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RESEARCH PAPER

CDO1 promoter methylation is associated with gene silencing and is a prognostic biomarker for biochemical recurrence-free survival in prostate cancer patients

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ABSTRACT

Molecular biomarkers may facilitate the distinction between aggressive and clinically insignificant prostate cancer (PCa), thereby potentially aiding individualized treatment. We analyzed *cysteine dioxygenase 1 (CDO1)* promoter methylation and mRNA expression in order to evaluate its potential as prognostic biomarker. *CDO1* methylation and mRNA expression were determined in cell lines and formalin-fixed paraffin-embedded prostatectomy specimens from a first cohort of 300 PCa patients using methylation-specific qPCR and qRT-PCR. Univariate and multivariate Cox proportional hazards and Kaplan-Meier analyses were performed to evaluate biochemical recurrence (BCR)-free survival. Results were confirmed in an independent second cohort comprising 498 PCa cases. Methylation and mRNA expression data from the second cohort were generated by The Cancer Genome Atlas (TCGA) Research Network by means of Infinium HumanMethylation450 BeadChip and RNASeq. *CDO1* was hypermethylated in PCa compared to normal adjacent tissues and benign prostatic hyperplasia ($P < 0.001$) and was associated with reduced gene expression ($\rho = -0.91$, $P = 0.005$). Using two different methodologies for methylation quantification, high *CDO1* methylation as continuous variable was associated with BCR in univariate analysis (first cohort: HR = 1.02, $P = 0.002$, 95% CI [1.01–1.03]; second cohort: HR = 1.02, $P = 0.032$, 95% CI [1.00–1.03]) but failed to reach statistical significance in multivariate analysis. *CDO1* promoter methylation is involved in gene regulation and is a potential prognostic biomarker for BCR-free survival in PCa patients following radical prostatectomy. Further studies are needed to validate *CDO1* methylation assays and to evaluate the clinical utility of *CDO1* methylation for the management of PCa.

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Introduction

Prostate cancer (PCa) is the most common cancer in the Western world among men. In the United States, 180,890 new cases and 26,120 tumor-related deaths have been predicted for 2016.[1] The high incidence coupled with a low mortality rate necessitates a careful clinical management in order to reduce mortality but avoid overtreatment. Despite the upsurge of novel molecular tests, therapeutic decisions are still and almost exclusively based on clinicopathological parameters. Given the commonly long natural course of PCa and the considerable morbidity of contemporary treatment options, therapy planning remains challenging, and additional information is highly desirable to estimate the biological potential of primary PCa.[2] Especially in low and intermediate risk cases (according to, e.g., D'Amico criteria), there is a pressing need for biomarkers that advocate either an active surveillance strategy or immediate active therapy.

So far, immunohistochemical biomarkers have not been proven successful and may not represent an ideal basis for a robust prognostic test,[3] whereas nucleic acid-based biomarkers appear more promising.[4] Changes in DNA promoter methylation of human genes are of great potential to serve as biomarkers, as they are one of the most common epigenetic alterations in malignant tumors. Furthermore, DNA is highly stable and can be extracted from different materials, e.g., formalin-fixed paraffin-embedded (FFPE) tissues and body fluids.[5] It has previously been shown that the methylation level of the *cysteine dioxygenase 1* (*CDO1*) promoter region is associated with metastasis in estrogen receptor-positive, lymph node-positive breast cancer patients receiving adjuvant anthracycline treatment.[6] This result was confirmed in a second, independent study.[7] Jeschke and colleagues[7] could demonstrate that *CDO1* silencing contributes to reactive oxygen species (ROS) detoxification capacity and leads to a resistance against ROS-generating chemotherapeutics including anthracyclines. Only recently, another study reported that *CDO1* promoter hypermethylation has a prognostic value in primary breast cancer patients.[8] Additional studies have shown hypermethylation of the *CDO1* promoter region in several solid tumors, such as esophageal, bronchial, urinary bladder, gastric, cholangiocarcinomas, or

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colorectal carcinomas.[9-11] Moreover, a correlation between *CDO1* promoter hypermethylation and poor survival has been demonstrated in clear cell renal cell carcinoma patients.[12]

CDO1 catalyzes the first important step in the cysteine catabolism, such as the oxidation of cysteine to cysteine sulfinic acid, and plays a key role in the taurin biosynthesis pathway.[13-15] Furthermore, *CDO1* has been suggested as a potential tumor suppressor gene.[9] However, to date, the role of *CDO1* in PCa remains unclear. In the present study, *CDO1* methylation and mRNA expression levels were analyzed, and their suitability as prognostic biomarker was investigated in 2 independent cohorts (one from University Hospital Bonn, Germany and another from The Cancer Genome Atlas (TCGA-http://cancergenome.nih.gov/)).

Results

The analytical performance of the *CDO1* quantitative methylation (QM) qPCR assay was tested using a DNA methylation dilution series of bisulfite-converted artificially methylated and unmethylated DNA. The analytical performance of the assay was accurate, specific, and reproducible over the whole range of 0–100% DNA methylation (Supplementary Fig. S1).

Correlation of CDO1 promoter methylation and mRNA expression in cell lines and prostatic tissue

The methylation status of the *CDO1* promoter in PCa cell lines (PC3, LNCaP, DUCaP, VCaP, DU145, 22Rv1) and in non-tumorigenic cell lines (HEK293T and BPH) was examined. High *CDO1* promoter methylation was observed in the cancer cell lines PC3 (88%), LNCaP (85%), DU145 (97%), and 22Rv1 (90%), whereas the *ETS-related gene (ERG)* translocation-positive PCa cell lines DUCaP (46%) and VCaP (37%), as well as the benign cell lines HEK293T (50%) and BPH (35%) showed a significantly lower level of *CDO1* promoter methylation (Fig. 1). Furthermore, the transcriptional level of *CDO1* mRNA in the aforementioned cell lines was examined by means of real-time RT-PCR. No expression of *CDO1* mRNA was detectable in the cancer cell lines PC3, LNCaP, DU145, and 22Rv1, whereas higher expression levels were observed in DUCaP and VCaP cells, as well as in the benign BPH cell line. HEK293T cells showed a weak *CDO1* mRNA expression (Fig. 1). The expression levels inversely correlated with the methylation status ($\rho = -0.91$, $P = 0.005$).

