DNA methylation of sarcosine dehydrogenase (*SARDH*) loci as a prognosticator for renal cell carcinoma

MEHRDAD MAZDAK[1*], HOSSEIN TEZVAL[1*], JANNE CARMEN CALLAUCH[1], NATALIA DUBROWINSKAJA[1], INGA PETERS[1], CARSTEN BOKEMEYER[2], JÖRG HENNENLOTTER[3], ARNULF STENZL[3], MARKUS A. KUCZYK[1] and JÜRGEN SERTH[1]

[1]Department of Urology, Hannover Medical School (MHH), D-30625 Hannover; [2]Department of Oncology, Hematology and Bone Marrow Transplantation, University Medical Center Hamburg-Eppendorf, D-20246 Hamburg; [3]Department of Urology, Eberhard-Karls University, D-72076 Tübingen, Germany

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Abstract. DNA methylation plays an important role in the genesis and progression of tumor diseases. To identify new DNA methylation markers possibly associated with the clinical characteristics of renal cell carcinoma (RCC), we investigated loci in the sarcosine dehydrogenase (*SARDH*) gene. SARDH is involved in the metabolism of the glycine-derivative sarcosine and is closely linked through a functional control loop. Statistical evaluation of methylation data and clinical characteristics of patients showed that kidney tumors with clinically aggressive features such as a high tumor stage, positive lymph nodes, distant metastases or a previously advanced tumor status exhibited significantly lower methylation of a locus in the *SARDH* gene. Moreover, *SARDH* methylation was found to be a significant prognostic factor for recurrence-free survival in RCC patients showing statistical independence from the clinical prognosticators, grade, stage and state of metastasis. In conclusion, the methylation status of the *SARDH*-CGI was identified as an independent prognostic candidate marker for RCC.

Introduction

Renal cell carcinoma (RCC) accounts for 90% of all cases of kidney cancer and kidney/renal pelvis cancer ranks seventh for men [5% of all new cancer diagnoses in 2016] and tenth for women [3% in 2016] (1,2). Epigenetic alterations, in particular DNA-methylation, are vital for tissue- and cell-specific differentiation of an organism and are stably passed on to daughter cells. DNA methylation which is important for the normal functioning of cells has also been demonstrated in many tumor entities and often shows an association with the pathogenesis as well as the progression of malignancies (3,4). In regards to RCC, tumor-specific hypermethylation and hypomethylation of numerous loci in a substantial number of genes have also been described (5,6). The role of DNA methylation in RCC has been a topic of past and present research aimed to improve our understanding of the molecular basis of the pathological changes concerning RCC (7,8). Due to the lack of valid biomarkers, DNA methylation alterations have been discussed as early diagnosticators, prognosticators, and predictors of RCC as well as for molecular differentiation of histological subtypes (9-11). Moreover, detection of increased promotor methylation for the estimation of patient prognosis is suggested by a substantial number of studies identifying candidate loci associated with worse survival in all RCC subtypes (12). Although the TCGA data set provides the basis for new developments in the research of novel markers, significant clinical advances in prognostic models have not been introduced in the past decade (13). Therefore, there is still a need for identification of new candidate markers with prognostic relevance. In addition, a comparatively low number of studies have been designed to analyze DNA methylation for prediction of targeted therapeutic outcome in the management of advanced RCC (14-17). Independent of the possible clinical application in terms of prognosis or prediction, the functional relevance of many alterations in regards to RCC development, progression as well as response to therapeutic approaches remains to be clarified for a large part of loci (18-21).

The present study aimed to ascertain whether DNA methylation alterations of the sarcosine dehydrogenase (*SARDH*) gene occur in RCC. The SARDH protein was recently described to undergo changes in prostate cancer (PCa) showing potential prognostic relevance. SARDH is a monomeric flavoprotein of the mitochondrial matrix. The enzyme catalyzes the oxidative demethylation of N-methylglycine (sarcosine) which is synthesized by the glycine N-methyltransferase (GNMT) catalyzed by the transfer of a methyl group from the donator S-adenosylmethionine to the amino acid glycine. It has been suggested that sarcosine is a possible marker for distinguishing healthy prostate tissue, localized PCa, and already

Correspondence to: Dr Jürgen Serth, Department of Urology, Hannover Medical School (MHH), 1 Carl Neuberg Street, D-30625 Hannover, Germany
E-mail: serth.juergen@mh-hannover.de

*Contributed equally

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metastasized tumors (22). Significantly elevated sarcosine levels have been found in 79% of the examined metastatic tumors, whereas only 42% of localized tumors exhibit elevated sarcosine concentrations. Benign prostatic tissues did not show any detectable sarcosine levels. Notably, alterations in the SARDH/GNMT system could be measured non-invasively in the urine by means of sarcosine measurements potentially offering efficient access to diagnostic relevant information. Moreover, the functional analysis suggested an effect of SARDH as well as GNMT protein levels on mortality and invasiveness of benign prostatic cells roughly comparable to effects by known oncogenic factors of PCa such as E26 family of transcription factors and the androgen receptor (22).

The *SARDH* gene is encoded on chromosome 9q34.2. Decreased expression has been detected in hepatocellular carcinoma and has been discussed as a potential prognostic marker (23-25). We investigated whether DNA-methylation alterations of *SARDH* as a potential surrogate of gene expression alterations and corresponding gene activation/inactivation can be found in RCC and cell line models of other human tumors. Although in human tumor cell lines overall high *SARDH* methylation values were detected, we found statistical robust associations of DNA hypomethylation with adverse clinical parameter and survival of patients.

