (*HOXD3*) with a few "outside" and "far out" values (Fig. 1g). Therefore, higher BPH/PCa discriminating methylation cutoffs were introduced for those four markers (20–27 %, Table 3) resulting in the reduced sensitivity of *PITX2* (0.36), *LGALS3* (0.41) and *HOXD3* (0.56), but not of *RASSF1* (0.81). In contrast, all BPH samples but one had significantly higher levels of *TDRD1* methylation (95–100 %) compared with the PCa samples (Fig. 1g). This gene may be classified as

Table 3 Sensitivity and specificity of DNA methylation markers

Gene (combination)	Sensitivity	Specificity	Methylation cutoff value (%)
PITX2	0.36	0.98	20
LGALS3	0.41	0.98	26
HOXD3	0.56	1.00	27
TDRD1	0.79	0.98	95
HAAO	0.80	0.98	2
RASSF1	0.81	0.98	24

a PCa-specific hypomethylation marker with the sensitivity and specificity (0.79 and 0.98, correspondingly, at the 95 % methylation cutoff) similar to those of *HAAO* and *RASSF1*. Finally, a combination of *HAAO* with *TDRD1* and *HOXD3* identified PCa with the highest sensitivity of 0.99 at the specificity of 0.95, failing to identify only one tumor, which apparently may be attributed to MI PCa cases (99 % *TDRD1* methylation and 1–10 % methylation of the rest of the markers). In conclusion, in a set of tumor samples containing >90 % of tumor DNA, *HAAO*, *RASSF1*, and *TDRD1* appeared to be highly PCa-specific methylation markers detecting ~80 % of PCa cases each at the methylation cutoff values of 2, 24 and 95 %, correspondingly.

Methylation of *PITX2*, *HOXD3*, *RASSF1* and *TDRD1* is associated with BCR

Next, we applied a Cox proportional hazard model to determine the relationship between DNA methylation and BCR. To check the possibility of a nonlinear relationship, the fit of a model with linear trend was compared with that of the nonlinear quadratic and restricted cubic splines-based (RCS four and five knots) models using a likelihood ratio test (Harrell 2002). A linear term was selected to characterize the association of the event risk with *PITX2*, *HOXD3* and *RASSF1* methylation (Fig. 2a–c, p values 0.0024–0.0196), as none of the three nonlinear models showed better performance (Fig. 2a–c, likelihood ratio test p values 0.1559–0.6790). As expected, the risk of BCR becomes higher with an increase in methylation levels of these genes (Fig. 2a–c). In contrast, a relation between *TDRD1* methylation and BCR is better described by a nonlinear quadratic model, as patients with completely methylated, or, alternatively, heavily hypomethylated *TDRD1* had worse clinical outcome (Fig. 2d, $p = 0.0339$ for overall effect; $p = 0.0296$ for a comparison with a linear term). No association was observed between BCR and methylation of *LGALS3* and *HAAO* (Fig. 2e–f, p values 0.0974–0.9983). Thus, patients with increased methylation levels of *PITX2*, *HOXD3* and *RASSF1* show a higher risk for BCR after RP, while *TDRD1* methylation is associated with BCR in a U-shaped fashion, with both heavily *TDRD1* hyper- and hypo-methylated tumors having worse prognosis.

Categorized methylation of *PITX2*, *HOXD3*, *RASSF1* and *TDRD1* is an independent predictor of BCR

To estimate the predictive value of DNA methylation, we categorized the methylation percentage of the markers showing a linear trend in association with BCR (*PITX2*, *HOXD3* and *RASSF1*) into groups with low and high methylation. Cox models were used to select cutoffs leading to the best dichotomization (highest likelihood) by comparing all possible dichotomizations (not shown). The best selected cutoffs were 24 % (*PITX2*), 25 % (*HOXD3*) and 50 % (*RASSF1*). For the categorization of *TDRD1* methylation showing nonlinear association with BCR into low (LM)/moderate (MM)/high (HM) methylation groups, the cutoffs 50 % (LM to MM) and 90 % (MM to HM) were introduced based on clinical arguments.

Patients with HM of *PITX2*, *HOXD3* and *RASSF1* showed a significantly higher risk for BCR as compared to patients with LM in univariate analysis (Table 4a, HR 3.08–4.21; Fig. 3a–c; p values 0.0005–0.0013) as well as after adjustment for pathological T stage, Gleason score, preoperative PSA level, surgical margin status and lymph node invasion (Table 4b, HR 2.80–3.96). Trichotomized *TDRD1* methylation was also significantly associated with BCR, as shown by both univariate (Table 4a, $p = 0.0135$; Fig. 3d, $p = 0.0080$) and multivariate (Table 4b; $p = 0.0066$) analysis. Pairwise uni- and multivariate analysis of *TDRD1* methylation revealed that patients with LM tumors had a significantly higher risk of BCR as compared to patients with MM tumors (Table 4a, b, HR 4.00–4.58; Fig. 3d, $p = 0.0021$), but not with HM tumors. HM patients showed an intermediate risk of BCR when compared with MM and LM groups, although pairwise comparisons with those groups were not statistically significant (Fig. 3d, p values 0.1245 and 0.1583). However, a combination HM + LM still had a worse prognosis compared with MM patients in both uni- and multivariate analysis (Table 4a, b, HR 2.85–3.01). Thus, the categorized methylation of each of the four markers is an independent predictor of BCR, with HM (*PITX2*, *HOXD3* and *RASSF1*) and, alternatively, LM and LM + HM (*TDRD1*) tumors demonstrating more aggressive epi-subtypes.