To examine the expression of *CDO1* mRNA in prostate tissue, real-time RT-PCR analysis of cDNAs derived from cancerous and normal adjacent tissue (NAT) from 16 PCa patients was performed. *CDO1* mRNA was shown to be downregulated significantly in the cancerous tissue compared to the NAT specimens ($P = 0.003$, Fig. 2a). The inverse correlation between *CDO1* mRNA expression and *CDO1* promoter methylation was confirmed in the PCa patient cohort from the TCGA Research Network (n = 498). The expression levels of *CDO1* mRNA decreased with increasing *CDO1* promoter methylation ($\rho = -0.642$, $P < 0.001$, Supplementary Fig. S2). Furthermore, *CDO1* mRNA expression was shown to be significantly higher in normal prostate tissue compared to the cancerous tissue ($P < 0.001$, Fig. 2b).

CDO1 promoter hypermethylation in prostate cancer compared to normal adjacent tissue and benign prostatic hyperplasia

In a pilot study, methylation levels of the *CDO1* promoter region were analyzed in 66 prostate tissue samples from 24 PCa patients [24 cancer samples, 23 NAT specimens, and 19 tissue samples with benign prostatic hyperplasia (BPH)]. *CDO1*

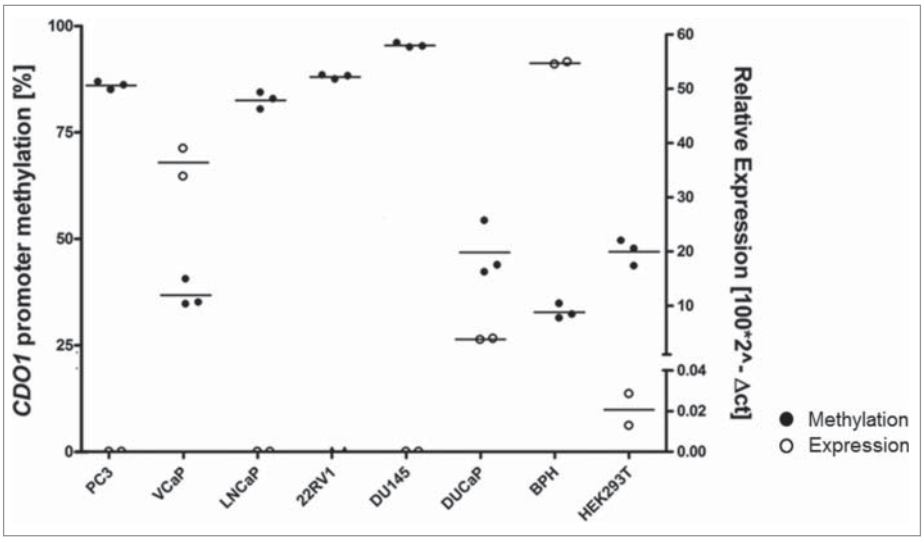

Figure 1. *CDO1* promoter methylation and mRNA expression in cell lines. Comparison of *CDO1* promoter methylation and mRNA expression in prostate cancer cell lines, a benign hyperplasia prostate cell line (BPH), and a benign human embryonic kidney cell line (HEK293T). Left Y-axis and black dots illustrate a high *CDO1* promoter methylation in the *ERG* translocation-negative prostate cancer cell lines PC3, LNCaP, 22Rv1, and DU145, whereas the *ERG* translocation-positive prostate cancer cell lines VCaP and DUCaP as well as the benign cell lines showed a markedly reduced *CDO1* promoter methylation. DNA methylation measurements were performed in 3 biological replicates. Each biological replicate was analyzed in triplicate measurement. Right Y-axis and open circles illustrate that only the VCaP, DUCaP, BPH, and HEK293T cell lines showed a detectable *CDO1* mRNA expression. Expression and methylation of *CDO1* correlated inversely. Expression measurements were performed in 2 biological replicates. Each biological replicate was analyzed in triplicate measurement.

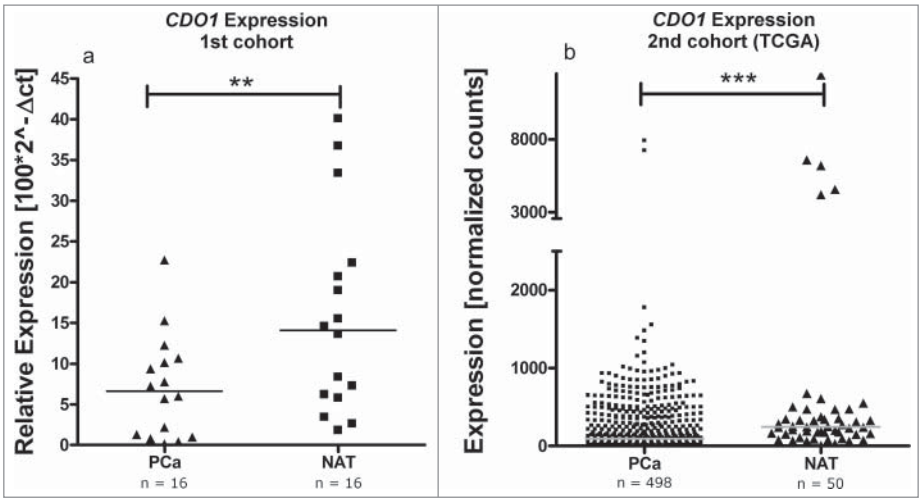

Figure 2. CDO1 mRNA expression in prostate cancer and normal adjacent tissue. A: In the first cohort, 16 prostate cancer (PCa) samples were compared with corresponding normal adjacent tissue (NAT). B: In the second (TCGA) cohort, 498 prostate cancer samples were compared to 50 NAT samples. *CDO1* mRNA expression in prostate cancer tissue was significantly lower compared to corresponding normal adjacent tissues. *P*-values refer to Wilcoxon–Mann–Whitney test (*** refers to *P*-values < 0.001, ** *P*-value < 0.01).