Materials and methods

Primary cells and tumor cell lines. Renal proximal tubular epithelial cells (RPTECs) were obtained from Lonza (Basel, Switzerland) and renal, urothelial and prostate cancer cell lines (ACHN, A498, 786-O, RCC-GS, RCC-HS/RCC-EW, RCC-MF, RT112, CLS-439, EJ28, 5637, T24, and PC-3) were purchased from Cell Line Services (CLS, Eppelheim, Germany). Cells were immediately after receipt cultured solely for the purpose of DNA extraction for a maximum number of 5 passages. Therefore, further authentication of cell lines after storage of DNA was not carried out. Note, that the renal cancer cell line RCC-HS has been meanwhile identified by the supplier to be identical to the renal cancer cell line RCC-EW.

Tissue samples. Fresh frozen tissue samples of 118 RCC tumor tissues were subjected to methylation analyses (Table I). The tissue samples were obtained between January 2001 and December 2005 at Eberhard Karls University of Tübingen by kidney surgery. The degree of differentiation (grading) and the histopathological subtype of each tumor sample were determined by two pathologists. Pathological tissue assessment, preparation, storage as well as oncological staging and grading beside to the data management have been described before (26). For analysis of tumor-specific hypermethylation a subset of 82 tumors for which tumor-adjacent normal tissues were available were subjected to statistical analysis. Follow-up data were available for a subset of 57 tumors and were used for survival analyses.

Isolation and conversion of DNA. Isolation of DNA and bisulfite conversion was performed as described previously (27).

Pyrosequencing. The *SARDH* gene is located on chromosome 9q34 between positions 136,528,684 and 136,605,077 according to the hg19 genomic assembly in the UCSC genome browser (28). For methylation analysis of *SARDH*, eight CpG sites, located between positions 136,568,091 and 136,568,135, were identified to be suitable for pyrosequencing. They were part of the CpG island designated as CpG island 37 and located in the gene body of *SARDH* adjacent to exon 12 or exon 1 of a putatively alternatively spliced transcript displayed in the genome browser. Pyrosequencing was carried out for relative quantitation of the CpG sites of interest applying the universal reverse primer concept (29). Primer sequences are specified as following: 5'-ATGGTTTATTTGAGGGATAGGTAGAA-3' (forward primer), 5'-GGGACACCGCTGATCGTTTAACTAAAAACC ACCTCTTTTCTTCCCAAA TC-3' (reverse universal primer) and 5'-GGTGTATTAGTTTGTTAGTAGTTTG-3' (sequencing primer). PCR and pyrosequencing were carried out as previously described (30).

Statistical methods. Clinicopathologic and experimental data were collected in a relational database. All statistical analyses were conducted by means of the statistical software packages R 3.03 and R Studio 1.0.136 (https://www.R-project.org/). Distributions of methylation values are presented by box plots using notches as an estimate of the median confidence interval. Statistical significance was assumed for P-values <0.05. For tumor-specific hypermethylation analysis paired normal and tumor tissue samples were analyzed using the two-sided paired t-test. Logistic regression was applied for comparison of independent tumor samples and analysis of possible associations with clinicopathological parameters. P-values, odds ratios (ORs) and 95% confidence intervals (Cis) were provided. Association of methylation and clinicopathologic parameters with recurrence-free survival was statistically evaluated using univariate Cox regression analysis presenting P-values, hazard-ratios (HRs) and 95% CIs. For the presentation of the univariate survival characteristic, a Kaplan-Meier plot is presented. With respect to the low number of patients in the survival subset of tumors, bivariate Cox regression was carried out in pairwise combinations of dichotomized methylation data and most relevant clinical covariates as a surrogate for multivariate survival analysis of data.

Results

Analysis of SARDH methylation in cancer cell lines models and primary normal cells. Twelve tumor cell lines and one primary cell as a model for normal renal tissue were tested for DNA methylation. We found increased methylation in large part in the tumor cell lines. All cell lines representing tumors from the kidney, bladder, and prostate demonstrated high levels of at least 70% up to about 95% relative methylation (Fig. 1). Renal proximal tubular epithelial cells (RPTECs) demonstrated a substantial methylation level of 50%.

Analysis of paired tissue samples for the detection of tumor-specific hypomethylation or hypermethylation. We investigated whether the comparison of *SARDH* loci of histopathologic normal tissues with paired tumoral tissue samples shows relevant alteration in DNA methylation. We found both, tumor-specific hypermethylation as well as hypomethylation in different subgroups of the tissue pairs, showing overall a heterogeneous representation for methylation alterations in the

Table I. Clinicopathological parameters of the RCC tumor samples using in univariate and bivariate logistic regressions.