A two-gene continuous DNA methylation model "*PITX2* × 0.020677 + *HOXD3* × 0.0043132" is a better predictor of BCR in comparison with the individual markers

We also investigated the correlation of *PITX2*, *HOXD3* and *RASSF1* methylation treated as a continuous variable with

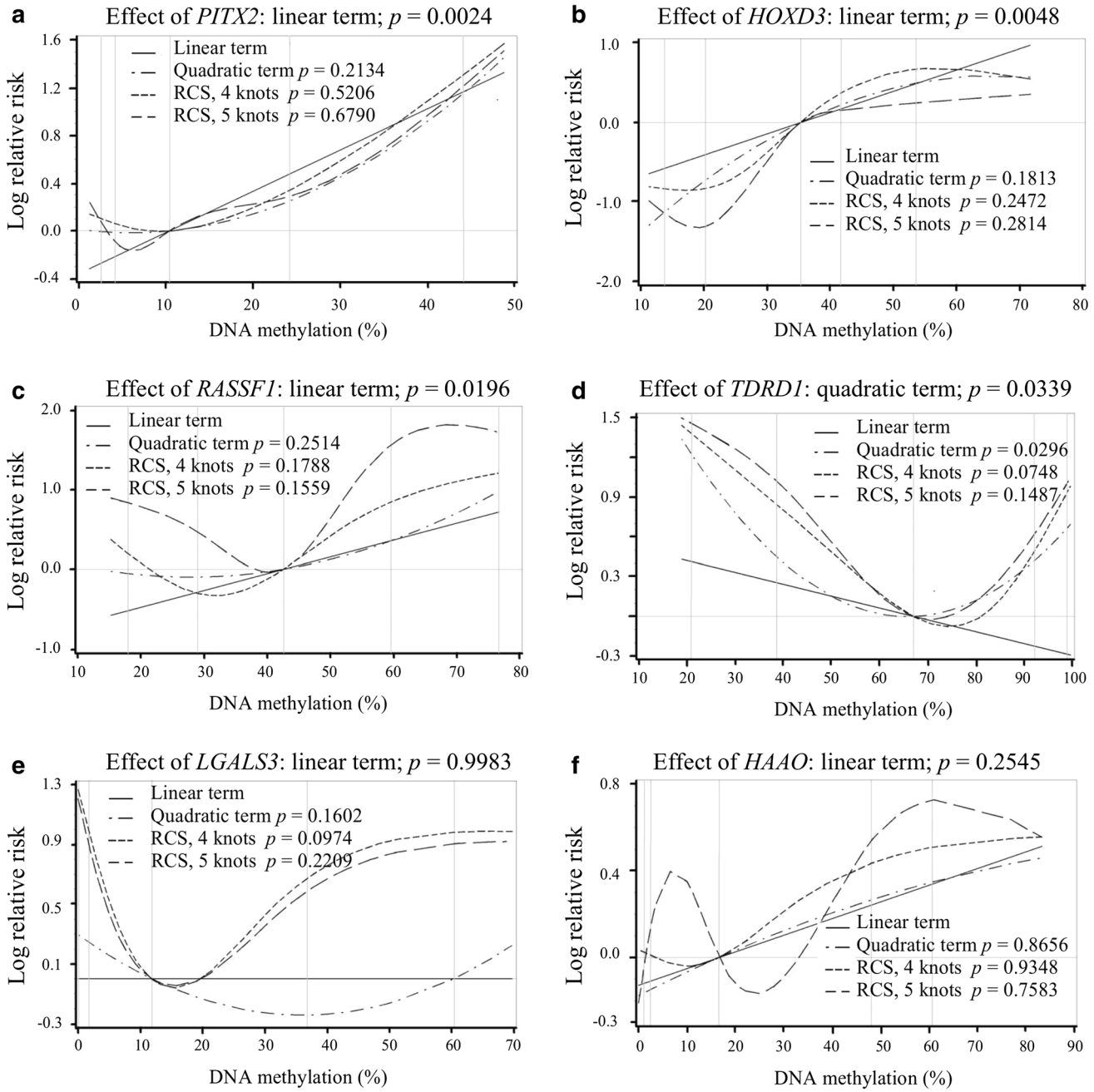

Fig. 2 Checking linearity of association between DNA methylation and biochemical recurrence. The fit of the nonlinear quadratic (quadratic term) and restricted cubic spline-based (RCS, four knots and RCS, five knots) models was compared with the fit of a model with linear trend (linear term) for *PITX2* (**a**), *HOXD3* (**b**), *RASSF1* (**c**), *TDRD1* (**d**), *LGALS3* (**e**) and *HAAO* (**f**). Likelihood ratio test *p* values are provided for each of the three comparisons. *Headlines* represent the overall effect of the best fitting model of the association between DNA methylation and event risk. *Vertical gray lines* denote percentiles 10, 25, 75, 90 and the median value. *P* likelihood ratio tests *p*-values

