The Prostate 66:1521–1534 (2006)

Quantitative Multi-Gene Expression Profiling of Primary Prostate Cancer

Uta Schmidt,[1] Susanne Fuessel,[1] Rainer Koch,[2] Gustavo B. Baretton,[3] Andrea Lohse,[1] Silke Tomasetti,[1] Susanne Unversucht,[1] Michael Froehner,[1] Manfred P. Wirth,[1] and Axel Meye[1]*

[1]*Department of Urology, Technical University of Dresden, Germany*
[2]*Institutes of Medical Informatics and Biometry, Technical University of Dresden, Germany*
[3]*Institute of Pathology, Technical University of Dresden, Germany*

BACKGROUND. This study describes the evaluation of the expression patterns of prostate-related transcripts in 106 matched prostate tissues from prostatectomies as predictors for prostate cancer (PCa).
METHODS. Quantitative PCR (QPCR) assays with site-specific hybridization probes were established for four housekeeping genes (*GAPDH, HPRT, PBGD, TBP)* and nine prostate-related genes (*AibZIP, D-GPCR, EZH2, PCA3, PDEF, prostein, PSA, PSCA, TRPM8*).
RESULTS. The relative mRNA expression levels of *AibZIP, D-GPCR, EZH2, PCA3, PDEF, PSA, TRPM8* (all $P < 0.001$) and *prostein* ($P = 0.019$) normalized to the *TBP* reference gene were significantly higher in malignant compared to non-malignant prostate tissues. Employing receiver-operating characteristic (ROC) analyses, *PCA3* was the best single tumor marker with the highest area-under-the-curve (AUC = 0.85). A multivariate logit model for the predictability of the tumor was developed, which employed the relative expression levels of *EZH2, PCA3, prostein*, and *TRPM8* and yielded an AUC of 0.90.
CONCLUSIONS. The transcript marker *PCA3* is a powerful predictor of primary PCa but the inclusion of *EZH2, prostein*, and *TRPM8* adds even more to the diagnostic power. The finding of a significantly higher mRNA expression of three different genes (*prostein, PSA, TRPM8*) in organ-confined tumors compared to non-organ-confined tumors as well as the multi-marker PCa prediction model developed in the retrospective model system on prostatectomies could be of clinical importance for diagnostic purposes, and should be verified in diagnostic biopsies. *Prostate 66: 1521–1534, 2006.* © 2006 Wiley-Liss, Inc.

KEY WORDS: **expression patterns; LightCycler technology; molecular tumor marker; primary prostate carcinoma; quantitative real-time PCR**

INTRODUCTION

In 2005, more than 230,000 new cases of prostate cancer (PCa) were diagnosed in the US, and approximately 30,000 men died of the disease [1]. It is still unclear at present which PCa can be classified as clinically important or clinically insignificant [2]. Therefore, it is necessary to develop means of early diagnosis that can reliably distinguish between benign and malignant forms of cancer in order to choose an appropriate treatment for the individual patient.

Uta Schmidt and Susanne Fuessel contributed equally to this work.

Grant sponsor: Deutsche Forschungsgemeinschaft.

Uta Schmidt's present address is Department of Physiological Chemistry, University of Ulm, Germany.
*Correspondence to: Axel Meye, Department of Urology, Faculty of Medicine, Technical University Dresden, Fetscherstrasse 74, D-01307 Dresden, Germany. E-mail: axel.meye@uniklinikum-dresden.de

Received 13 April 2006; Accepted 14 June 2006
DOI 10.1002/pros.20490
Published online 18 August 2006 in Wiley InterScience (www.interscience.wiley.com).

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The most common way of stratification before surgery is the determination of serum levels of prostate specific antigen (PSA), clinical stage and Gleason score (GS) of the biopsy specimens and, lately, the PSA doubling time using validated nomograms [3,4]. Up to now, no reliable parameter exists for the prediction of organ-confined disease (OCD), the form of PCa that is curable.

Several authors have reported on the prediction of PCa malignancy using transcript profiles assessed by expression analyses using various microarrays or quantitative PCR (QPCR) [5–9]. Interestingly, two microarray studies describing a 5-gene classifier [10] and a 17-gene metastasis signature [11] did not identify well-known tumor-associated genes, thereby provoking the demand for validation studies with large cohorts of patients. Many molecular markers described so far display only a certain level of correlation with PCa presence, disease progression, risk of recurrence, prediction of response to therapy and/or disease-free survival [12]. Although much effort has been invested, no single molecular marker has experienced widespread use, and the GS remains the sole relevant marker for the assessment of clinically significant PCa until now [13]. In general, extensive validation studies on the evaluation of the clinical impact of these potential markers are needed. Therefore, we have chosen the following nine markers for a retrospective validation study using QPCR on matched specimens of malignant and non-malignant prostate tissues of 106 PCa patients.

The pre-selection of the following biomarkers was based on the descriptions of (i) a prostate-associated expression pattern, (ii) a postulated up-regulation of the mRNA levels in PCa, and (iii) a validated gene and transcript structure.

The androgen-induced bZIP gene (*AibZIP*; synonym *CREB3L4*) is located on chromosome 1q21, a region often affected by amplification in PCa [14]. The gene product is characterized by a region of basic amino acids (the so-called ''bZIP domain'') and shows homology to cAMP-responsive element binding protein/activating transcription factors. Immunohistochemical staining of biopsies samples indicated an over-expression in PCa tissue versus non-malignant prostate cells [14].

The Dresden G protein-coupled receptor (*D-GPCR*; synonym: *OR51E1*) is specifically expressed in prostate, and significantly up-regulated in PCa [15]. *D-GPCR* shares 57% identity with the *PSGR* protein (synonym: *OR51E2*), another prostate-specific G protein-coupled receptor [16–18]. G protein-coupled receptors are involved in signaling pathways contributing amongst others to the regulation of a variety of pathological effects [19].

The polycomb group enhancer of zeste homolog 2 gene (*EZH2*) was originally identified through gene expression analyses as frequently over-expressed gene in metastatic and hormone-refractory PCa [20]. Functional studies showed that *EZH2* is a oncogene and its gene locus is specifically amplified in different primary cancers including PCa [21]. The protein functions as a transcriptional repressor and may play a role in the development of the metastatic phenotype [22,23].

The prostate cancer antigen 3 (*PCA3*; synonym: differential display code 3 gene, *DD3*), is an early and specific indicator for PCa [24–26]. The transcript is prostate-specifically expressed, and is highly up-regulated in PCa tissue compared with non-malignant prostate tissue indicating a high diagnostic potential [24–26].

The prostate-derived Ets factor (*PDEF*) acts both as an androgen-independent transcriptional activator of the *PSA* promoter and as an enhancer of the androgen receptor-mediated action on the *PSA* promoter [27]. It was shown to physically interact with the androgen receptor and its counterpart, the homeobox gene *NKX-3.1* [28]. The expression pattern of *PDEF* was shown to correlate with invasive potential in human breast cancers [29]. Corresponding data on PCa are lacking so far.