Parameters	All RCCs n (%)
Total cases	118 (100)
Histology	
ccRCC	82 (69.5)
papRCC	23 (19.5)
Chrom. RCC	3 (2.5)
Mixed histology	6 (5.1)
Other	4 (3.4)
No RCC	0 (0)
Sex	
Female	41 (34.7)
Male	77 (65.3)
Age	
Median	64.5
Range (min-max)	(35-91)
Metastasis	
M0	92 (78)
M+	26 (22)
Na	0 (0)
Lymph node metastasis	
N0	103 (87.3)
N+	15 (12.7)
Na	0 (0)
T-classification	
pT1	11 (9.3)
pT1a	34 (28.8)
pT1b	22 (18.6)
pT2	7 (5.9)
pT3	5 (4.2)
pT3a	10 (8.5)
pT3b	24 (20.3)
pT3c	3 (2.5)
pT4	1 (0.8)
Na	1 (0.8)
Differentiation	
G1	23 (19.5%)
G1-2	15 (12.7%)
G2	61 (51.7%)
G2-3	9 (7.6%)
G3	10 (8.5%)
Na	Na
State of disease[a]	
Localized disease	61 (51.7)
Advanced disease	56 (47.5)
Na	1 (0.8)
State of disease[b]	
Localized disease	64 (54.2)
Advanced disease	53 (44.9)
Na	1 (0.8)
Paired samples	
No. of patients	82 (69.5)

RCC, renal cell carcinoma; ccRCC, clear cell renal cell carcinoma; papRCC, papillary; chrom., chromophobe; met., metastasis; Na, not available. [a]Localized and advanced disease defined as pT≤2, N0, M0 and G1 or G1-2 or pT≥3 and/or N+, M+ or G2-3 or G3. [b]Localized and advanced disease defined as pT≤2, N0, M0 or pT≥3 and/or N+, M+.

tumors (Fig. 2). Thus, paired sorted difference analysis of methylation alterations demonstrated a tumor-specific increase in methylation of more than 10% for ~15% of the tumors, while an estimated one-third of the tumors showed hypomethylation of more than -20% relative methylation (Fig. 3). Correspondingly, paired t-test analysis for comparison of mean relative methylation values for the tumor (39.2%) and corresponding normal tissue group (40.9%) did not demonstrate a significant difference (two-sided paired t-test, P=0.084).

Statistical association of SARDH methylation and histological and clinicopathological parameters of the tumors. To identify possible statistical associations of *SARDH* methylation with clinicopathological parameters we carried out bivariate logistic regression analyses following dichotomization of tumors if necessary. The mean relative methylation values, P-values, odds ratios (ORs) and 95% confidence intervals (CIs) are summarized in Table II.

Distant metastasis. Comparison of relative methylation values corresponding to the tumors showing distant metastasis (M+) with primary non-metastatic tumors (M0) demonstrated a substantial hypomethylation for the M+ group (Fig. 4). Relative mean methylation values of 33.2% (M+) and 42.4% (M0) were observed showing a significant substantial difference between both groups (bivariate logistic regression, P=0.003, OR=0.938, 95% CI 0.897-0.975) while age was not detected as a significant parameter in the bivariate model.

Lymph node status. We analyzed relative methylation of *SARDH* in relation to lymph node status (Fig. 5) and found that patients with lymph node metastases (N1) had a statistically significantly lower methylation with a mean value of 32.9% in comparison with tumors from patients without lymph node metastasis (N0), who exhibited a mean value of 41.5% (bivariate logistic regression, P=0.020, OR=0.946, 95% CI 0.902-0.990). Age was no significant parameter in the bivariate statistical model.

Tumor stage. Patients showing high-tumor stages (T3-T4) showed a significantly reduced *SARDH* methylation of 35.5% (Fig. 6) when compared with low-stage tumors (T1-T2) demonstrating mean relative methylation of 43.1% (bivariate logistic regression, P=0.002, OR=0.944, 95% CI 0.908-0.977). The covariate age was not detected as a significant parameter.