BCR in PCa1 ($n = 71$) cohort. Of the three genes, only *HOXD3* and *PITX2* appeared to be significant predictors of BCR in univariate analysis (Table 5a, HR 1.03–1.04), but not when adjusted for pathological T stage, Gleason score, preoperative PSA, surgical margin status and lymph node invasion (Table 5a, *p* values 0.0648–0.0979). Next, a regularized linear regression modeling was performed to create a methylation multivariate model for BCR-free survival. A cross-validated two-gene methylation model *PITX2* × 0.020677 + *HOXD3* × 0.0043132 showed a higher hazard ratio compared with the individual genes in univariate analysis (Table 5a; HR 4.85 vs. 1.03 and 1.04 for

Table 4 Univariate and multivariate Cox regression analysis for BCR (DNA methylation as a categorical variable)

Variable	HR	95 % CI	*p* value
(a) Univariate analysis			
PITX2 LM versus HM (>24 %)	3.25	1.61–6.57	0.0010
HOXD3 LM versus HM (>25 %)	4.21	1.63–10.92	0.0031
RASSF1 LM versus HM (>50 %)	3.08	1.49–6.36	0.0024
TDRD1 LM versus MM (>50 %) versus HM (>90 %)	–	–	0.0135
MM versus HM	2.26	0.82–6.24	0.1146
LM versus HM	0.57	0.26–1.25	0.1589
MM versus LM	4.00	1.56–10.23	0.0038
MM versus LM + HM	3.01	1.23–7.35	0.0157
LM + MM versus HM	1.13	0.52–2.47	0.7558
Pathological T stage 2–3a versus 3b–4	2.88	1.46–5.67	0.0022
Gleason score 2–7 versus 8–10	2.03	0.97–4.26	0.0611
Preoperative PSA continuous	1.01	1.00–1.03	0.0416
Positive surgical margins	1.62	0.83–3.17	0.1602
Lymph node invasion	1.96	0.75–5.07	0.1675
(b) Multivariate analysis PITX2 and covariates			
PITX2 LM versus HM (>24 %)	3.96	1.72–9.09	0.0012
Pathological T stage 2–3a versus 3b–4	1.66	0.67–4.14	0.2735
Gleason score 2–7 versus 8–10	2.59	1.11–6.03	0.0276
Preoperative PSA continuous	1.01	0.99–1.02	0.4634
Positive surgical margins	1.10	0.53–2.30	0.7987
Lymph node invasion	2.04	0.57–7.36	0.2764
HOXD3 and covariates			
HOXD3 LM versus HM (>25 %)	3.44	1.23–9.59	0.0181
Pathological T stage 2–3a versus 3b–4	1.58	0.66–3.77	0.3075
Gleason score 2–7 versus 8–10	2.18	1.01–4.71	0.0478
Preoperative PSA continuous	1.01	0.99–1.02	0.4510
Positive surgical margins	1.29	0.64–2.60	0.4750
Lymph node invasion	0.90	0.28–2.91	0.8576
RASSF1 and covariates			
RASSF1 LM versus HM (>50 %)	2.80	1.32–5.93	0.0071
Pathological T stage 2–3a versus 3b–4	2.06	0.83–5.11	0.1171
Gleason score 2–7 versus 8–10	2.17	0.96–4.92	0.0635
Preoperative PSA continuous	1.01	0.99–1.02	0.5122
Positive surgical margins	1.44	0.69–3.00	0.3352
Lymph node invasion	1.35	0.42–4.33	0.6163
TDRD1 and covariates			
TDRD1 LM versus MM (>50 %) versus HM (>90 %)	–	–	0.0066
MM versus HM	1.78	0.60–5.25	0.2963
LM versus HM	0.39	0.15–1.01	0.0536
MM versus LM	4.58	1.72–12.15	0.0023
MM versus LM + HM	2.85	1.15–7.10	0.0242
LM + MM versus HM	0.83	0.34–2.04	0.6877
Pathological T stage 2–3a versus 3b–4	1.87	0.76–4.58	0.1703
Gleason score 2–7 versus 8–10	2.93	1.18–7.25	0.0204
Preoperative PSA continuous	1.01	0.99–1.02	0.3141
Positive surgical margins	1.53	0.75–3.12	0.2448
Lymph node invasion	1.13	0.34–3.79	0.8429

Hazard Ratio (HR) >1 (<1) indicates higher (lower) risk for the second group

% % of methylation, *CI* confidence interval, *HM* high methylation, *LM* low methylation and *MM* moderate methylation

HOXD3 and *PITX2*). Besides, it was the only continuous DNA methylation model predicting BCR independently of pathological T stage, Gleason score, preoperative PSA, surgical margin status and lymph node invasion (Table 5a; HR 3.08).