methylation levels were significantly higher in the carcinomatous tissues (mean = 32.3%; 95% CI [22.3–42.3%]) compared to BPH (mean = 1.9%; 95% CI [1.2–2.6%]) and NAT samples (mean = 3.1%; 95% CI [1.3–4.9%]; $P < 0.001$ each, Fig. 3a). *CDO1* promoter methylation did not differ significantly between hyperplastic and normal prostate tissue. Data from the TCGA cohort (498 cancer samples, 50 NAT specimens) confirmed that *CDO1* methylation was significantly higher in cancerous tissue compared to normal prostate tissue ($P < 0.001$, Fig. 3b).

Association of CDO1 methylation with clinicopathological parameters and BCR-free survival

Tissue samples from a first cohort of 300 PCa patients after radical prostatectomy were analyzed. Androgen receptor (AR) and Ki-67 expression of this cohort have previously been reported.[16,17] ERG expression was assessable for 211 patient samples and resulted in 65 (30.8%) ERG-positive and 146 (69.2%) ERG-negative specimens. Tissue samples were further analyzed for *CDO1* promoter methylation. The median *CDO1* methylation was 22.3% (mean = 25%, 95% CI: [23.0–27.5%]) with a range from 0% to 88% (25% percentile: 7.1%, 75% percentile: 39.4%). As continuous variable, *CDO1* promoter methylation positively correlated with the International Society of Urological Pathology (ISUP) Gleason Grading Groups[18] (r = 0.231, $P < 0.001$), pT-category ($P = 0.003$, Wilcoxon–Mann–Whitney test), and the proliferation marker Ki-67 (r = 0.216, $P = 0.006$; Table 1) However, pre-surgical prostate-specific antigen (PSA) levels, age, AR, and ERG expression did not correlate with *CDO1* promoter methylation (Table 1).

Follow-up information was available for 260 of the 300 patients of the first cohort. Univariate Cox proportional hazard

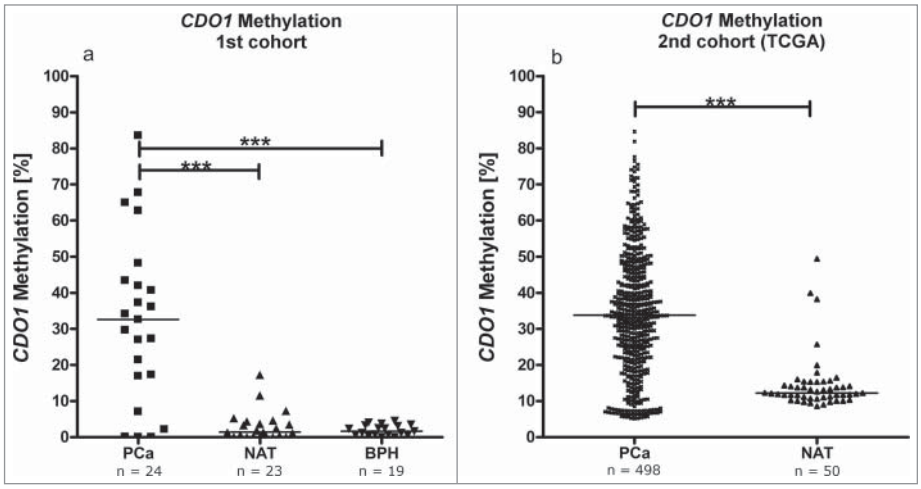

Figure 3. *CDO1* promoter methylation in prostate cancer and normal adjacent tissue. A: In a pilot study, 24 prostate cancer (PCa) samples were compared to 23 normal adjacent (NAT) and 19 benign hyperplasia (BPH) tissue samples. B: In a second (TCGA) cohort, 498 prostate cancer samples were compared to 50 normal adjacent tissue samples. *CDO1* promoter methylation in prostate cancer tissue was significantly higher compared to corresponding normal adjacent and benign prostate hyperplasia tissue. *P*-values Wilcoxon–Mann–Whitney test (*** refers to *P*-values < 0.001).

Table 1. Associations/correlations of *CDO1* promoter methylation with clinicopathological parameters in 2 independent cohorts of prostate cancer patients. The associations/correlations of percentage*CDO1* methylation with the following clinicopathological parameters were tested with Pearson correlation: age (continuous), pre-surgical PSA (continuous), ISUP Gleason grading group (1–5), Ki-67 (continuous), AR (weak/negative, moderate, strong); ERG expression (negative vs. positive) and pT category (pT2 vs. pT3) were tested using Wilcoxon–Mann–Whitney test.