PSA has emerged as the most important serum tumor marker for PCa [30]. Although the role of serum PSA and its different forms in the diagnosis and follow-up of PCa is unequalled, the importance of the quantification of its transcripts in prostate tissues is not yet clear [31,32].

Prostate stem cell antigen (*PSCA*) is a cell surface protein that maps to chromosome region 8q24, a region harboring also the *MYC* proto-oncogene which is frequently amplified in human cancers [33,34]. Gains of 8q are associated with the development of metastatic disease and an increased risk of dying from PCa making this chromosomal region one of the candidate regions for PCa progression prediction [35]. The levels of *PSCA* protein expression were reported to increase with high GS, advanced stage and bone metastasis [36–39].

Prostein was identified as a prostate-specifically expressed gene using a cDNA library subtraction strategy [40] and expression analysis using microarrays [41]. Its expression both at mRNA and protein levels is highly restricted to the prostate [40,42,43].

TRPM8 (synonym: *trp-p8*) was described as a transcript specifically expressed in prostate and PCa [42]. The gene product shows high homology to the transient receptor potential (trp) family of Ca^{2+} channel proteins [44,45]. A possible role of *TRPM8* in the regulation of intracellular Ca^{2+} levels with effects on cell motility and cell proliferation as well as in different

signaling pathways contributing to PCa progression was proposed [7].

The aim of this study was to evaluate whether one of the nine proposed single markers or a combination of them are predictors for the presence of PCa in a retrospective model setting. After careful assessment of the potential of the different prostate-associated and/or PCa-relevant candidates for comparative analyses, standardized and validated measurements of transcript levels were performed. In addition, the power of the single markers for predicting localized disease was assessed what could be of potential for diagnostic purposes on native biopsies. In order to choose a suitable reference gene for prostate tissue pairs, the mRNA expression levels of four different housekeeping genes were determined in parallel.

MATERIALS AND METHODS

Tumor Patients and Cell Lines

This study was approved by the internal institutional review board of the Technical University of Dresden. Matched pairs of malignant (tumor, Tu) and non-malignant (tumor-free, Tf) tissue samples were examined from 106 patients with primary PCa from whom we had obtained informed consent before surgery.

All patients were treated by radical prostatectomy (RPE) in the Department of Urology at the Technical University of Dresden. The patients' median age was 64 years (range 48–78). The serum levels of PSA were determined the day before surgery (Axsym System; Abbott Diagnostics, Wiesbaden, Germany) and ranged between 1.3 and 57.2 ng/ml (median 8.3 ng/ml). To exclude distant metastasis, a bone scan was performed in cases with a PSA level $\geq$10 ng/ml. All patients were cM0. None of the patients received neoadjuvant hormonal treatment. Tissue specimens were collected and snap frozen directly after RPE and stored in liquid nitrogen until further use.

The histopathological examination of corresponding autologous specimens (Tu and Tf samples), which were simultaneously embedded in paraffin was performed according to the UICC classification system. According to this system, 59 (56%) patients had organ-confined disease (OCD, pT2) and 47 (44%) had non organ-confined disease (NOCD, pT3 and pT4). Ninety-two (87%) patients had a negative lymph node status (pN0), whereas 14 (13%) were pN1. Using the GS system, the sample population was divided into low grade PCa (GS 2–6; n = 28 (26%)), intermediate grade PCa (GS = 7; n = 51 (48%)) and high grade PCa (GS 8–10; n = 27 (26%)).

Of the 106 investigated patients 77 had no PSA relapse after surgery (median follow-up of 32 months), whereas 10 developed a PSA relapse (defined as a PSA value of $\geq$0.2 ng/ml) and 29 received adjuvant treatment.

The prostate cell lines DU 145 (HTB-81), LNCaP (CRL-1740), 22Rv1 (CRL-2505), PC-3 (CRL-1435), and BPH-1 (ACC143) were purchased from ATCC (Manassas, VA) and from DSMZ (Braunschweig, Germany) and were cultivated according to the providers' recommendations.

RNA Isolation and cDNA Synthesis

Only Tu tissue samples with a percentage of $\geq$70% tumor cells in the epithelial cell population and Tf tissue samples with less than 5% of tumor cells in the epithelial cell population were included. The autologous non-malignant tissue was collected as far as possible from the palpable site of the PCa.

Tissue samples were cut with a cryo-microtome into 50–60 slices of 10 μm thickness which were immediately placed in lysis buffer (DCT solution; Invitek, Berlin, Germany). Subsequently, total RNA was extracted by standard procedures (Spin Tissue RNA Mini Kit and Invisorb Spin Cell RNA Mini kit; Invitek). The purity and integrity of the RNA were assessed by UV spectrophotometry and by agarose gel electrophoresis.

Two portions of 500 ng of total RNA were employed in a reverse transcription reaction using Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) and random hexamer primers (Amersham GE Healthcare, Freiburg, Germany) following the manufacturer's instructions. Both cDNA samples were pooled and diluted 1:5. Aliquots of 20 μl of these diluted samples were stored at 4°C until further use. In cases, where 1 μg of total RNA was not available for cDNA synthesis, less total RNA was employed. All raw expression data were primarily normalized to the respective amount of total RNA.

Quantitative Real-Time PCR (QPCR)

Based on the LightCycler (LC) technology (Roche, Mannheim, Germany), quantitative real-time PCR (QPCR) assays were established in order to quantify the mRNA expression of four different housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH;* hypoxanthine phosphoribosyltransferase, *HPRT*, porphobilinogen deaminase, *PBGD;* TATA box binding protein, *TBP*) and 9 prostate-related transcripts (*AibZIP, D-GPCR, EZH2, PCA3, PDEF, prostein, PSA, PSCA, TRPM8*). The amount of a specific PCR product was determined by the quantification of fluorescence signals of site-specific hybridization probes or with TaqMan probes (only for *GAPDH, PCA3*, and *PDEF* assays) (Table I).