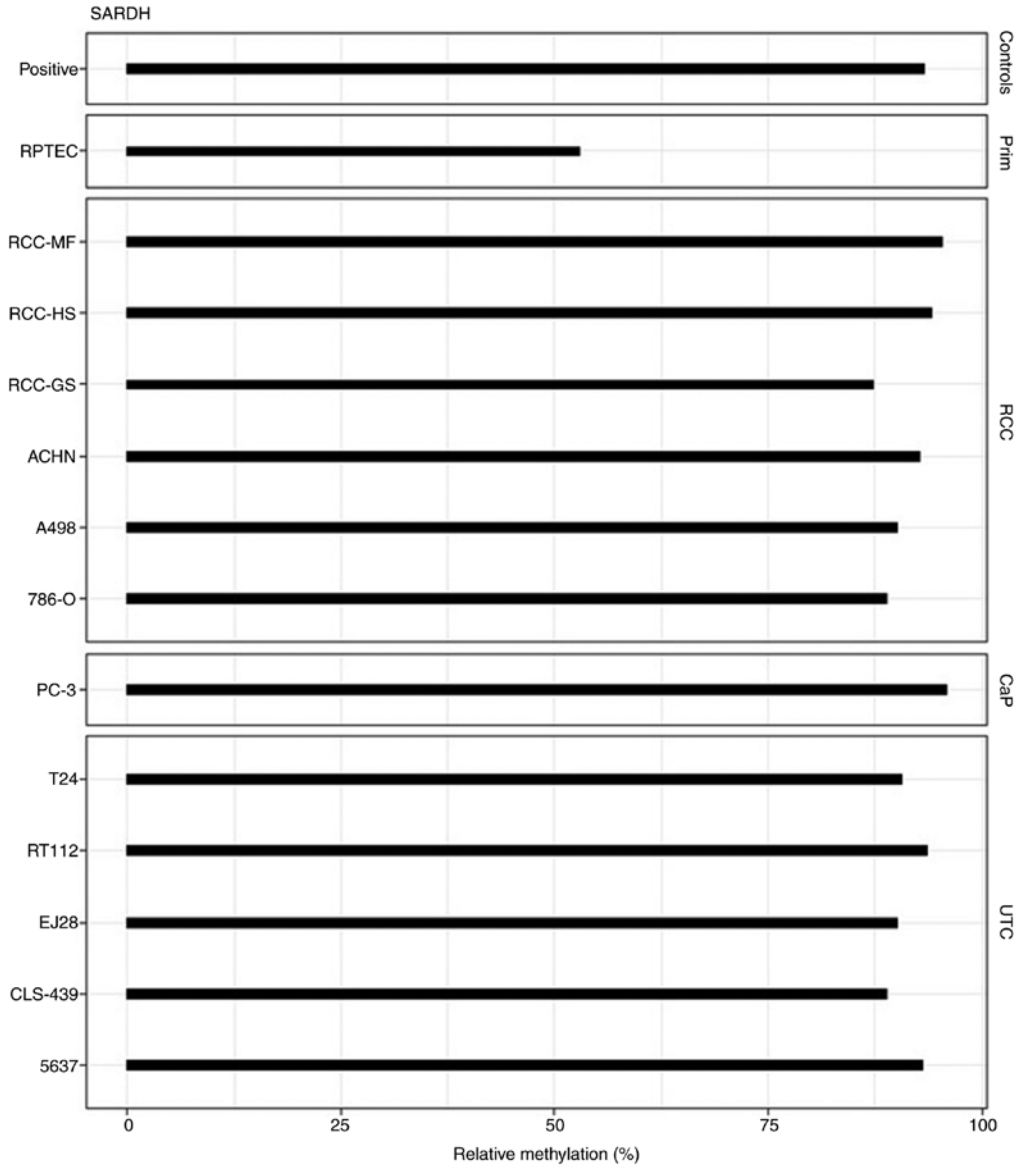

Figure 1. Pyrosequencing analysis of SARDH methylation in controls, primary cells as well as renal cancer, prostate and bladder cancer cell lines. RPTECs, renal proximal tubular epithelial cells; SARDH, sarcosine dehydrogenase.

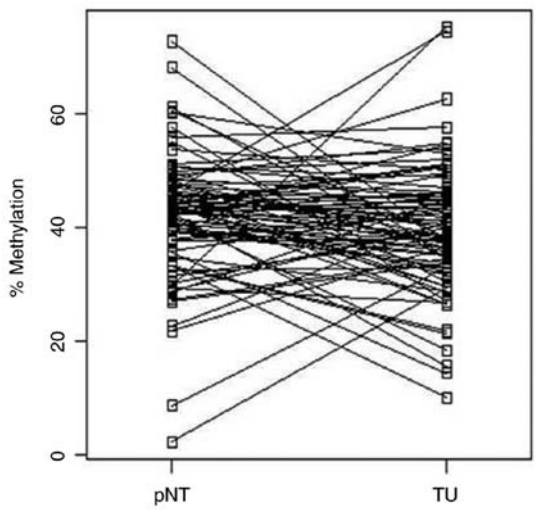

Figure 2. Hypermethylation detection and analysis of SARDH in paired normal (pNT) and tumor tissue (TU) samples. SARDH, sarcosine dehydrogenase.

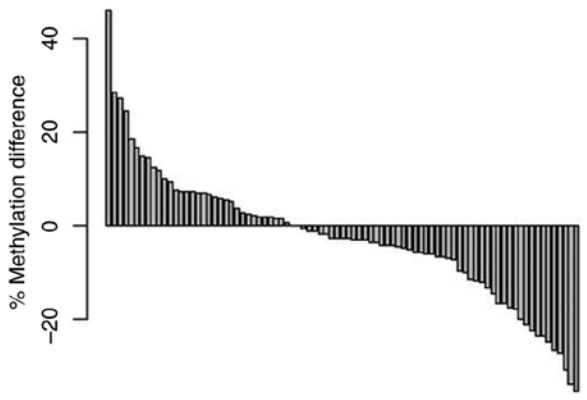

Figure 3. Assorted paired difference plot for pairwise methylation differences.

Comparison of localized and advanced tumors. Dichotomization of tumors into a localized tumor group (pT2, N0,

Table II. Overview of the comparison of *SARDH* methylation levels in regards to the different tumor characteristics.

SARDH methylation	Relative methylation (%) (mean values of the categories)	P-value	OR[a]	95% CI
Grade of tumor		0.260	0.976	0.936-1.017
G1-2	41.0			
G3	36.9			
Stage of tumor		0.002	0.944	0.908-0.977
T1-T2	43.1			
T3-T4	35.5			
Lymph node status		0.020	0.946	0.902-0.990
N0	41.5			
N1	32.9			
Distant metastases		0.003	0.938	0.897-0.975
M0	42.4			
M1	33.2			
State of disease		<0.001	0.919	0.879-0.956
Localized	44.8			
Advanced[b]	34.9			

In each comparison, the methylation values of category 1 were compared to the values of category 2. [a]The odds ratio is only available for comparison of independent groups using logistic regression. [b]Localized means pT ≤2, lymph node (N) and metastasis (M) negative (N0/M0) and grading (G) 1-2. Progressed means pT ≥3 and/or N1, M1 or G 2-3. OR, odds ratio; CI, confidence interval.