	First cohort					Second cohort				
	Patients [n]	Median m*CDO1* [%]	*P*-value	m*CDO1*low	m*CDO1*high	Patients [n]	Median m*CDO1* [%]	*P*-value	m*CDO1*low	m*CDO1*high
Patients [n]	300	22.0		183 (61.0%)	117 (39.0%)	498	33.9		252 (50.6%)	246 (49.4%)
Patients with follow up	260					417				
Mean/median follow-up [months]	66/63					22/16				
Range [months]	0–145					1–113				
Age			0.55[¥]					0.009[¥]		
≤60 [years]	75 (25.1%)			57 (76.0%)	18 (24.0%)	224 (45.0%)			121 (54.0%)	103 (46.0%)
>60 [years]	224 (74.9%)			126 (56.3%)	98 (43.8%)	274 (55.0%)			131 (47.8%)	143 (52.2%)
Pre-surgical PSA (ng/ml)			0.40[¥]					0.003[¥]		
0–4	27 (9.4%)	17.5		19 (70.4%)	8 (29.6%)	53 (10.7%)	34.6		25 (47.2%)	28 (52.8%)
4–10	173 (60.3%)	22.1		101 (58.4%)	72 (41.6%)	286 (57.8%)	33.1		154 (53.8%)	132 (46.2%)
>10	87 (30.3%)	22.4		56 (64.4%)	31 (27.9%)	156 (31.5%)	36.7		72 (46.2%)	84 (53.8%)
pT category			0.003[*]					0.011[*]		
pT2	206 (68.7%)	20.2		136 (66.0%)	70 (33.9%)	351 (86.5%)			189 (53.8%)	162 (46.2%)
pT3	94 (31.3%)	29.1		47 (50.0%)	47 (50.0%)	55 (13.5%)			21 (38.2%)	34 (61.8%)
ISUP Gleason grading group[†]			<0.001[¥]					<0.001[¥]		
1 (<7)	163 (56.2%)	17.6		113 (69.3%)	50 (30.7%)	45 (9.0%)	24.9		32 (71.1%)	13 (28.9%)
2 (3+4)	53 (18.3%)	23.3		32 (60.4%)	21 (39.6%)	147 (29.5%)	33.2		78 (53.1%)	69 (46.9%)
3 (4+3)	23 (7.9%)	31.6		10 (43.5%)	13 (56.5%)	101 (20.3%)	33.7		52 (51.5%)	49 (48.5%)
4 (=8)	35 (12.1%)	30.7		16 (45.7%)	19 (54.3%)	64 (12.9%)	35.2		30 (46.9%)	34 (53.1%)
5 (>8)	16 (5.5%)	34.1		5 (31.3%)	11 (86.8%)	141 (28.3%)	36.8		60 (42.6%)	81 (57.4%)
ERG expression			0.70[*]							
Negative	146 (48.7%)	26.5		78 (53.4%)	68 (46.6%)					
Positive	65 (21.7%)	27.5		33 (50.8%)	32 (49.2%)					
Unknown	89 (29.7%))	11.5		72 (80.9%)	17 (19.1%)					
Ki67										
Weak/negative	110 (67.5%)		0.006[¥]	70 (63.6%)	40 (36.4%)					
Moderate	45 (27.6%)			24 (53.3%)	21 (46.7%)					
Strong	8 (4.9%)			2 (25.0%)	6 (75.0%)					
AR										
Weak/negative	12 (7.4%)		0.064[¥]	4 (33.3%)	8 (66.7%)					
Moderate	34 (20.9%)			19 (55.9%)	15 (44.1%)					
Strong	117 (71.8%)			73 (62.4%)	44 (37.6%)					

[*]Wilcoxon–Mann–Whitney test.
[¥] Pearson correlation.
[†]Numbers in brackets refer to traditional Gleason scores.

Table 2. Univariate and multivariate Cox proportional hazard analyses of BCR-free survival in the first cohort of prostate cancer patients (n = 260) after radical prostatectomy. DNA methylation was quantified by means of quantitative methylation real-time PCR.

	Univariate Cox		Multivariate Cox	
Clinicopathological parameter / biomarker	Hazard ratio [95% CI]	*P*-value	Hazard ratio [95% CI]	*P*-value
Tumor stage (pT3 and pT4 vs. pT2)	2.60 [1.51–4.49]	<0.001	1.19 [0.61–2.32]	0.62
ISUP grading group	2.00 [1.63–2.42]	<0.001	1.99 [1.56–2.49]	<0.001
Surgical margin (R1 vs. R0)	2.46 [1.41–4.29]	0.002	1.43 [0.75–2.74]	0.27
Nodal status (pN1 vs. pN0)	1.68 [0.67–4.22]	0.27	0.42 [0.14–1.31]	0.13
Pre-surgical PSA level	1.01 [1.00–1.02]	0.11	1.00 [0.83–1.00]	0.86
ERG expression (ERG-positive vs. ERG-negative)	0.78 [0.40–1.51]	0.46	0.95 [0.46–1.99]	0.90
Age	1.01 [0.96–1.06]	0.70	1.00 [0.93–1.05]	0.71
CDO1 methylation (continuous variable)	1.02 [1.01–1.03]	0.002	1.01 [1.00–1.03]	0.15
CDO1 methylation (m*CDO1*$_{high}$ vs. m*CDO1*$_{low}$)	2.32 [1.34–4.02]	0.003	#	#

CDO1 methylation as dichotomized variable was not included in the multivariate Cox proportional hazard analysis.

analysis showed a strong association between *CDO1* promoter methylation (as continuous variable) and biochemical recurrence (BCR)-free survival (*P*=0.002, hazard ratio (HR) = 1.02, 95% CI [1.01–1.03], Table 2). *CDO1* promoter methylation levels dichotomized at the median revealed a significant prognostic value in Kaplan-Meier (*P* = 0.011) and univariate Cox proportional hazard analysis (*P* = 0.013, HR = 2.07, 95% CI [1.17–3.67]). Using an optimized cut-off, *CDO1* promoter methylation was further dichotomized into m*CDO1*$_{low}$ (<30% methylation, n = 161) and m*CDO1*$_{high}$ (>30% methylation, n = 99) groups. In univariate Cox proportional hazards analysis, high *CDO1* promoter methylation showed a significantly higher risk for PSA recurrence compared to patients with low *CDO1* promoter methylation (*P* = 0.003, HR = 2.32, 95% CI [1.34–4.02]). This finding was confirmed in a Kaplan-Meier analysis (Fig. 4a, *P*=0.002; median BCR-free survival for m*CDO1*$_{low}$: 68.8 months and for m*CDO1*$_{high}$: 60.6 months). In a multivariate survival analysis including age, TNM, surgical margin, ISUP grading group, ERG-status, and pre-surgical PSA, however, *CDO1* methylation failed to qualify as independent prognostic factor (Table 2).