These results were validated in an independent cohort PCa2 ($n = 52$) which differed from PCa1 by the tumor DNA content in DNA samples (>90 % tumor DNA in PCa1 samples versus mixed DNA from tumor and benign adjacent cells in PCa2), as well as by a shorter follow-up period (Table 1, median follow-up of 1.58 years vs. 11.50 years in PCa1). Like in PCa1, both *PITX2* and the two-gene model, but not *HOXD3*, had a significant association with the risk of BCR in univariate analysis, with the two-gene model showing a tenfold higher hazard ratio (Table 5b, HR 11.83 vs. 1.06 for *PITX2*). In multivariate analysis, none of the factors analyzed were associated with BCR (Table 5b, *p* values 0.1372–0.8824), presumably due to a short follow-up period in that group (Table 1, median follow-up 1.58 years). In conclusion, continuous methylation of *PITX2* and the continuous two-gene methylation model *PITX2* × 0.020677 + *HOXD3* × 0.0043132 were associated with the risk of BCR in two independent cohorts of high-risk PCa patients, with the later model showing higher HR (4.85–11.83 vs. 1.04–1.06 for *PITX2*). A two-gene model was also an independent predictor for BCR in the context of known prognostic clinico-pathological variables in the PCa1 cohort.

Discussion

We have developed a two-step quantitative multiplexed nested MSP procedure to measure quantitatively and reliably promoter methylation of *HAAO*, *HOXD3*, *LGALS3*, *PITX2*, *RASSF1* and *TDRD1*. In the first step, the six markers were co-amplified from the same DNA template independently of their methylation status, which significantly reduced the amount of precious bisulfite-converted DNA template needed for the analysis, generating at the same time a sufficient amount of the DNA targets for methylation/unmethylation-specific primers to reduce

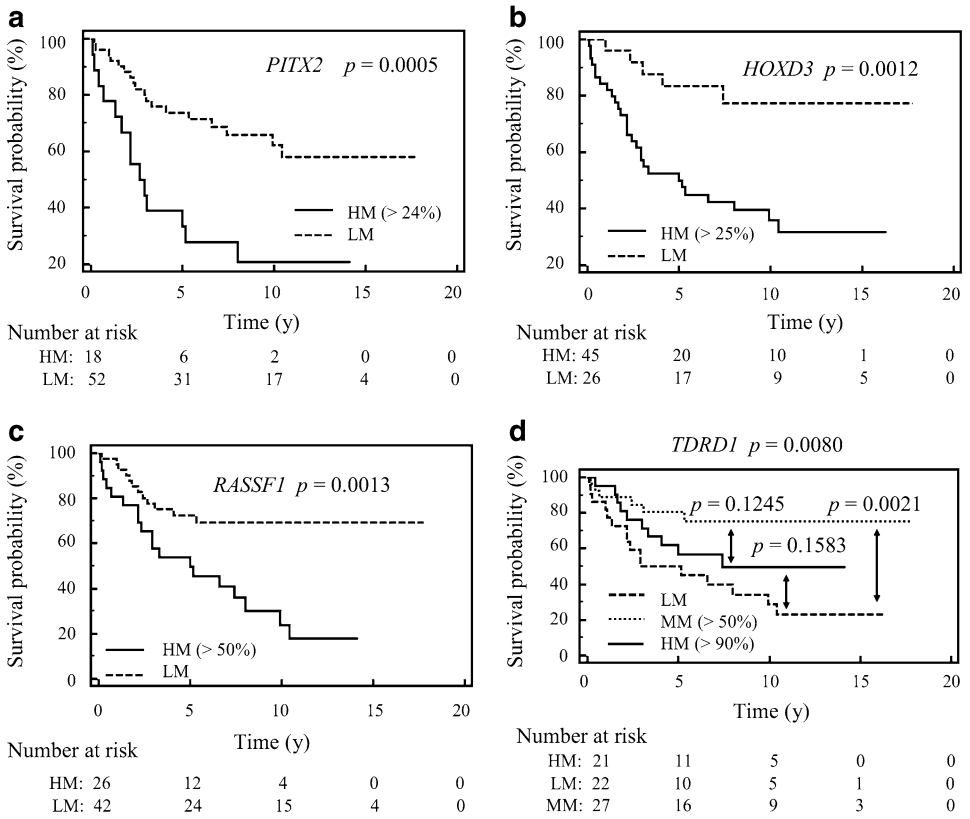

Fig. 3 Kaplan–Meier survival plots for patient methylation subgroups. The curves show BCR-free survival of patients from low (LM) and high (HM) methylation groups of *PITX2* (**a**), *HOXD3* (**b**), *RASSF1* (**c**) and from the LM, MM (moderate methylation) and HM groups of *TDRD1* (**d**) from PCa1 cohort. (%) DNA methylation cutoff value in %, *p* log-rank test *p*-value

false priming at the stage of quantification. In the second step, the quantification of DNA methylation was made more reliable (compared with the assays using the external references like the *ACTB* gene) by including plasmid standards for each gene, corresponding to fully methylated (M) or unmethylated (U) promoter regions after bisulfite conversion.