TABLE I. Primers and Hybridization Probes for QPCR Assays

Gene name [synonym] (length of QPCR fragment)	Acc. no./ gene ID*	Primer/probe	Sequence 5′-3′	Nucleotide position
PBGD	NM_000190/	PBGD for	GCTgCAACggCggAA	173–187
[HMBS]	3145	PBGD rev	CCTgTggTggACATAgCAATgATT	307–330
(158 bp)		PBGD FL	TCgCATACAgACggACAgTgTggTg-FL	244–268
		PBGD LC	LC Red640-CAACATTgAAAgCCTCgTACCCTgg-PH	270–294
HPRT	NM_000194/	HPRT for	ATCAgACTgAAgAgCTATTgTAATgACCA	383–411
[HPRT1]	3251	HPRT rev	TggCTTATATCCAACACTTCgTg	591–613
(231 bp)		HPRT FL	AgACTTTgCTTTCCTTggTCAggCAgT-FL	516–542
		HPRT LC	LC Red640-AATCCAAAgATggTCAAggTCgCAAgC-PH	545–571
TBP	NM_003194/	TBP Linja for	gAATATAATCCCAAgCggTTTg	803–824
(226 bp)	6908	TBP Linja rev	ACTTCACATCACAgCTCCCC	1028–1009
		TBP Linja FL	TTTCCCAgAACTgAAAATCAgTgCC-FL	886–862
		TBP Linja LC	LC Red640-TggTTCgTggCTCTCTTATCCTCATg-PH	860–835
AibZIP	NM_130898/	AibZIP for	TgTAggCCTTATCTCCATCCA	664–684
[CREB3L4]	148327	AibZIP rev	gCTACggTgCCTgCTCTg	789–772
(126 bp)		AibZIP FL	ggCAgCTCACTgACCATgCAggA-FL	744–722
		AibZIP LC	LC Red640-TCAggCACCATAAATgCTgggCTC-PH	720–697
D-GPCR	AY698056/	Olf R78 for	ggTCACACATTCCTTCCATACg	22–43
[OR51E1]	143503	Olf R78 rev	AAgAAATATATACATgggCTCATgCA	287–262
(266 bp)		Olf R78 FL	TCAgTTCTggTTggCCTTCCCATT-FL	167–190
		Olf R78 LC	LC Red640-TgCTCCCTCTACCTTATTgCTgTgCT-PH	192–217
EZH2	NM_004456/	EZH2 for	gCCAgACTgggAAgAAATCTg	171–191
(277 bp)	2146	EZH2 rev	TgTgTTggAAAATCCAAgTCA	447–427
		EZH2 FL	AACCTCTTgAgCTgTCTCAgTCgCA-FL	261–237
		EZH2 LC	LC Red640-TACTCTgATTTTACACgCTTCCgCC-PH	234–221
PCA3	AF103907/	DD3 for	TgTTTTTgCACATTTCCAgC	402–421
[DD3]	50652	DD3 521rev	gggCgAggCTCATCgAT	521–505
(120 bp)		DD3 Taq	6FAM-AgAAATgCCCggCCgCCATC-XT-PH	478–497
PDEF	NM_012391/	PDEF Ex4 for	CTggATgAAAgAgCggACTTC	1054–1074
[SPDEF]	25803	PDEF Ex5 rev	TTgAggAACTgCCACAggTg	1182–1163
(129 bp)		PDEF Taq Ex5	6FAM-CgAggTggACTCATCATgCTCCggTg-XT-PH	1152–1129
Prostein	NM_033102/	Pro for	gCCAggATCTgAgTgATgAgA	49–69
[SLC45A3]	85414	Pro rev	gTTCAggCACTCCAgAACTg	252–233
(204 bp)		Pro FL	CggTCCAgCTTCTCAgCCCA-FL	131–112
		Pro LC	LC Red640-gCTCAACACCTgCTgCTgTggg-PH	110–89
PSA	NM_001648/	PSA 494 for	TgCCCACTgCATCAggAACA	230–249
[KLK3]	354	PSA Gao rev	CATCACCTggCCTgAggAATC	387–367
(158 bp)		PSA FL	ATTTCAggTCAgCCACAgCTTCCC-FL	308–331
		PSA LC	LC Red640-CACCCgCTCTACgATATgAgCCTCC-PH	333–357
PSCA	NM_005672/	PSCA for	CCCTgCAgCCAggCACT	60–76
(133 bp)	8000	PSCA rev	AggCCAACTgCgCggAT	192–176
		PSCA FL	CCTgCAggCAgTCCTCgTTgCTC-FL	131–109
		PSCA LC	LC Red640-CCTgggCTTTgCAggAgTAgCACA-PH	107–84
TRPM8	NM_024080/	Trp-P8 for	ACgCTTgTgTACCggAATCT	1547–1566
[trp-p8]	79054	Trp-P8 rev	CgAgTAATAggAgACACgTCg	1713–1693
(167 bp)		Trp-P8 FL	TTTCCAgACAAACgTgAggAgggC-FL	1618–1595
		Trp-P8 LC	LC Red640-CATTATAggAATTCTTggCgATCTgCA-PH	1592–1566

FL, fluorescence dye fluorescein; Red640, fluorescence dye LC Red640; PH, phosphorylated 3′-end; FAM, 6-Carboxy-fluorescein; XT, 6-Carboxytetramethylrhodamin.
*www.ncbi.nlm.nih.gov

The PCR assays were performed using 2 μl of the diluted cDNA. The kits ''LC FastStart DNA Master Hybridization Probes'' (Roche) and ''RoboGene *GAPDH* Real-Time Reagent Mix'' (Roboscreen, Leipzig, Germany) were used for amplification.

All measurements were performed with aliquots of the same cDNA dilutions within short time periods to ensure standardized and comparable conditions. All QPCR assays were carried out at least twice as independent PCR runs for each cDNA sample. Samples were measured for a third time if differences of >30% occurred. The means of all measurements were used for further calculations. Positive (cDNA from the PCa cell line LNCaP) and negative controls (without template) were measured in each of the PCR runs.

The mRNA copy number of a single marker was calculated in relation to the amplification product amounts of external standards. Quantity standard curves were generated employing LC capillaries storage-stable coated with amounts of 10^1–10^7 molecules of HPLC-calibrated PCR fragments [46]. All generated quantity standard curves of the single markers were compared with regard to their reproducibility and reliability. The transcript amounts were calculated by the automated analysis mode of the LC-software 3.5.

Relative expression levels of prostate-related markers were obtained by normalization to the reference gene (zmol transcripts of the marker per zmol transcripts of the reference gene).

Statistics and Correlation of the Quantification Results to the Clinical Data

Patients were subdivided into groups according to the clinically relevant parameters (GS, T, and N stage). Statistical analyses were performed using the SAS software (SAS Institute, Inc., Cary, NC) and SPSS software (SPSS, Inc., Chicago, IL).

From each patient's material, one value for the Tu specimen and one value for the autologous Tf specimen per transcript were generated. Because these two values resulted from the same patient, a paired analysis with corresponding values could be taken into consideration. However, since the experimental unit is not the patient but the single tissue specimen, and the intra-individual correlation proved negligible, unpaired analyses were also used.

Since the relative expression levels were not distributed normally, these values were log-transformed, and tested for Gaussian distribution again. The log-transformed relative expression levels were sufficiently normally distributed and therefore used for comparisons employing Student's *t*-test. The log-transformed relative mRNA expression levels of the different markers were compared between matched pairs of Tu and Tf specimens using the paired Student's *t*-test. An unpaired homoscedastic *t*-test was used to evaluate the suitability of the mRNA expression levels of single markers to differentiate between the specific clinical parameter groups in Tu samples.