Results were validated in an independent second cohort including PCa patients from the TCGA Research Network (n = 498). In this cohort, the median *CDO1* promoter methylation was 34% (mean = 34.1%) with a range from 5% to 85% (25% percentile: 22.2%, 75% percentile: 46.3%). Compared to the first cohort, patients from the second cohort were younger and a shorter follow-up period was available (follow-up data available for 417 patients). Furthermore, the distribution of the ISUP grading group was notably different (Table 1). However, correlations between *CDO1* promoter methylation and the ISUP Gleason Grading Group (r = 0.175, *P* < 0.001) as well as the T-category (*P* = 0.011) were confirmed (Table 1).

Additionally, correlations between *CDO1* methylation and age (r = 0.117, *P* = 0.003) as well as the pre-surgical PSA level (r = 0.132; *P* = 0.003) were observed (Table 1). Univariate Cox proportional hazards analysis revealed a significant correlation between *CDO1* promoter methylation (as continuous variable) and BCR (*P* = 0.032, HR = 1.02, 95% CI [1.00–1.03]) (Table 3). Due to the different methodology used for methylation quantification and the different clinicopathological characteristics of the TCGA cohort, a new optimized cut-off for dichotomizing

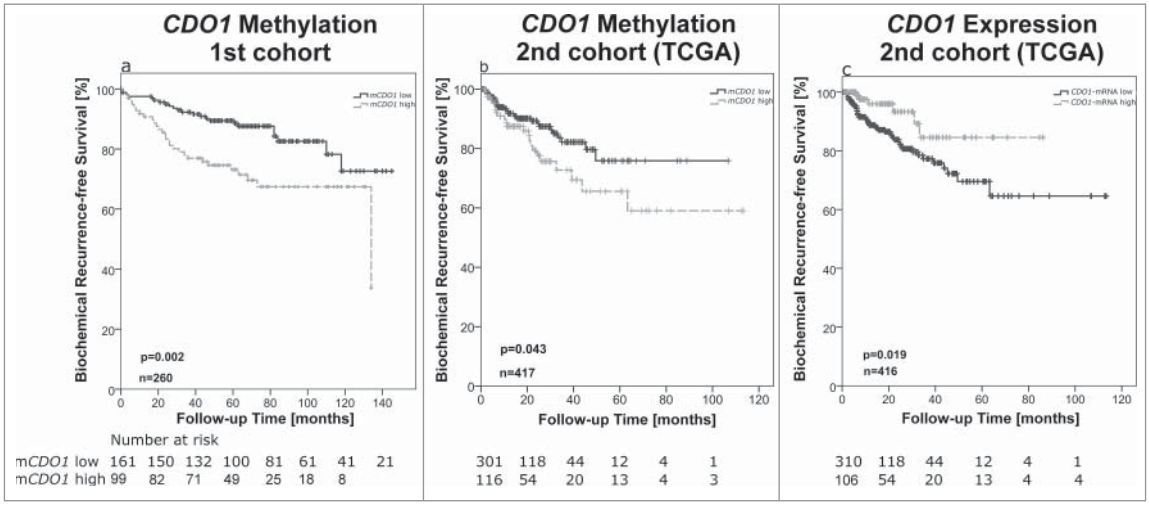

Figure 4. Kaplan-Meier analysis of BCR-free survival in prostate cancer patients. A: *CDO1* promoter methylation was dichotomized into m*CDO1*$_{low}$ (<30% methylation, n = 161) and m*CDO1*$_{high}$ (>30% methylation, n = 99) groups. In a first cohort comprised of 260 prostate cancer patients from the University Hospital Bonn, a significant association between *CDO1* promoter hypermethylation and BCR-free survival after radical prostatectomy was observed. B: In 417 prostate cancer patients from the TCGA cohort, the prognostic value of *CDO1* methylation was confirmed. *CDO1* promoter methylation was dichotomized into m*CDO1*$_{low}$ (<43 .0% methylation, n = 301) and m*CDO1*$_{high}$ (>43% methylation, n = 116) groups. C: The expression value of *CDO1* mRNA also showed a significant prognostic value in prostate cancer patients from the TCGA cohort. *CDO1* expression was dichotomized into *CDO1*-mRNA$_{low}$ (n = 310) and *CDO1*-mRNA$_{high}$ (n = 106) groups.

Table 3. Univariate and multivariate Cox proportional hazard analyses of BCR-free survival in the second (TCGA) cohort of prostate cancer patients (n = 410) after radical prostatectomy. DNA methylation was quantified by means of the Infinium HumanMethylation450 BeadChip.