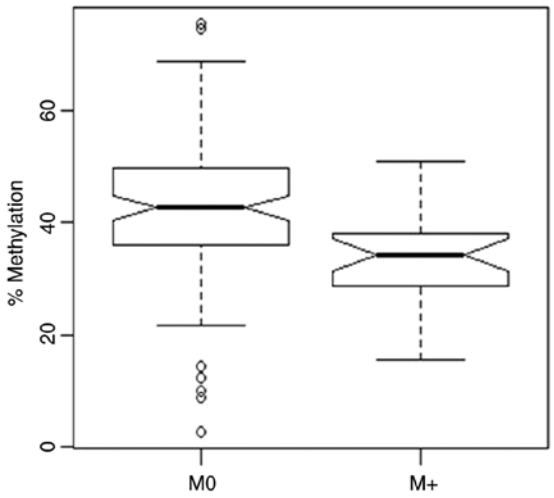

Figure 4. Assorted paired difference plot for pairwise M0 vs. M1 primary cancer (bivariate logistic regression, P=0.003, OR=0.938, 95% CI 0.897-0.975). M0, primary non-metastatic tumors; M1, distant metastasis; OR, odds ratio; CI, confidence interval.

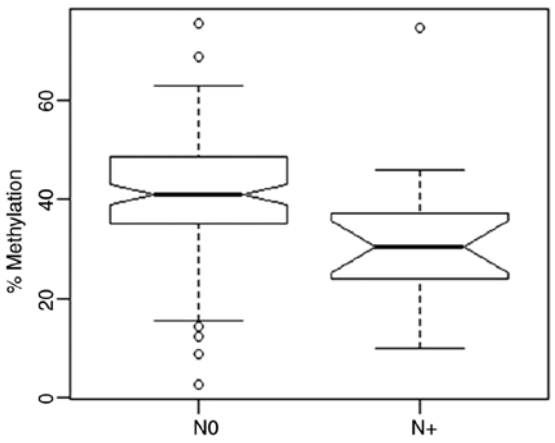

Figure 5. Assorted paired difference plot for pairwise lymph node status N0 vs. N1 (bivariate logistic regression, P=0.020, OR=0.946, 95% CI 0.902-0.990). N0, without lymph node metastasis; N1, with lymph node metastasis; OR, odds ratio; CI, confidence interval.

M0, G1-2) and an advanced tumor group (pT ≥3 and/or N1, M1 or G 2-3) for bivariate statistical comparison revealed significantly lower methylation values of 34.9% for advanced tumors in comparison to localized tumors showing a mean value of 44.8% (Fig. 7, bivariate logistic regression, P<0.001, OR=0.919, 95% CI 0.879-0.956). No significance was observed for the covariate age in bivariate analysis.

Comparison of low- and high-grade tumor groups. Comparing tumor groups including G1-G2 classifications for the low-grade and G2-3 or higher classifications for the high-grade tumor group exhibited no statistically significant difference in mean relative methylation (Fig. 8). The corresponding values were 41.0 and 36.9% for the low- and high-grade groups (bivariate logistic regression, P=0.260, OR=0.976, 95% CI 0.936-1.017). In addition, age demonstrated no significant effect on methylation in the bivariate model.

Comparison of normal and tumor samples. The analysis of the paired tissue samples showed that on average both the paired normal tissue samples (pNT) and the corresponding tumor samples (TU) exhibited high methylation demonstrating

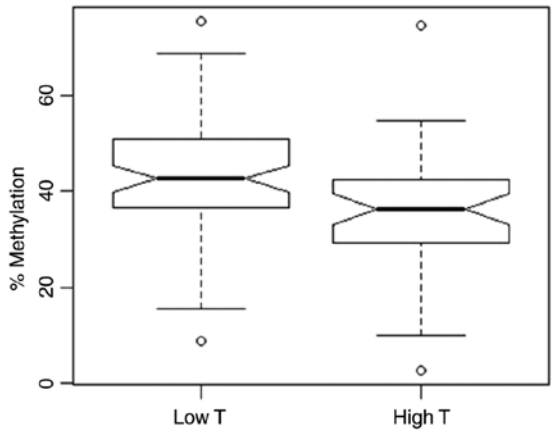

Figure 6. Assorted paired difference plot for pairwise differentiation: T1,T2 (low) vs. T3,T4 (high) stage of cancer (bivariate logistic regression, P=0.002, OR=0.944, 95% CI 0.908-0.977). OR, odds ratio; CI, confidence interval.

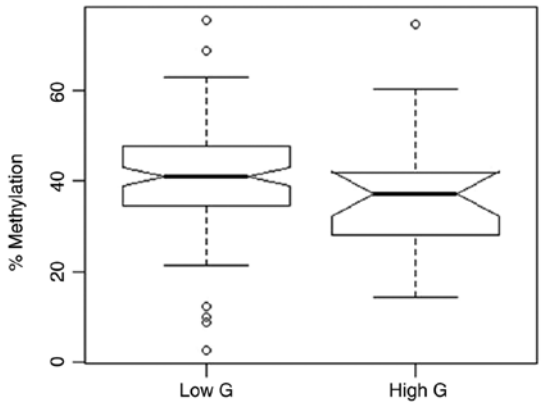

Figure 8. SARDH methylation analysis in the category low-grade G1 vs. high-grade G3 tumors (bivariate logistic regression, P=0.260, OR=0.976, 95% CI 0.936-1.017). SARDH, sarcosine dehydrogenase; OR, odds ratio; CI, confidence interval.