Furthermore, receiver-operating characteristic (ROC) curves were calculated in order to assess the diagnostic power of each separate variable univariately and for the multivariate diagnostic rule by the area under curve (AUC) of the ROC curve. The ROC curve shows sensitivity and specificity of the binary diagnostic decision for varying cut points based on a single quantitative diagnostic variable or based on a multivariate diagnostic rule. The AUC of the ROC is an estimation of the rate of correct diagnoses. The 95% confidence limits of the AUC values were calculated as described [47]. Differences between ROC curves were evaluated using a Wald test.

The multivariate diagnostic rules are based on optimized logistic regression models comprising optimal sets of competing variables and optimal cut points for each variable. All variables were divided into two to four classes by varying and optimizing cut points. The validity and quality of the resulting logit models were judged by Hosmer and Lemeshow goodness-of-fit tests [48], by the Akaike information criterion [49], by *P*-values of each regression parameter, and by the estimated AUC. The latter was performed by both varying cut points as well as with continuous data over the predicted absolute probability for the origin of the tissue specimen for tumor prediction. This probability can be calculated for each individual case by a simple addition of regression parameters depending on the original values of the variables and subsequent transformation from the logit model into probability.

An extern validation of the logit model in the sense of prospective application on independent series of patients has not been carried out, but as an alternative, the cross-validated estimation of the AUC is given. This based on a one-step approximation by always elimination of one case from the sample, estimation of the model parameters from the remaining sample, use of the resulting model on the removed case, and finally averaging of all errors of prediction.

RESULTS

Standardization of the Real-Time QPCR Assays

Highly sensitive real-time QPCR assays based on the LC technology were established for the transcript quantification of the nine prostate-related and the four reference genes. The run-to-run performance of each assay was assessed performing at least 23 PCR runs per

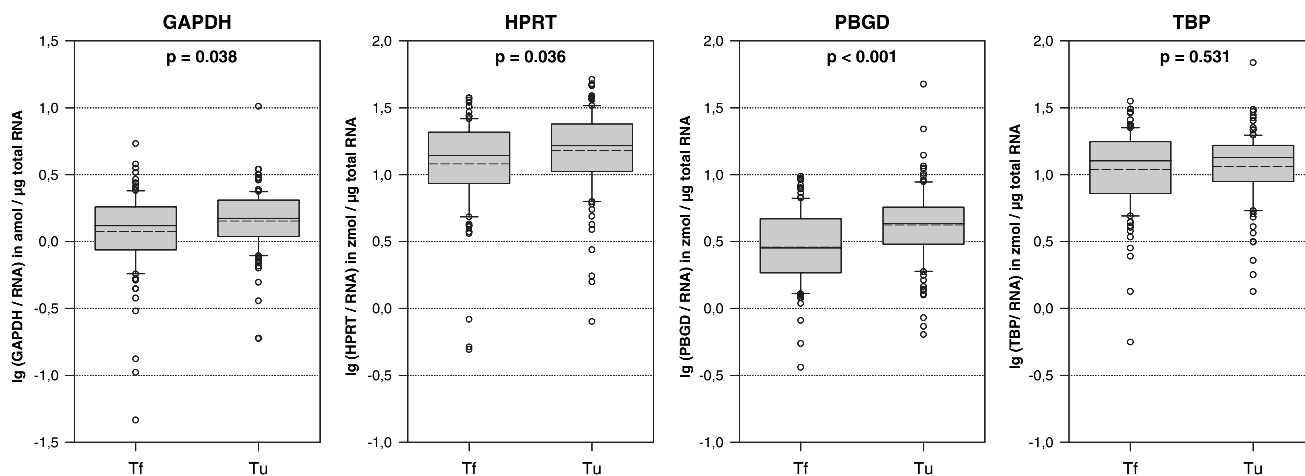

Fig. 1. Boxplots of mRNA expression levels of different reference genes. Shown is the distribution of the log-transformed transcript levels of four different reference genes (normalized to the amount of RNA) in corresponding malignant (Tu) and non-malignant (Tf) prostate tissue samples of 106 patients with primary PCa. The boxes within the plots represent the 25–75th percentiles. The mean values are depicted as dashed and the medians as solid lines. White circles indicate outlier values outside of the 10th and 90th percentiles. Statistical differences were calculated by an unpaired *t*-test.

gene. Thereby, the use of capillaries coated with external DNA standards was of particular importance for improving the reproducibility and lowering the detection and quantification limits of each particular assay. Taken together, the mean slopes of the regression curves (measured vs. coated molecule numbers) for the reference and prostate-related genes ranged from 0.984 (*GAPDH*) to 1.149 (*TBP*). The correlation for these curves was at least 99.9%, standard deviations for all 13 QPCRs were <10% in average (range 4%–18%). The median slope of the quantity standard curves ranged from −3.575 to −3.341 indicating a good PCR performance for all tests (data not shown).

Choice of a Suitable Reference Gene for Prostate Tissues

Before comparing the relative mRNA expression levels of a particular prostate-related gene in Tu and Tf tissues, it was sought to evaluate the usefulness of four different reference genes (*GAPDH*, *HPRT*, *PBGD*, *TBP*) for the intended analyses. Therefore, the log-transformed mRNA expression levels of these housekeeping genes in Tu specimens, which had been normalized before to the amount of total RNA per cDNA reaction were compared with the ones of the Tf specimens.

In the analyzed patient cohort, significant differences for the commonly used housekeeping genes *GAPDH* ($P = 0.038$), *HPRT* ($P = 0.036$), and *PBGD* ($P = 0.00003$) were assessed by unpaired *t*-test. The only housekeeping gene being not differentially expressed between Tu and Tf tissues was *TBP* ($P = 0.531$) (Fig. 1). Therefore, expression levels of *TBP* were used for normalization.

Differential Expression of Prostate-Related Genes

Median relative expression levels of the prostate-related genes in Tu and Tf specimens were calculated (Table II). For both Tu and Tf specimens, the genes with the lowest and the highest median relative mRNA expression levels were *EZH2* and *PSA*, respectively. All prostate-related genes showed a range of the relative expression levels over two to five orders of magnitude in both Tu and Tf tissues. In comparison to that, for all PCa-associated transcript markers, except for *EZH2*, the relative expression levels in the five prostate-derived cell lines were one to three orders of magnitude lower than in the Tu tissue specimens (Table II). The log-transformed relative mRNA expression levels were significantly higher in Tu than in Tf tissues for *AibZIP*, *D-GPCR*, *EZH2*, *PCA3*, *PDEF*, *PSA*, *TRPM8* (all $P < 0.001$), and *prostein* ($P = 0.018$; paired *t*-test).