The procedure was used to quantify methylation of the marker genes in tissue samples from patients diagnosed with BPH and from PCa patients with high risk of cancer recurrence. Since far from all high-risk patients defined by a clinical stage ≥T3a, a biopsy Gleason score of 8–10 and/or a serum PSA level >20 ng/ml develops a fatal disease (Cooperberg et al. 2008; Spahn et al. 2010a, b), these patients represent an excellent group for the identification of the new biomarkers enabling better risk stratification in comparison with the conventional clinico-pathological parameters.

As observed previously (Chung et al. 2008), cancer cell lines usually exhibit higher levels of CpG-island hypermethylation compared with primary cancers, which may be a result of higher heterogeneity in tumor samples, as well as of their contamination by adjacent nonmalignant cells. In our study, the PCa cell cultures also showed mainly polar methylation values (100 or 0 %), while median methylation values in the PCa samples did not exceed 50 %, despite the fact that the analyzed samples contained >90 % of tumor cells. This suggests that far from all tumor cells contain hypermethylated copies of a definite gene. Of the six markers analyzed, *HAAO* and the hypomethylation marker *TDRD1* showed the highest PCa specificity, demonstrating at the same time a high sensitivity. Other hypermethylation markers may be attributed to a group of genes which are moderately methylated in benign prostate tissue, but change their methylation pattern in tumors to a higher degree and/or intensity. Diagnostic specificity of such markers may be increased by assessing a higher methylation cutoff below which their methylation is considered cancer insignificant. Correspondingly, *RASSF1* showed sensitivity similar to that of *HAAO* and *TDRD1* at the methylation cutoff value of 24 %, and a three-gene signature *HAAO*/*HOXD3*/*TDRD1* demonstrated the highest sensitivity of 0.99 at the specificity of 0.95 at the cutoff values of 2, 27 and 95 %. However, the sensitivity of the signature may be lower in biopsy samples containing various numbers of (and sometimes predominantly) noncancerous cells. The diagnostic potential of the proposed assay requires additional validation in a range of samples, including plasma and urine.

While *PITX2* emerged as an independent predictor of BCR in a previous study (Weiss et al. 2009), to our knowledge, we are the first to report a significant association between BCR and categorized *HOXD3*, *RASSF1* and *TDRD1* methylation after adjustment for

Table 5 Univariate and multivariate Cox regression analysis for BCR (DNA methylation as a continuous variable)

Variable	HR	95 % CI	*p* value
(a) PCa1 cohort (training)			
Univariate analysis			
RASSF1	1.02	0.99–1.03	0.1175
HOXD3	1.03	1.01–1.04	0.0080
PITX2	1.04	1.02–1.10	0.0050
PITX2 × 0.020677 + *HOXD3* × 0.0043132	4.85	2.03–11.60	0.0003
Multivariate analysis			
HOXD3 and covariates			
HOXD3	1.02	0.99–1.04	0.0979
Pathological T stage 2–3a versus 3b–4	3.27	1.62–6.60	0.0010
Gleason score 2–7 versus 8–10	1.27	0.67–2.40	0.4669
Preoperative PSA continuous	1.02	0.99–1.03	0.0708
Positive surgical margins	2.90	1.30–6.48	0.0096
Lymph node invasion	1.08	0.28–4.22	0.9103
PITX2 and covariates			
PITX2	1.03	0.99–1.05	0.0648
Pathological T stage 2–3a versus 3b–4	2.98	1.45–6.14	0.0030
Gleason score 2–7 versus 8–10	1.35	0.71–2.55	0.3628
Preoperative PSA continuous	1.01	0.99–1.03	0.1180
Positive surgical margins	2.46	1.14–5.33	0.0225
Lymph node invasion	1.12	0.28–4.52	0.8650
Two–gene model and covariates			
PITX2 × 0.020677 + *HOXD3* × 0.0043132	3.08	1.05–9.02	0.0405
Pathological T stage 2–3a versus 3b–4	3.26	1.54–6.88	0.0010
Gleason score 2–7 versus 8–10	1.50	0.77–2.93	0.2331
Preoperative PSA continuous	1.01	0.99–1.03	0.1431
Positive surgical margins	2.33	1.05–5.16	0.0372
Lymph node invasion	1.05	0.26–4.23	0.9489
(b) PCa2 cohort (validation)			
Univariate analysis			
RASSF1	1.02	0.99–1.06	0.2041
HOXD3	1.05	0.99–1.09	0.0558
PITX2	1.06	1.02–1.10	0.0073
PITX2 × 0.020677 + *HOXD3* × 0.0043132	11.83	2.15–65.04	0.0045
Multivariate analysis			
Two–gene model and covariates			
PITX2 × 0.020677 + *HOXD3* × 0.0043132	7.49	0.53–106.54	0.1372
Pathological T stage 2–3a versus 3b–4	1.96	0.30–12.63	0.4805
Gleason score 2–7 versus 8–10	0.92	0.32–2.69	0.8824
Preoperative PSA continuous	0.98	0.85–1.13	0.7681
Positive surgical margins	–	–	–
Lymph node invasion	2.75	0.33–22.94	0.3503