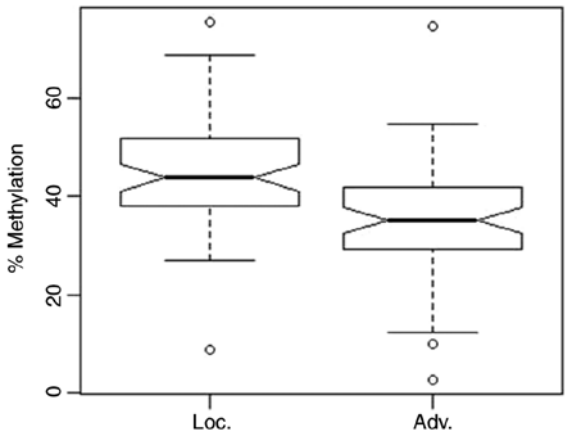

Figure 7. Assorted paired difference plot for pairwise localized (Loc.) vs. advanced (Adv.) disease cancer (bivariate logistic regression, P<0.001, OR=0.919, 95% CI 0.879-0.956). OR, odds ratio; CI, confidence interval.

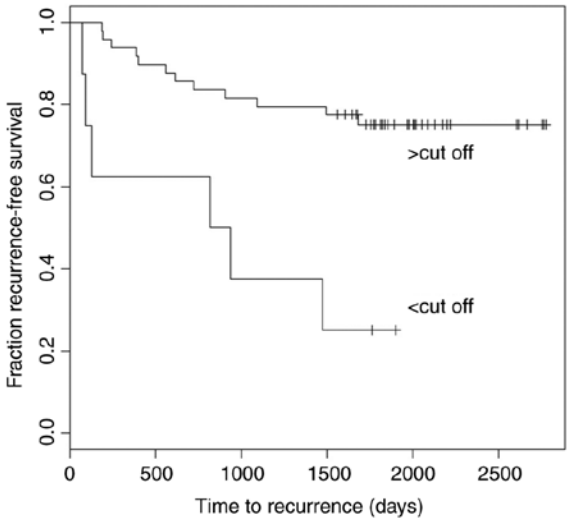

Figure 9. Kaplan-Meier survival curves analysis; recurrence-free survival of patients in the univariate model. The upper line shows patients with high SARDH methylation (>31.7%), and the lower line shows patients with low SARDH methylation (≤31.7%). Highly methylated patients had a significantly longer recurrence-free survival than patients with low methylation (P<0.005). SARDH, sarcosine dehydrogenase.

no statistically significant difference between pNT and TU samples (Fig. 2).

Statistical association of SARDH methylation with recurrence-free survival. We evaluated the known prognostic factors of distant metastasis (M), lymph node status (N), the degree of tumor differentiation (G), tumor stage (T) as well as *SARDH* methylation for their association with the recurrence-free survival of patients. Univariate Cox regression analysis demonstrated that *SARDH* methylation, distant metastases (M1), positive lymph nodes (N1), poor tumor differentiation (G3) and high tumor stage (T3) were significant parameters for poorer outcome as indicated by high hazard ratios (HRs) of ~3.2 to 14 (Table III). Kaplan Meier analysis revealed that patients with *SARDH* methylation below the statistical determined optimum threshold value of 31.7% were at higher risk for early recurrence (P<0.005, Fig. 9) showing a median time to recurrence of 878 days (28.8 months). In contrast, patients exhibiting higher *SARDH* methylation demonstrated in less than half of cases a recurrence event within the maximum observation period.

Whether *SARDH* methylation provides prognostic information independent of the analyzed clinical parameters was investigated using pairwise bivariate Cox regression analyses to circumvent statistical limitations due to insufficient patient numbers in subsets needed for multivariate Cox regression analysis.

Pairwise comparison with states of distant metastasis, lymph node metastasis, grade, stage, diameter, age and sex of the patients revealed that methylation remained a significant parameter in all bivariate regression models showing remarkably constant low HR values between 0.04 and 0.2 (Table IV).

In silico analysis of SARDH methylation using TCGA KIRC data. To validate our results obtained by pyrosequencing analysis we used *in silico* analysis of the KIRC dataset from TCGA. We found seven evaluable loci exhibiting in

Table III. Evaluation of patient recurrence-free survival in the univariate model.

Recurrence-free survival[a]	P-value	HR	95% CI
Optimal threshold[b]	0.000095	0.09	0.031-0.315
Distant metastasis (M0 vs. M1)	0.001241	5.55	1.962-15.720
Lymph node status (N0 vs. N1)	0.203067	2.29	0.640-8.193
Grade of the tumor (G1-2 vs. G3)	0.000123	9.32	2.982-29.140
Stage of the tumor (T1 vs. T3)	0.004072	6.43	1.806-22.880

[a]Calculated statistics: Cox regression model with Cox proportional hazard. [b]The optimal threshold was with 31.7% methylation. HR, hazard ratio; CI, confidence interval.

Table IV. Results of the pairwise bivariate survival analyses.

Recurrence-free survival[a]	P-value	HR	95% CI
Methylation vs. distant metastasis (M0/M1)			
Methylation	0.0001506	0.09	0.024-0.305
Distant metastasis	0.0015724	6.24	2.005-19.410
Methylation vs. lymph node status (N0 vs. N1)			
Methylation	0.0004066	0.10	0.027-0.356
Lymph node status	0.9928786	0.99	0.238-4.153
Methylation vs. grading (G1-2/G3)			
Methylation	0.0134751	0.20	0.056-0.717
Grading	0.0059307	5.83	1.660-20.450
Methylation vs. tumor stage (T1/T3)			
Methylation	0.0088421	0.19	0.057-0.663
Stage of tumor	0.0319277	4.41	1.137-17.080
Methylation vs. dichotomized tumor diameter			
Methylation	0.0003234	0.04	0.007-0.230
Tumor diameter	0.1358545	3.03	0.706-13.000
Methylation vs. dichotomized patient age			
Methylation	0.0001626	0.05	0.010-0.235
Age	0.1281683	0.34	0.083-1.368

[a]Calculated statistics: Cox regression model with Cox proportional hazard.

two cases statistically significant tumor-specific hypermethylation (cg27114512, cg14163119) and in one case hypomethylation (cg13824009) using paired t-test and Bonferroni-Hochberg correction in the genome-wide analysis (Table V). However, a detailed view using assorted paired difference plots showed that all of the identified loci include hypermethylated and hypomethylated subsets in varying proportions.