	Univariate Cox		Multivariate Cox	
Clinicopathological parameter / biomarker	Hazard ratio [95% CI]	*P*-value	Hazard ratio [95% CI]	*P*-value
Tumor stage (pT3 and pT4 vs. pT2)	4.25 [2.37–7.61]	<0.001	2.49 [1.30–4.79]	0.006
ISUP grading group	1.69 [1.34–2.13]	<0.001	1.54 [1.17–2.02]	0.002
Surgical margin (R1 vs. R0)	1.49 [0.87–2.56]	0.15	0.89 [0.45–1.77]	0.73
Nodal status (pN1 vs. pN0)	1.84 [1.00–3.36]	0.048	1.41 [0.73–2.73]	0.31
Pre-surgical PSA level	1.04 [1.02–1.05]	<0.001	1.02 [1.00–1.04]	0.060
ERG expression (ERG-positive vs. ERG-negative)	N/A			
Age	1.02 [0.98–1.06]	0.39	1.01 [0.96–1.05]	0.82
CDO1 methylation (continuous variable)	1.02 [1.00–1.03]	0.032	1.01 [0.99–1.03]	0.38
CDO1 methylation (m$CDO1_{high}$ vs. m$CDO1_{low}$)	1.73 [1.01–3.00]	0.046	#	#
CDO1 mRNA (continuous variable)	0.99 [0.99–1.00]	0.074	#	#
CDO1 mRNA (m$CDO1_{high}$ vs. m$CDO1_{low}$)	0.38 [0.16–0.88]	0.024	#	#

Variables were not included in the multivariate Cox proportional hazard analysis.

CDO1 promoter methylation was introduced, which allocated patients to m$CDO1_{low}$ (<44% methylation, n = 301) and m$CDO1_{high}$ (>44% methylation, n = 116) groups. In univariate Cox proportional hazards analysis high *CDO1* promoter methylation showed a significantly higher risk for PSA recurrence compared to patients with low *CDO1* promoter methylation ($P = 0.046$, HR = 1.73, 95% CI [1.01–2.96]). This finding was confirmed in a Kaplan-Meier analysis of BCR-free survival in patients stratified by *CDO1* methylation levels ($P = 0.043$, Fig. 4b).

Additionally, survival analyses with *CDO1* transcriptome data (RNA-Seq V2) from the TCGA PCa patient series were performed. In univariate Cox proportional hazards analysis, elevated *CDO1* mRNA expression (as continuous variable) showed a trend toward a favorable prognosis ($P = 0.072$, HR = 0.99, 95% CI [0.99–1.00], Table 3). After dichotomization using an optimized cut-off ($CDO1_{low}$, n = 310; $CDO1_{high}$, n = 106), *CDO1* mRNA expression levels assessed by RNA-Seq analysis qualified as a significant prognostic factor ($P = 0.024$, HR = 0.38, 95% CI [0.16–0.88], Fig. 4c and Table 3). However, in a multivariate Cox proportional hazards analysis including ISUP grading group, age, pre-surgical PSA level, and TNM categories, dichotomized CDO1 mRNA levels failed to reach statistical significance.

Discussion

In the present study, hypermethylation of the *CDO1* promoter region was demonstrated to be significantly associated with BCR and aggressive tumor behavior in a population-based prostatectomy cohort. Aberrant hypermethylation was further shown to be tumor-specific and to associate with *CDO1* gene silencing. These results were confirmed in an independent PCa patient cohort from the TCGA Research Network. The biomarker's excellent performance is supported by the fact that it was substantiated using 2 different technologies (methylation-specific qPCR / first cohort, Infinium HumanMethylation450 / second cohort). However, in multivariate survival analysis including Gleason Grading Group, pre-surgical PSA level, and TNM categories, only the Gleason Group remained a statistically significant prognostic parameter. Accordingly, *CDO1* methylation and mRNA expression failed to qualify as independent prognostic factors.

CDO1 catalyzes the oxidation of cysteine to cysteine sulfinic acid (CSA).[14] In 2 large metabolome studies, cysteine levels in PCa were shown to be significantly higher compared to NAT specimens.[19,20] Downregulation of CDO1 expression by promoter hypermethylation in cancerous prostate tissue compared to NAT is therefore entirely in line with results taken from the metabolome data. However, functional analyses are required to elucidate whether *CDO1* is under direct epigenetic control *via* DNA methylation or if the association of methylation and reduced transcription is due to an indirect effect. In serum and urine, levels of cysteine were shown to be associated with PCa recurrence.[21] This is in keeping with the presented data, which show that *CDO1* hypermethylation in PCa associates with BCR. Since *CDO1* methylation positively correlates with aggressive tumor behavior, hypermethylation and subsequent repression of CDO1 could lead to elevated cysteine levels.[22,23] Cysteine further is a source for the biosynthesis of glutathione,[24,25] a major antioxidant molecule which is decreased in the presence of CDO1.[23] In a previous study, glutathione was significantly increased in PCa compared to NAT.[19] This suggests that CDO1 is able to reduce antioxidant capacity.[7] In contrast, epigenetic *CDO1* gene silencing in PCa may foster the ability of cancer cells to adapt to oxidative stress by enhancing the antioxidant capacity.[7] This might explain the highly significant correlation between *CDO1* promoter methylation and the ISUP Gleason Grading Group, since the Gleason Score has already been shown to correlate with hypoxia markers,[26] and hypoxia leads to a production of reactive oxygen species (reviewed in[27]). High-grade PCa cells might therefore potentially benefit from a downregulation of CDO1 and a consequently enhanced antioxidant capacity. These data indicate that epigenetic *CDO1* gene silencing might be partly driven by increased ROS levels in cancer cells to enhance their antioxidant capacity. Moreover, *CDO1* promoter methylation might potentially serve as predictive biomarker for docetaxel (DTX) treatment in PCa patients. DTX is mainly applied as first line chemotherapy in patients with castration-resistant, advanced PCa. In a large clinical trial, DTX in combination with androgen deprivation therapy (ADT) has recently shown promising results in patients with metastatic, hormone-sensitive tumors.[28] Predictive biomarkers might help to identify subgroups of patients who benefit from an addition of DTX to ADT. One molecular mechanism of DTX causing endothelial dysfunction

is DTX-induced ROS formation.[29-33] Here, low *CDO1* methylation may be a useful marker to stratify patients for DTX treatment comparable to anthracycline treatment in breast cancer patients.[7] However, this is highly speculative and further studies are needed to test this hypothesis.