clinico-pathological factors. For *PITX2* and *HOXD3,* the selected cutoffs separating HM from LM groups (24 and 25 %, correspondingly) were similar to those discriminating between BPH and PCa with the specificity of 0.98–1.00 (20 and 27 %, correspondingly), while for *RASSF1,* the former was almost two times higher than the latter (50 vs. 24 %). This implies that PCa-specific methylation of *PITX2* and *HOXD3* is significantly associated with the worse outcome, while that of *RASSF1* still needs further stratification, as mostly heavily *RASSF1*-methylated tumors are associated with BCR.

In case of the hypomethylation marker *TDRD1*, moderately methylated tumors were associated with better outcome in comparison with LM and LM + HM tumors. *TDRD1* is known to be significantly overexpressed in PCa tumors containing translocation of the *TMPRSS2* gene to the ETS transcription factor gene *ERG* (*TMPRSS2-ERG* gene fusion), which has been reported in up to 2/3 of PCas (Jhavar et al. 2008). Overexpression of *ERG* in *TMPRSS2*:*ERG*-positive PCa and binding of the ERG transcription factor upstream of the *TDRD1* transcription start site induces a loss of DNA methylation at the *TDRD1* promoter-associated CpG island (Paulo et al. 2012; Kacprzyk et al. 2013). Taking into account that *TDRD1* methylation is a marker of the presence of the *TMPRSS2-ERG* gene fusion in a tumor, *TDRD1* methylation > (<) 90 % may denote *TMPRSS2-ERG*-negative (positive) tumors in the study cohort analyzed here. This hypothesis needs further experimental confirmation; besides, it does not explain a nonlinear fashion of the association between *TDRD1* methylation and BCR (i.e., both heavily *TDRD1* hypo- and hyper-methylated tumors show worse outcome compared with moderately methylated tumors), because ERG immunopositivity is not recognized as an important prognostic factor in PCa (Xu et al. 2014).

Due to the fact that the artificial dichotomization of continuous variables may lead to a considerable loss of power and incomplete correction for confounding factors (Naggara et al. 2011), we have also investigated the correlation between DNA methylation treated as a continuous variable and BCR. Both *PITX2* and *HOXD3* continuous methylation showed a significant linear association with BCR in univariate analysis; however, a two-gene continuous methylation model *PITX2* × 0.020677 + *HOXD3* × 0.0043132 outperformed any individual methylation marker in BCR prediction in both uni- and multivariate analysis.

Radical prostatectomy is regarded as an efficient cure for patients with clinically localized PCa. Still, up to 20 % of patients treated with RP experience BCR within 5 years of surgery (Roehl et al. 2004). Nevertheless, about 60 % of the high-risk PCa patients defined by a clinical stage ≥T3a, a biopsy Gleason score of 8–10 and/or a serum PSA level > 20 ng/ml experience a metastasis-free survival after RP of at least 15 years, demonstrating that not all patients in this group have a poor prognosis (Spahn et al. 2010a, b). The consideration of molecular and (epi) genetic

characteristics of the tumor could sufficiently improve currently existing predictive models based on clinical and pathological features. Still, the evidence on the prognostic utility of the majority of methylation markers in PCa presented in the literature is rather inconclusive (reviewed by Chao et al. 2013). Until now, *PITX2* was the only methylation marker with a reported prognostic value independent of clinico-pathological parameters in two large cohorts of PCa patients (Weiss et al. 2009; Bañez et al. 2010). However, in those studies, continuous *PITX2* methylation was dichotomized into high and low methylation groups. In contrast, we were able to build a continuous two-gene *PITX2* + *HOXD3* methylation model which appeared to be a better predictor of BCR-free survival compared with the single markers *PITX2* and *HOXD3*. The model proved to be valid in two independent cohorts PCa1 and PCa2 despite the fact that the second cohort had a shorter follow-up period and the samples from that cohort were represented by a mixture of benign and malignant cells, while the samples from the first cohort comprised mainly tumor cells. The validity of the model in a cohort with a short follow-up period seems especially important, since the majority of clinically meaningful BCR occurs mainly during 3–5 years following RP (our unpublished data). Besides, unlike single-gene markers, the continuous two-gene model added independent prognostic information to known clinico-pathological parameters such as preoperative PSA, final Gleason score, pT stage and surgical margin status. The latter finding, however, needs further validation, as neither the methylation model nor clinico-pathological parameters showed any significant association with BCR in PCa2 in multivariate analysis, presumably due to a limited number of observations. The two-gene model also needs further validation in intermediate- and low-risk patient groups, as well as in a set of diagnostic biopsy cores.