Statistical analysis for a possible association of methylation of loci and clinical parameters of patients using univariate logistic regression analysis revealed that two loci (cg21122774, cg14163119), annotated to exon 1 and intron 8 respectively, showed high ORs for distant metastasis as well as high stage and high-grade tumors, indicating that higher methylation demonstrated an association with unfavorable clinicopathology (Table V). In contrast, cg13709982 and cg27114512 (adjacent to exon 13), as well as cg13824009 (adjacent to exon 17), exhibited low ORs indicating a possible association of hypomethylated tumors with adverse clinical parameters (Table V). Univariate survival analysis for cg21122774 showed that hypermethylated tumors were associated with a worse survival of patients. Similar to the analysis of clinicopathological parameters, cg13709982 and cg13824009 showed a significant association of hypomethylated loci with worse clinical outcome of patients (Table V).

Discussion

Metabolic as well as expression studies have postulated sarcosine dehydrogenase (*SARDH*) as an effector of tumor progression both in prostate and hepatocellular carcinoma (22-25). Here we investigated whether DNA methylation

Table V. *In silico* validation of *SARDH* methylation results using TCGA KIRC data.

Genomic position		Clinicopathology[a]							
		T		N		M		G	
Label	Start position	P-value	OR	P-value	OR	P-value	OR	P-value	OR
cg13824009	135,537,683	<0.001	0.007	0.795	1.399	0.0562	0.052	4.291	0.001
cg14524643	135,538,907	0.095	0.077	0.338	0.236	0.928	1.190	0.059	0.053
cg13709982	135,557,560	0.002	0.010	0.902	1.181	0.451	0.269	0.002	0.011
cg27114512	135,557,966	0.047	0.052	0.687	1.788	0.267	0.126	0.022	0.032
cg14163119	135,571,032	0.016	36.467	0.534	0.405	0.008	111.286	0.063	16.419
cg14360014	135,593,537	0.226	0.282	0.767	0.743	0.518	2.234	0.812	1.272
cg21122774	135,594,817	0.003	17.526	0.264	0.345	<0.001	79.832	0.003	18.937

Results are shown exclusively for cg-loci demonstrating tumor-specific hypermethylation. Specification and genomic positions of CpG sites refer to the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) assembly. Only P-values considered as significant are shown for statistical associations obtained in hypermethylation, epigenetic gene silencing, and clinicopathological parameter analyses. [a]Univariate logistic regression for methylation comparison of dichotomized subsets of tumors for detection of statistical association with high- (≥T3) and low-stage (<T3), positive or negative state of lymph node (N) and distant metastasis (M) as well as low- (<G3) and high-grade (≥G3) tumor subsets.

alterations of *SARDH* occur in renal cell carcinoma (RCC) and can be utilized as a potential prognosticator for the clinical course of patients.

Comparing RCC with paired normal tissue samples demonstrated a heterogeneous pattern of methylation alterations. While overall high methylation values of approximately 30 to 60% were observed, subsets of tissue pairs showed hypermethylation as well as hypomethylation and a large part of tissues demonstrated low or no alteration in the loci investigated. Thus neither hypomethylation nor hypermethylation in these loci seemed to be associated with the development of RCC.

Notably, the TCGA KIRC dataset included seven evaluable CpG sites from which cg27114512 and cg14163119 showed clear hypermethylation while cg13824009 demonstrated hypomethylation. The hypermethylated sites are located to exon 1 and 2 of the long transcript while the hypomethylated CpG site is annotated to the region of exon 12 in case of the long transcript as well as to exon 1 of an alternative shorter transcript. Taking into account that this region is also adjacent to the CpG island analyzed in part by our pyrosequencing analysis, the *in silico* analysis of KIRC data obviously support our results.

We also statistically investigated whether tumor subgroups stratified for the most important histological and clinical parameters exhibit methylation alterations of *SARDH*. First, we found no significant difference in mean methylation comparing the clear cell and papillary histological entities (data not shown). In contrast, methylation in primary tumors without distant metastasis (M0) and metastasized tumors (M+) showed a clear *SARDH* hypomethylation of the M+ tumors which was independent of the covariate age of the patients, giving rise to the assumption that hypomethylation of this locus may be indicative of an adverse clinical outcome of patients. In line, hypomethylation, in addition, turned out to be significantly associated with lymphogenic metastasis, high stage of tumors as well as the state of progressive tumors. *In silico* validation analyzing the KIRC data also demonstrated that hypomethylation at exon 12 was associated with the positive status of distant metastasis as well as high-stage and high-grade tumors, thus supporting our findings for the adjacent loci measured in our study. Interestingly, hypermethylated loci identified in the KIRC *in silico* analysis neighboring exon 1 and 2 were also found to be associated with the positive status of distant metastasis.