ERG translocation may lead to demethylation or inhibition of *CDO1* hypermethylation as suggested by methylation levels of the prostatic cell lines. Only the benign BPH cell line and the malignant *ERG* translocation-positive VCaP and DUCaP cell lines showed strongly reduced methylation levels and a subsequently higher expression of CDO1, whereas all other PCa cell lines exhibited high *CDO1* methylation levels and complete silencing of the gene. However, no correlation between ERG expression and *CDO1* methylation could be found in the PCa patient cohort. Possibly, within the physiological context, a lower methylation of the *CDO1* promoter in *ERG* translocation-positive patients is in some cases mimicked by a ROS driven hypermethylation. Thus, in statistical analyses, a correlation between *ERG* translocation and lower *CDO1* promoter methylation is lost due to too low sample numbers. Börno et al. reported that *ERG*-negative samples show significantly higher numbers of differentially methylated regions compared to *ERG*-positive or normal samples.[34] This is in line with the shown methylation data from cell lines. Additionally, other studies demonstrated that ROS-induced oxidative stress is involved in site-specific hypermethylation of promoter regions of tumor suppressor genes (reviewed in[35]).

However, a few limitations of the study need to be mentioned. A major limitation of this retrospective study is the use of radical prostatectomy specimens. Further studies will have to analyze the prognostic value of *CDO1* promoter methylation in (pre-operative) biopsies and/or liquid biopsies (circulating tumor cells/free DNA) of PCa patients, and ultimately a prospective validation of its suitability as a prognostic biomarker for treatment planning is needed. Furthermore, the cut-offs used for dichotomization of *CDO1* promoter methylation ought to be validated in independent cohorts. While the use of 2 different methodologies for methylation analyses strengthen the evidence of the prognostic value of *CDO1* methylation on the one hand, a short-term implementation of a respective biomarker test into clinical routine is impeded, and an additional clinical and analytical performance evaluation is required. Furthermore, the fact that *CDO1* methylation did not qualify as independent prognostic factor in multivariate analyses lowers its potential utility as a clinically useful test. Currently, no prognostic methylation biomarkers for PCa patients are applied in clinical routine. However, several sound studies have suggested promising prognostic methylation markers such as *PITX2*,[36,37] the methylation panel *AOX1*/*C1orf114*/*HAPLN3*,[38] or GABRE∼miR-452∼miR-224[39] promoter hypermethylation (for review see[40]). A limitation of the TCGA data employed in this study was the relatively short follow-up, which might explain the decreased prognostic value compared to the patients included in the first cohort.

In summary, the association of *CDO1* promoter methylation with downregulation of the respective transcript and the correlation with an adverse prognosis for PCa patients both underline the biological relevance of *CDO1* as a potential tumor suppressor gene. Further studies on a functional level are indispensable to evaluate the potential biological significance of CDO1 in PCa and its suitability as a predictive biomarker for response to DTX treatment.

Materials and methods

Ethics statement

The present study was approved by the Institutional Review Board of the University Hospital of Bonn, Germany (vote no. 071/14), which waived the need for written informed consent from the participants. All experiments were performed in accordance with the relevant guidelines and regulations.

Patients

Pilot study: A case control group with 66 tissue samples from 24 radical prostatectomy specimens including 24 PCa, 23 NAT, and 19 BPH tissue samples was included. In addition, fresh frozen tumor and NAT obtained from 16 PCa patients were included.

First cohort: The first arm of the study enclosed 300 patients who had undergone radical prostatectomy for clinically organ-confined PCa in the surgical center at the University Hospital Bonn, Germany between 2000 and 2008. To avoid a selection bias, all available tissue samples were taken in consecutive order. A total of 163 (54.3%), 76 (25.3%), and 53 (17.7%) patients had a Gleason score of <7, $=7$, and >7, respectively. Median follow-up time after radical prostatectomy was 63 months (range 0 to 145 months) and the median age was 65 y (range 45 to 83 years; Table 1). BCR was defined by post-surgical PSA levels exceeding 0.2 ng/mL from nadir and was assessable for 277 patients. During the observation time, 54 (19.5%) patients suffered from BCR after a median time of 26 months after prostatectomy.

Second cohort: For confirmation of the results from the first cohort, an independent series of 498 PCa patients with complete follow-up and clinicopathological data was evaluated. The results from the validation cohort shown here are entirely based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/). Overall, a Gleason score of <7, 7, and >7 was recorded for 45 cases (9.0%), 250 cases (50.2%), and 205 cases (41.2%), respectively. The median follow-up time after prostatectomy was 16 months. Within the observation time, 58/431 (13.5%) patients suffered from BCR after a median time of 16 months, and 8/498 (1.6%) patients died after a median time of 11 months.

Sample preparation and bisulfite conversion

For methylation analysis, FFPE specimens were processed using the InnuCONVERT Bisulfite All-In-One Kit (Analytik Jena, Germany) as previously described.[5]

For analytical performance evaluation of the assay, a dilution series of bisulfite-converted artificially methylated DNA (CpGenome^{TM} Universal Methylated DNA; Merck Millipore, Darmstadt, Germany) and unmethylated DNA (peripheral blood lymphocytes (PBL) DNA, Roche Applied Science, Mannheim, Germany) was used. UV spectrophotometry was

carried out using a Nanodrop ND-1000 spectral photometer (Nanodrop Technologies, Wilmington, DE, USA). For calculation of the DNA concentration, the multiplication factor 33 was used for single-strand DNA (bisulfite DNA).