In conclusion, our study identifies a two-gene continuous DNA methylation model which can be used as a molecular tool for better stratification of high-risk PCa patients relative to the risk of BCR.

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Conflict of interest None.

References

Ahmed H, Cappello F, Rodolico V, Vasta GR (2009) Evidence of heavy methylation in the galectin 3 promoter in early stages of prostate adenocarcinoma: development and validation of a methylated marker for early diagnosis of prostate cancer. Transl Oncol 2:146–156

Aus G, Abbou CC, Bolla M, Heidenreich A, Schmid HP, van Poppel H, Wolff J, Zattoni F (2005) EAU guidelines on prostate cancer. Eur Urol 48:546–551

Bañez LL, Sun L, van Leenders GJ, Wheeler TM, Bangma CH, Freedland SJ, Ittmann MM, Lark AL, Madden JF, Hartman A et al (2010) Multicenter clinical validation of PITX2 methylation as a prostate specific antigen recurrence predictor in patients with post-radical prostatectomy prostate cancer. J Urol 184:149–156

Chao C, Chi M, Preciado M, Black MH (2013) Methylation markers for prostate cancer prognosis: a systematic review. Cancer Causes Control 24:1615–1641

Chung W, Kwabi-Addo B, Ittmann M, Jelinek J, Shen L, Yu Y, Issa JP (2008) Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling. PLoS ONE 3:e2079

Cooperberg MR, Cowan J, Broering JM, Carroll PR (2008) High-risk prostate cancer in the United States, 1990–2007. World J Urol 26:211–218

De Smet C, Loriot A (2013) DNA hypomethylation and activation of germline-specific genes in cancer. Adv Exp Med Biol 754:149–166

Donninger H, Vos MD, Clark GJ (2007) The *RASSF1*A tumor suppressor. J Cell Sci 120:3163–3172

Freedland SJ (2011) Screening, risk assessment, and the approach to therapy in patients with prostate cancer. Cancer 117:1123–1135

Friedman J, Hastie T, Tibshirani K (2010) Regularization paths for generalized linear models via coordinate descent. J Stat Softw 33:1–22

Harbeck N, Nimmrich I, Hartmann A, Ross JS, Cufer T, Grützmann R, Kristiansen G, Paradiso A, Hartmann O, Margossian A et al (2008) Multicenter study using paraffin-embedded tumor tissue testing *PITX2* DNA methylation as a marker for outcome prediction in tamoxifen-treated, node-negative breast cancer patients. J Clin Oncol 26:5036–5042

Harrell FE (2002) Regression modeling strategies. With applications to linear models logistic regression and survival analysis. Springer, New-York

Heidenreich A, Bastian PJ, Bellmunt J, Bolla M, Joniau S, van der Kwast T, Mason M, Matveev V, Wiegel T, Zattoni F et al (2014) EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and local treatment with curative intent-update 2013. Eur Urol 65:124–137

Jhavar S, Reid A, Clark J, Kote-Jarai Z, Christmas T, Thompson A, Woodhouse C, Ogden C, Fisher C, Corbishley C et al (2008) Detection of *TMPRSS2-ERG* translocations in human prostate cancer by expression profiling using Gene Chip Human Exon 1.0 ST arrays. J Mol Diagn 10:50–57

Kacprzyk LA, Laible M, Andrasiuk T, Brase JC, Börno ST, Fälth M, Kuner R, Lehrach H, Schweiger MR, Sültmann H (2013) ERG induces epigenetic activation of Tudor domain-containing protein 1 (TDRD1) in ERG rearrangement-positive prostate cancer. PLoS ONE 8:e59976

Kron KJ, Liu L, Pethe VV, Demetrashvili N, Nesbitt ME, Trachtenberg J, Ozcelik H, Fleshner NE, Briollais L, van der Kwast TH et al (2010) DNA methylation of HOXD3 as a marker of prostate cancer progression. Lab Invest 90:1060–1067

Liu L, Kron KJ, Pethe VV, Demetrashvili N, Nesbitt ME, Trachtenberg J, Ozcelik H, Fleshner NE, Briollais L, van der Kwast TH et al (2011) Association of tissue promoter methylation levels of APC, TGFβ2, *HOXD3*, and *RASSF1*A with prostate cancer progression. Int J Cancer 129:2454–2462

Loriot A, Boon T, De Smet C (2003) Five new human cancer-germline genes identified among 12 genes expressed in spermatogonia. Int J Cancer 105:371–376

Mahapatra S, Klee EW, Young CY, Sun Z, Jimenez RE, Klee GG, Tindall DJ, Donkena KV (2012) Global methylation profiling for risk prediction of prostate cancer. Clin Cancer Res 18:2882–2895