Furthermore, the ratios Tu:Tf of the relative expression levels were calculated for each variable of each matched tissue pair (Fig. 2). These ratios are another means of assessing the differential expression between the two corresponding samples of each patient. The highest ratios of over-expression (Fig. 2) were observed for *PCA3* (median 37.5-fold) and *TRPM8* (median 3.7-fold). *D-GPCR*, *EZH2*, *PDEF*, and *AibZIP* showed a nearly two-fold up-regulation in Tu related to Tf in the paired analyses. In contrast, the transcripts of *PSA*, *prostein*, and *PSCA* displayed median Tu:Tf ratios in the range of 1.4–1.0 (Fig. 2).

TABLE II. Relative Transcript Levels of Prostate-Related Genes in Prostate Tissues and Prostate-Derived Cell Lines

	Prostate tissues		Prostate cell lines				
Gene*	Malignant (Tu) Median (min to max)	Non-malignant (Tf) Median (min to max)	LNCaP Mean	22Rv1 Mean	PC-3 Mean	DU145 Mean	BPH-1 Mean
AibZIP	24.5 (3.36 to 74.3)	13.7 (0.52 to 59.2)	9.863	5.901	1.108	0.758	0.298
PCA3	35.4 (0.04 to 389)	0.57 (0.02 to 213)	0.100	0.030	0.001	0.001	0.001
D-GPCR	3.81 (0.04 to 136)	1.55 (0.02 to 29.9)	0.195	0.003	0.003	0.001	0.002
EZH2	1.06 (0.35 to 5.86)	0.53 (0.13 to 19.9)	2.143	6.423	1.714	6.036	2.405
PDEF	23.3 (2.01 to 54.1)	12.9 (0.17 to 59.0)	5.168	3.950	1.382	0.035	0.004
Prostein	16.8 (1.63 to 90.7)	16.3 (0.27 to 84.9)	1.135	0.860	0.101	0.030	0.021
PSA	367 (18.9 to 1350)	226 (0 to 1685)	5.384	1.997	n.d.	n.d.	n.d.
PSCA	2.16 (0.02 to 732)	1.89 (0.01 to 158)	0.063	0.204	0.007	0.032	0.097
TRPM8	35.9 (0.18 to 428)	9.37 (0.03 to 77.7)	1.124	0.004	0.017	n.d.	0.001

max, maximum; min, minimum; n.d., (transcript) not detectable.
*Data for the prostate-related genes are given for the measured relative expression levels (zmol gene/zmol *TBP*).

Univariate and Multivariate Analyses for the Prediction of Malignant Prostate Tissue

In order to assess the diagnostic power of the individual prostate-related transcripts, ROC curves were generated, and the AUC were calculated for every single parameter. *PCA3* is the marker with the highest AUC value (0.85) indicating the best performance as a single tumor marker (Table III, Fig. 3). As an example, choosing a sensitivity of 95%, this would result in a specificity of 46%, a positive predictive value of 64%, and a negative predictive value of 91% when using a cut off value of 0.4 zmol *PCA3*/zmol *TBP*. *EZH2* and *TRPM8* also had AUC values of more than 0.80 thus performing better than the other single markers (Table III).

Furthermore, the data were analyzed with regard to a multivariate model comprising the most suitable genes. After having analyzed the contribution of each marker, a logit model was developed. This model based on the expression levels of *EZH2*, *PCA3*, *prostein*, and *TRPM8* and yielded an AUC of 0.90 (Table IV, Fig. 3).

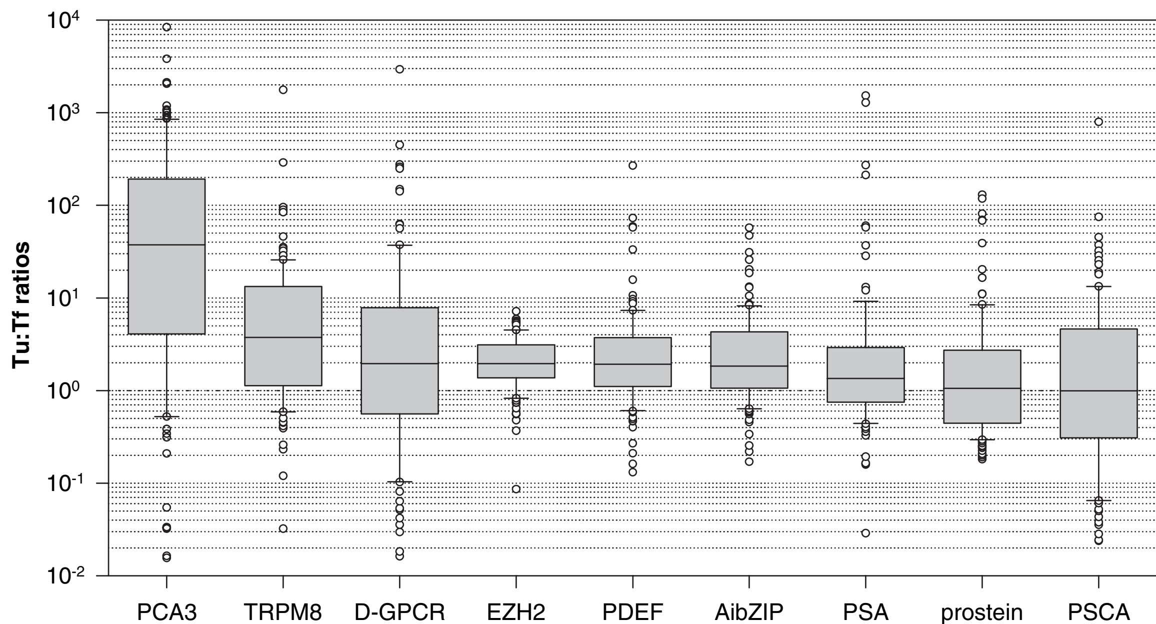

Fig. 2. Differential expression of prostate-related genes in non-malignant (Tf) and malignant (Tu) prostate tissues. For estimation of the individual expression of the prostate-related genes (normalized to transcript amounts of *TBP*) the Tu:Tf ratios of the paired tissue specimens were calculated. The boxes within the plots represent the 25–75th percentiles. The medians are depicted as solid lines. White circles indicate outlier values outside of the 10th and 90th percentiles.

TABLE III. Calculation of AUC Values for the Relative Expression Levels of Prostate-Related Genes by ROC Analyses

Gene*	AUC	SE	95% CI
AibZIP	0.767	0.078	0.615–0.919
D-GPCR	0.645	0.069	0.510–0.781
EZH2	0.814	0.082	0.654–0.974
PCA3	0.851	0.085	0.685–1.018
PDEF	0.765	0.078	0.612–0.918
Prostein	0.554	0.059	0.438–0.669
PSA	0.630	0.066	0.501–0.760
PSCA	0.516	0.059	0.401–0.631
TRPM8	0.813	0.082	0.652–0.974

SE, standard error; 95% CI, 95% confidence interval.
*Data for the prostate-related genes are given for the measured relative expression levels (zmol gene/zmol *TBP*).