Quantification of CDO1 DNA methylation

Quantitative methylation-specific qPCR: The DNA methylation of *CDO1* was analyzed by a QM real-time PCR assay located in the putative *CDO1* promoter region. The assay was performed using an AB 7500 Fast Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA, USA). A region of the *CDO1* promoter within the CpG island was amplified by one pair of methylation-unspecific primers (Supplementary Fig. 3). By means of 2 probes that bind specifically and competitively to methylated (*CDO1*-P-M) and unmethylated (*CDO1*-P-U) alleles, respectively, the methylation of this region was quantified accurately in a single-tube PCR reaction. Primer and probe sequences are given in Supplementary Tab S1. Thermal cycling was initiated with a first denaturation step at 95°C for 15, min followed by 45 cycles of 95°C for 15 s, 60°C for 2 s, and 55°C for 60 s. All reactions were performed in triplicates applying 25 ng bisulfite converted template DNA. Methylation values for each sample were determined using ΔΔCT method: $\Delta CT = CT_{CDO1\text{-P-U}} - CT_{CDO1\text{-P-M}}$, $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{calibrator}$; methylation of *CDO1* $= 100/(1+2^{(\Delta\Delta CT)})$.

Methylation BeadChip: The methylation data generated by the TCGA Research Network (http://cancergenome.nih.gov/) were created by means of the Infinium HumanMethylation450 BeadChip (Illumina, Inc., San Diego, CA, USA). HumanMethylation450 data of level 2 were downloaded directly from the TCGA webpage. The tab-delimited, ASCII text (.txt) files included background-corrected methylated (bead_M) and unmethylated (bead_U) summary intensities as extracted by the R package 'methylumi'. The two bead pairs (cg12880658, cg16265906) in proximity to the locus of the QM qPCR assay were selected. Methylation values for each bead pair were calculated by the formula 100% × bead_M / (bead_M + bead_U). The mean methylation values from both bead pairs were computed und used for analysis.

Immunohistochemistry

Immunohistochemical staining of Ki67 and AR have previously been described.[16,17] ERG staining was performed in the immunohistochemistry laboratory of the Institute of Pathology, University Hospital of Bonn. Immunohistochemical staining in the tissue sections was performed using the LabVision Autostainer 480S system (Thermo Scientific, Waltham, MA, USA) along with the Thermo Scientific Reagents and the N-Histofine® DAB-3S detection kit. The PT-Module was used for dewaxing and epitope retrieval (pH 6.0 at 99°C for 20 min). The following antibodies and dilutions were used: Ki-67, clone MIB-1 (Dako, Glostrup, Denmark; 1:500); androgen receptor, clone AR441 (Dako A/S, Glostrup, Denmark; 1:400); ERG, clone EPR3864 (Biologo, Kronshagen, Germany; 1:100). Evaluation of the slides was performed by 2 experienced pathologists (GK, VS).

Cell lines

Six PCa cell lines (PC3, LNCaP, DUCaP, VCaP, DU145, 22Rv1) and the non-tumorigenic cell lines HEK293T and BPH were analyzed. Cell lines were authenticated using Multiplex Cell Authentication by Multiplexion GmbH (Heidelberg, Germany) as previously described.[41] The SNP profiles matched known profiles or were unique. Cell lines were grown in accordance to the instructions from the American Type Culture Collection.

mRNA expression analysis

CDO1 mRNA expression was analyzed in cell lines as well as in fresh frozen tumor and NAT specimens. Total RNA was isolated from cell pellets and fresh frozen tissues by means of the RNAeasy® Kit (Qiagen, Hilden, Germany) following the "animal tissue" protocol. First-strand cDNA synthesis was conducted with oligo-dT primers and the SuperScript® III RT kit (ThermoFisher Scientific, Darmstadt, Germany) according to the manufacturer's protocol. A total of 500 ng RNA were used in a total reaction volume of 20 μl.

Five nanograms of cDNA were used in a final PCR volume of 20 μl. All real-time RT-PCR assays were measured in duplicates using an AB 7500 Fast Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA, USA). Oligonucleotide sequences are given in Supplementary Tab. S1. Expression levels of *CDO1*-mRNA were normalized to human *G6PD*- and *SDHA*-mRNA used as internal control.

The mRNA data provided by the TCGA Research Network (http://cancergenome.nih.gov/) were generated by means of the Illumina HiSeq 2000 RNA Sequencing Version 2 analysis (Illumina, Inc., San Diego, CA, USA). mRNA expression data of level 3 were downloaded directly from the TCGA webpage. The tab-delimited ASCII text files included the normalized results for the expression of a gene. Counts per genes were calculated using the SeqWare framework via the RSEM algorithm.[42]

Statistics

Statistical analyses were performed using SPSS, Version 21 (IBM SPSS Statistics). Wilcoxon–Mann–Whitney tests were used to evaluate the statistical significance between *CDO1* methylation and clinicopathological parameters. The Pearson correlation coefficient was used for bivariate correlation analysis. BCR-free survival was defined as the time from prostatectomy to BCR or last contact. Univariate survival analyses were conducted according to univariate Cox proportional hazards and Kaplan-Meier analyses. *P*-values refer to Wald test and log-rank test, respectively. Two-sided *P*-values were reported and P-values < 0.05 were considered statistically significant. The association of methylation and mRNA expression was visualized using the MEXPRESS software tool.[43]

Disclosure of potential conflicts of interest

Dimo Dietrich is co-inventor and owns patents on methylation biomarkers and related technologies. These patents are commercially exploited by

Epigenomics AG. Dimo Dietrich receives inventor's compensation from Epigenomics AG. Dimo Dietrich is a consultant for AJ Innuscreen GmbH (Berlin, Germany), a 100% daughter company of Analytik Jena AG (Jena, Germany), and receives royalties from product sales. The other authors declare that they have no conflict of interest.

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