Maier S, Nimmrich I, Koenig T, Eppenberger-Castori S, Bohlmann I, Paradiso A, Spyratos F, Thomssen C, Mueller V, Nährig J et al (2007) DNA-methylation of the homeodomain transcription factor *PITX2* reliably predicts risk of distant disease recurrence in tamoxifen-treated, node-negative breast cancer patients–technical and clinical validation in a multi-centre setting in collaboration with the European Organisation for Research and Treatment of Cancer (EORTC) Patho biology group. Eur J Cancer 43:1679–1686

Naggara O, Raymond J, Guilbert F, Roy D, Weill A, Altman DG (2011) Analysis by categorizing or dichotomizing continuous variables is inadvisable: an example from the natural history of unruptured aneurysms. AJNR Am J Neuroradiol 32:437–440

Nelson WG, De Marzo AM, Yegnasubramanian S (2009) Minireview: epigenetic alterations in human prostate cancers. Endocrinology 150:3991–4002

Pan J, Chen J, Zhang B, Chen X, Huang B, Zhuang J, Mo C, Qiu S (2013) Association between *RASSF1*A promoter methylation and prostate cancer: a systematic review and meta-analysis. PLoS ONE 8:e75283

Park JY (2010) Promoter hypermethylation in prostate cancer. Cancer Control 17:245–255

Paulo P, Ribeiro FR, Santos J, Mesquita D, Almeida M, Barros-Silva JD, Itkonen H, Henrique R, Jerónimo C, Sveen A et al (2012) Molecular sub typing of primary prostate cancer reveals specific and shared target genes of different ETS rearrangements. Neoplasia 14:600–611

Pellacani D, Kestoras D, Droop AP, Frame FM, Berry PA, Lawrence MG, Stower MJ, Simms MS, Mann VM, Collins AT et al (2014) DNA hypermethylation in prostate cancer is a consequence of aberrant epithelial differentiation and hyperproliferation. Cell Death Differ 21:761–773

Phé V, Cussenot O, Rouprêt M (2010) Methylated genes as potential biomarkers in prostate cancer. BJU Int 105:1364–1370

Ploussard G, Epstein JI, Montironi R, Carroll PR, Wirth M, Grimm MO, Bjartell AS, Montorsi F, Freedland SJ, Erbersdobler A et al (2011) The contemporary concept of significant versus insignificant prostate cancer. Int Braz J Urol 37:405–406

Roehl KA, Han M, Ramos CG, Antenor JA, Catalona WJ (2004) Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: longterm results. J Urol 172:910–914

Schulz W (2005) Qualified promise: DNA methylation assays for the detection and classification of human cancers. J Biomed Biotechnol 2005:227–229

Spahn M, Joniau S, Gontero P, Fieuws S, Marchioro G, Tombal B, Kneitz B, Hsu CY, Van Der Eeckt K, Bader P et al (2010a) Outcome predictors of radical prostatectomy in patients with prostate-specific antigen greater than 20 ng/ml: a European multi-institutional study of 712 patients. Eur Urol 58:1–7

Spahn M, Kneitz S, Scholz CJ, Stenger N, Rüdiger T, Ströbel P et al (2010b) Expression of microRNA-221 is progressively reduced in aggressive prostate cancer and metastasis and predicts clinical recurrence. Int J Cancer 127:394–403

Swanson GP, Hussey MA, Tangen CM, Chin J, Messing E, Canby-Hagino E, Forman JD, Thompson IM, Crawford ED, SWOG 8794 (2007) Predominant treatment failure in post prostatectomy patients is local: analysis of patterns of treatment failure in SWOG 8794. J Clin Oncol 25:2225–2229

Swanson GP, Yu C, Kattan MW, Hermans MR (2011) Validation of postoperative nomograms in prostate cancer patients with long-term follow-up. Urology 78:105–109

Thompson IM, Tangen CM, Paradelo J, Lucia MS, Miller G, Troyer D, Messing E, Forman J, Chin J, Swanson G et al (2009) Adjuvant radiotherapy for pathological T3N0M0 prostate cancer significantly reduces risk of metastases and improves survival: long-term followup of a randomized clinical trial. J Urol 181:956–962

Weiss G, Cottrell S, Distler J, Schatz P, Kristiansen G, Ittmann M, Haefliger C, Lesche R, Hartmann A, Corman J et al (2009) DNA methylation of the *PITX2* gene promoter region is a strong independent prognostic marker of biochemical recurrence in patients with prostate cancer after radical prostatectomy. J Urol 181:1678–1685

Xu B, Chevarie-Davis M, Chevalier S, Scarlata E, Zeizafoun N, Dragomir A, Tanguay S, Kassouf W, Aprikian A, Brimo F (2014) The prognostic role of ERG immunopositivity in prostatic acinar adenocarcinoma: a study including 454 cases and review of the literature. Hum Pathol 45:488–497