Because a linearity of the quantitative variables in the logit for tumor tissue could not be assumed without further ado, all variables were divided into two to four classes resulting in different cut points to distinguish between Tu and Tf tissues (Table IV). The Hosmer and Lemeshow goodness-of-fit test yielded a value of $P = 0.47$ indicating a good performance of this model.

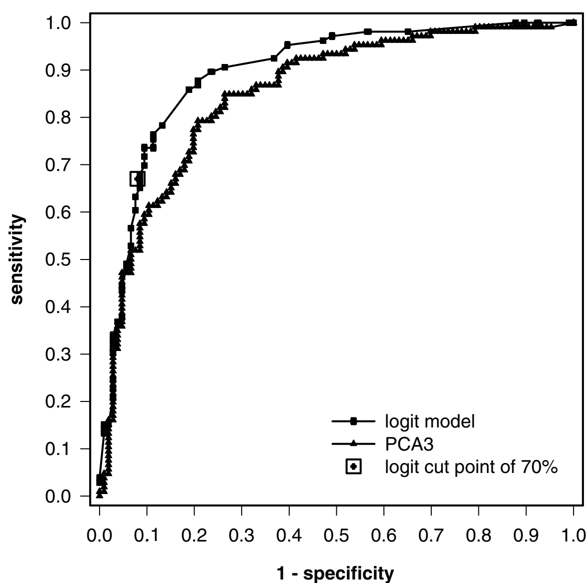

Fig. 3. Comparison of the univariate model for *PCA3* with the logit model based on the 4-gene signature. ROC curves are shown for *PCA3* as single marker (AUC = 0.85) in comparison to the multivariate logit model comprising *EZH2*, *PCA3*, *prostein* and *TRPM8* (AUC = 0.90). The both ROC curves are significantly different ($P = 0.0015$). For the marked cut-point of 70% (indicated by a square) of the predicted probability for malignant tissue, the sensitivity of the logit rule results to 67.0%, and the specificity results to 91.5%.

Bootstrap cross-validated estimation of the AUC yielded 0.87. At the example sensitivity of 95%, the model's specificity would be 61%, the positive predictive value (PPV) 71%, and the negative predictive value (NPV) 93%.

The result of the pure quantitative prediction model represents a defined probability and replaces therefore PPV and NPV. Taking patient no. 1 as a representative example for the multivariate prediction model (Table IV), using the diagnostic rule, a probability of 90.9% for the presence of malignant cells was calculated at the relative expression levels of the 4 marker candidates in the Tu sample (*prostein*/*TBP* = 17.02, *EZH2*/*TBP* = 1.76, *TRPM8*/*TBP* = 29.11, *PCA3*/*TBP* = 23.77). Moreover, considering the measured values of the corresponding Tf tissue (*prostein*/*TBP* = 2.01, *EZH2*/*TBP* = 1.17, *TRPM8*/*TBP* = 1.86, *PCA3*/*TBP* = 0.396), a predicted probability of being malignant was calculated at 6.9%, that is, a predicted probability of 93.1% for being non-malignant. For the 106 analyzed Tu samples a mean probability for the presence of malignant cells of 74.7% (median 80.5%) was observed.

Using a Wald test, the contrast between the univariate model (relative mRNA expression levels of *PCA3* only) and the logit model was calculated to be significant ($P = 0.0015$, Bonferroni adjustment) indicating a better performance of the multivariate model [47].

Subgroup Analysis and Correlation With Relevant Clinico-Pathological Parameters

The log-transformed relative mRNA expression levels of the investigated prostate-related genes of the Tu tissue samples were subsequently analyzed for associations with GS, T, and N stage of the affected patients they originated from.

When comparing the different GS categories with each other, only one of the genes showed increasing relative expression levels in the Tu specimens with rising GS. *D-GPCR* was the only gene with continuously rising relative expression levels starting from low GS to high GS (not significant, data not shown). The mRNA expression of *PSA* ($P = 0.026$) and *prostein* ($P = 0.032$) decreased in a ladder-like fashion from low GS to high GS tumors (data not shown). For both markers the relative mRNA expression differed significantly between intermediate and high-grade tumors (*prostein*: $P = 0.016$, *PSA*: $P = 0.001$), but not between low-grade and intermediate-grade tumors. The relative expression levels of *PCA3* also decreased with rising grade, however these differences were not significant (data not shown). As for the relative expression levels of the other five prostate-related

TABLE IV. Diagnostic Rule of a Logit Model for the Prediction of a Prostate Tumor

Gene	Regression coefficient	SE	P	OR	95% CI	Value for calculation example	Logit contribution for example
Constant	−2.605	0.597					−2.605
Prostein							
<13.5 (ref.)	0						
13.5 to <32	−1.332	0.532	0.0123	0.264	0.09–0.72	17.02	−1.332
≥32	−1.963	0.593	0.0009	0.140	0.04–0.43		
EZH2							
<1.2 (ref.)	0						
≥1.2	1.765	0.532	0.0009	5.84	2.17–17.91	1.76	1.765
TRPM8							
<6 (ref.)	0						
6 to <19	1.597	0.659	0.0154	4.94	1.42–19.26		
19 to <42	2.258	0.772	0.0034	9.57	2.23–46.83	29.11	2.258
≥42	2.965	0.848	0.0005	19.39	3.82–109.0		
PCA3							
<0.4 (ref.)	0						
0.4 to <20	1.253	0.604	0.0381	3.50	1.12–12.45		
20 to <47	2.212	0.800	0.0057	9.13	2.01–47.97	23.77	2.212
≥47	2.903	0.799	0.0003	18.23	4.06–95.63		
						Logit =	2.298

The calculation example is for patient no. 1. In this case, the probability p for malignant tissue results from the transformation.
$p = \exp(\text{logit})/[1 + \exp(\text{logit})] = \exp(2.298)/[1 + \exp(2.298)] = 0.909 = 90.9\%$.
SE, standard error; p, tail-area probability of the test statistic; OR, odds ratio; 95% CI, 95% confidence interval.
Cut points used are given as measured relative expression levels (zmol gene/zmol *TBP*).

genes, no significant differences between the three GS categories were found.

Since the extent of the PCa is the most relevant criterion for a therapeutic decision, the mRNA expression patterns of the prostate-related markers were compared between the Tu specimens derived from OCD and NOCD. Remarkably, significantly higher relative expression levels were found for *prostein*, *PSA*, and *TRPM8* in OCD compared to NOCD (unpaired *t*-test; Fig. 4). The relative expression levels of *D-GPCR* increased continuously from OCD to NOCD, whereby the lowest transcript levels were found in Tf tissue samples. In comparison to that, the observed up-regulation of *PCA3* and *AibZip* in the Tu specimens compared to the Tf specimens was independent from T stage (data not shown). Significant differences for *AibZIP*, *EZH2*, *PCA3*, *PDEF* (all $P < 0.001$), and *D-GPCR* ($P = 0.012$) were found between NOCD and Tf tissues but not between OCD and NOCD.

Concerning the lymph node status, no significant differences between relative mRNA expression levels in the Tu samples from pN0 and pN1 patients were observed for any of the genes. However, the relative *D-GPCR* expression levels were higher in Tu samples of pN1 patients than in those of pN0 patients or than in Tf samples ($P = 0.15$).

Correlation of Gene Expression With Treatment Failure

Comparing the relative expression levels of single prostate-related genes with the patients' outcome, no significant differences were assessed between patients with undetectable PSA after surgery (n = 77) and patients who developed a PSA relapse (n = 10). In contrast, statistically significant differences were observed between the relative expression levels of *AibZIP* ($P = 0.049$), *PDEF* ($P = 0.01$), *prostein* ($P = 0.006$), and *PSA* ($P = 0.04$) in the Tu tissues of patients without a PSA relapse and patients who had received adjuvant therapy since they had NOCD at the time of surgery (n = 29). As for all these genes, the relative gene expression levels were lower in the patients with adjuvant treatment (data not shown).

DISCUSSION

The present study describes the characterization of mRNA expression patterns of nine prostate-related genes in a cohort of 106 patients with primary PCa to find single candidates or marker combinations useful for the prediction of PCa. Additionally, a subset of three genes specifically up-regulated in OCD compared to

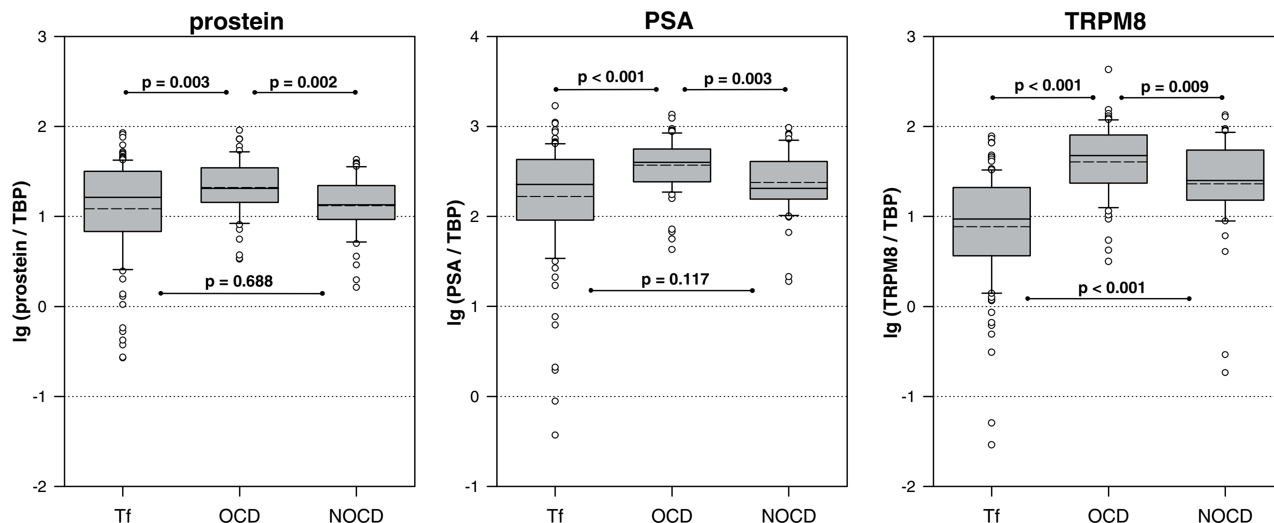

Fig. 4. Correlation of relative mRNA expression levels of *prostein*, *PSA* and *TRPM8* with T-stage. The log-transformed relative expression levels of *prostein*, PSA and TRPM8 were compared between Tf (n = 106), OCD (n = 59) and NOCD (n = 47) by an unpaired *t*-tests. The boxes within the plots represent the 25–75th percentiles. The mean values are depicted as dashed and the medians as solid lines. White circles indicate outlier values outside of the 10th and 90th percentiles.

NOCD was identified which could be of potential impact on the predictability of OCD.

Four different housekeeping genes (*GAPDH*, *HPRT*, *PBGD*, *TBP*) were evaluated for their usefulness in this comparative approach. Among others, these genes had been described in a study aiming at identifying a reference gene that can replace multiple gene measurements [50,51]. In contrast to the data for a limited number (n = 16) of matched tissue pairs (Tu and Tf) from different tumor entities presented in the study by de Kok et al. [50,51], we identified *TBP* as the only one out of the four housekeeping genes that is not differentially expressed between matched Tu and Tf prostate tissues.

Moreover, the results of comparative QPCR studies of tissue pairs using *TBP* for normalization could be influenced also by malignancy-associated changes of the autologous tumor-free prostate tissue specimen. A trained pathologist systematically collected the Tf samples used for this study from the prostate gland distant from the location of the PCa. Moreover, comparing the relative expression levels in another patient cohort (22 tissue pairs) for the same nine prostate-associated transcript markers, no significant differences were found between normalization to *TBP* and the prostate-associated gene of the androgen receptor indicating the usefulness of *TBP* (data not shown). However, future studies should evaluate the role of malignancy-associated changes as well as the heterogeneous composition of PCa tissues in detail [52].

Recently, a number of reports insisted on the use of multiple markers for the detection and the stratification of PCa according to their aggressiveness, the prediction of prognosis and/or on the validation of new markers using independent patient cohorts [5–7]. Most of the studies were performed by genome-wide expression analyses using microarrays with the intent to identify new molecular PCa markers and to characterize expression signatures specific for particular PCa subgroups. Several of these promising candidates emerged from gene expression profiling were selected for our comparative QPCR study such as *EZH2* and *TRPM8* which were described as prognostic PCa markers of disease progression and relapse [7,20]. Specific gene signatures for the prediction of metastasis and clinical outcome were identified and proven as useful adjuncts to histological examination in some of the genome-wide microarray studies [10,11].

Furthermore, a recent report described—based on the definition of a stem cell-like gene expression profile—a ''death-from-cancer'' expression signature analyzed by microarray studies which predicted also a therapy failure for 10 different tumor entities including PCa [53]. Based on a previously characterized multi-gene expression profile [54] a 11-gene set was identified which showed a reliable prognostic power to predict a recurrence for different carcinoma types. Remarkably, none of these genes was known to be specifically expressed in the prostate or altered in PCa. This 11-gene signature also measured by QPCR is a more uniform therapy-outcome predictor across multiple data sets compared with the individual genes [54].

Only few studies describing PCa prediction models which based on altered expression patterns of different

genes were performed by QPCR—a transcript quantification method exhibiting an obviously higher sensitivity than microarray expression analyses. One QPCR study on gene expression profiling of clinically localized PCa identified 46 out of selected 291 genes being differentially expressed between PCa relative to normal prostate [55]. These analyses initially employed different pools of prostate tissues before measuring the expression levels of pre-selected genes in a few paired samples of malignant and normal prostate specimens. Interestingly, a 4-gene expression model was deduced that discriminated between PCa patients with and without relapse [55]. As the most promising single markers which were up-regulated in PCa compared to normal prostate emerged *CDKN2A*, *GRPR*, and *PCA3* with AUC values of 0.969, 0.88, and 0.829, respectively [55]. Employing the same strategy, 19 out of 37 quantified genes with significant differential expression in hormone-refractory PCa in comparison to localized PCa were identified as potentially usable for the estimation of tumor progression or therapeutic decisions [56].

Also for the discrimination between benign prostatic hyperplasia (BPH) and PCa the expression patterns of selected genes were analyzed by QPCR and validated by immunohistochemistry revealing promising candidates such as δ-catenin, PSMA, hepsin, *PCA3*, and GALNT3 as potentially suitable markers for the diagnosis and management of PCa [57]. When combining the expression data for the last four of the mentioned genes in a logistic regression model an excellent predictive power for the discrimination between BPH and PCa was attained [6].

For a retrospective validation study, we employed matched tissue specimens of—in comparison to other reports—a relatively large cohort of 106 patients with primary PCa for quantitative mRNA measurements in order to develop a model for PCa prediction. The data on the differential expression of 9 selected prostate-related genes gives the evidence in favor of *PCA3* as the most suitable single marker since it performed best in our ROC analysis. In our study cohort the median *PCA3* transcript level was >37-fold higher in Tu specimens compared to the corresponding Tf specimens (Fig. 2). The expression data for *PCA3* support other reports describing the enormous diagnostic potential of *PCA3* based on the high specificity and sensitivity (reviewed in [26]). The usefulness of the relative expression levels of *PCA3* as a single PCa marker does perform as well as or even better than conventional serum PSA testing [58] as indicated by a specificity of 46% at a desired sensitivity of 95%. Nevertheless, the validity of the individual transcript markers should be re-assessed carefully since numerous of studies used unmatched tissue specimens and smaller patient cohorts. For example, *PSCA* did not exhibit any differential mRNA expression between the matched prostate tissue pairs in our analyses which is contrary to other reports [36–39].

The developed multivariate diagnostic rule for the prediction of PCa is based on optimized logistic regression models applying two to four subclasses for each gene by varying and optimizing cut points (Table IV). The cut points had to be optimized since the analyses of the continuous data indicated a non-linearity of the relative expression levels of the included genes. This multivariate PCa prediction model using the proposed 4-gene expression signature (*EZH2*, *PCA3*, *prostein*, *TRPM8*) seems to be preferable in comparison to single markers and should be re-evaluated in a suitable prospective study using diagnostic prostate biopsies. Interestingly, three of the four genes contributing to the prediction model displayed an evident discriminative power when used as single marker (Table III) whereas *prostein* seemed to be essential for the marker combination.

Additionally, the relative expression levels for three of the selected genes (*prostein*, *PSA*, *TRPM8*) appeared useful for the predictability of OCD (Fig. 4). Remarkably, *TRPM8* represents an attractive single marker since its expression levels might not only allow the discrimination between OCD and NOCD but also showed significantly higher expression levels in the Tu tissue specimens derived from NOCD compared to Tf specimens (Fig. 4). Independently, this observation indicates that the mentioned three genes are up-regulated already in localized primary PCa, whereas this over-expression is lost again in advanced tumor stages. A putative association with the transition to an androgen-independent growth could be assumed since these three genes are expressed in androgen-dependent manner [30,40,45]. Interestingly, one study by Fromont et al. also revealed a linear decrease of the transcript levels of several genes from pT2 to pT3 and to hormone-refractory PCa suggesting a validation of these candidates with regard to the prediction of OCD [56].

Only a limited number of studies for PCa, all using paraffin-embedded prostate biopsies reported the applicability of genetic profiling including the determination of multiple DNA polymorphisms for PCa risk estimation [59]. In comparison, gene expression array studies for PCa diagnostic and prognostic purposes, which were usually conducted on RPE specimens have emphasized several promising marker candidates, but are limited by the detection sensitivity regarding the single gene markers.

Our retrospective data for the model of PCa tissue pairs derived from RPE excisions indicate a potential for different applications using frozen prostate biopsies.

Preliminary data from an ongoing pilot study indicate the transferability of the QPCR-based expression profiling to frozen biopsy specimens collected for histological diagnosis of the PCa. The RNA amount extracted from one half of a freshly frozen biopsy was sufficient for robust QPCRs for at least 10 different genes. For most of the selected genes similar relative expression levels as in the RPE specimens were assessed (unpublished data).

Further on, the extension of the transcript pattern by other potential biomarker transcripts including PCA-1, TRMPSS2, Hepsin, and PSGR is planned [6,18,60,61].

Moreover, future prospective studies for diagnostic biopsies should also compare the diagnostic significance of a transcript gene signature as described in this study in comparison to the significance of other biomarkers including GSTP1, AMACR based on the detection at the DNA or the protein levels as described (reviewed in [61]).

In summary, a multivariate tumor prediction model based on a 4-gene signature was developed applying a standardized and sensitive QPCR profiling of nine genes known for their prostate-specific or PCa-associated mRNA expression in matched tissue samples derived from 106 prostatectomized patients. This tumor-predictive combination of markers and the identified up-regulation of three candidates particularly in organ-confined PCa should be re-evaluated in prospective studies, for example, on diagnostic prostate biopsies. The validation of the usefulness of these specific expression signatures for the prediction of tumor presence and especially of OCD might facilitate therapeutic decisions in the future.

ACKNOWLEDGMENTS

We thank Mrs. R. Kranz from our laboratory (Department of Urology) for her excellent technical assistance. We thank Dr. M. Haase and Dr. M. Toma (Institute of Pathology, Technical University Dresden) for the histopathological re-evaluation of prostate tissue pairs. We are indebted to Dr. M. Kotzsch and Mrs. A. Zobjack for their thorough tissue asservation, and Dr. K. Kraemer for critical reading of the manuscript. This study was supported by a grant from the Deutsche Forschungsgemeinschaft (Me-1649, to A.M., M.F., and G.B.).

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