In line, our survival analyses indicated that *SARDH* methylation may serve as an independent predictor of recurrence-free survival. Although our limited patient cohort did not permit multivariate Cox regression analysis, our surrogate multiple pairwise bivariate Cox regression clearly revealed methylation to be independent of all of the clinical covariates remaining a highly significant parameter in the statistical models and showing remarkable alteration of patient hazards. *In silico* univariate survival analysis of KIRC data also demonstrated association both hypermethylated and hypomethylated loci with the recurrence-free survival of patients. Thus, similar to the characteristics of associations observed for clinicopathological parameters, loci in the region of exon 1-7 preferably demonstrated association of hypermethylation with worse clinical prognosis while loci in the region of exon 7-17 adjacent to the two annotated CpG islands showed an association of hypomethylation with adverse outcome.

Thus, overall, the comparison of our pyrosequencing-based results with TCGA KIRC data for seven loci distributed over the genomic region of *SARDH* revealed that statistical associations with clinicopathology as well as the survival of patients do agree well for the loci showing closest proximity to the location of our analysis. On the other hand, the possible relevance of *SARDH* methylation for clinical outcome of patients together with the heterogeneity of methylation as indicated by the TCGA KIRC data point to the necessity of gene-wide methylation analyses in future studies.

Moreover, our primary analysis of various human tumor cell lines representing renal, bladder and prostatic cancers evenly demonstrated high relative methylation values for *SARDH*. Considering that we observed high methylation and corresponding low mRNA expression for all renal tumor cell lines but only a part of bladder cancer cell lines (data not shown), detailed methylation, expression, and re-expression analysis will be required for future functional analyses.

Interestingly, the mechanism of sarcosine-induced tumor progression to date has neither been elucidated for prostate cancer (31) nor other human malignancies have been functionally analyzed in detail. Thus, making molecular assumptions of the consequences of *SARDH* DNA-methylation alterations in RCC appear to be difficult at the current time point.

On the other hand, our statistical analyses of clinical data point to a contribution of *SARDH* to the progression of RCC and support previous studies underlining the relevance of the gene for human cancers.

In conclusion, our analyses in human cancer cell lines of prostate, bladder and renal tumors showed uniformly high methylation values of 70-95% for cancer cells and 50% for normal renal epithelial tubular cells, which supports the role of DNA methylation as an important epigenetic alteration in these human malignancies. We carried out DNA methylation analyses of CpG islands (CGIs) of the sarcosine dehydrogenase (*SARDH*) gene, which is involved in the metabolism of the amino acid derivative sarcosine. Interestingly, we were able to discover that the *SARDH*-CGIs analyzed by us showed strong methylation in human tumor and peritumoral tissues of the kidney. On the other hand DNA hypomethylation in tumors was statistically associated with more aggressive tumor behaviors. Aggressive carcinomas are thus characterized by a hypomethylation of the *SARDH*-CGIs characterized here. The hypomethylation of the *SARDH*-CGIs is a strong influencing factor which increases the risk of an unfavorable course of disease about four to five times. The comparison of papillary and clear-cell tumors showed no methylation differences. However, both patients with high tumor stages (T3) and patients with positive lymph nodes (N1) had significantly lower methylation values than patients with low tumor stages (T1) and without lymph node involvement (N0). Hypomethylation of the *SARDH*-CGIs was also associated with the patient clinicopathological status with regard to remote metastasis (M) and tumor status. The patients presenting with metastasis (M1) had a lower *SARDH* methylation than those without distant metastases (M0). Patients with a significant hypomethylation of *SARDH* had an approximately 2-fold increased risk of distant metastasis (Table II). Thus, the methylation status of the *SARDH*-CGIs analyzed means a statistically detectable risk-change in its clinicopathological outcome.

Whether the methylation status of *SARDH* also affects the survival of a patient or not was another question addressed in our study. First, in the univariate analysis of recurrence-free survival using the Cox proportional hazard model, patients with low *SARDH* methylation showed a significantly shortened recurrence-free survival as opposed to those with high methylation. The low-methylated patients had a 5-fold increase in their disease compared to highly methylated patients. In the survival time analysis, patients who had already metastasized (M1) when diagnosed were included. These patients have a higher risk of a re-emergence of their disease, which makes the statement concerning the independence of the marker to be tested more difficult. On the other hand, metastatic patients are treated surgically, thus the effect of the marker on survival is also of great importance for this collective. For our investigation, this meant that the independence of our marker had to be checked by additional multivariate survival analysis. Due to the low cohort size, this was carried out by means of paired combinations of bivariate survival time analyzes. On the basis of the largely constant HRs and P-values (Table IV), it can be concluded that the methylation status of the *SARDH*-CGIs has a knock-on effect on a patient survival. Remarkably, the pairwise bivariate survival analyses revealed that *SARDH* is an independent factor for RCC, which correlates significantly with the course of the disease, independently of the classical clinical prognostic factors (distance metastasis, lymph node status, degree of differentiation, tumor stage, tumor diameter and age of the patient).

It is to be assumed that *SARDH* plays an important role in the clinical behavior of RCC and the recurrence-free survival of patients. The exact functions and connections would have to be evaluated in the future using appropriate functional models. If *SARDH* can also prove to be an important factor of influence for RCC in follow-up studies, it would certainly be possible to routinely test the patients for their *SARDH*-gene methylation. Methylations are easy and fast to detect, which makes the introduction of a clinical test procedure feasible.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HT and JS played the main role in the conception and design of this research. JCC, ND, MM, AS and JH carried out the acquisition of the data as the main participant colleagues. JS served as the main researcher in the analysis and interpretation of the data. The main responsibility for drafting and contribution in writing of the manuscript were conducted by JCC, MM and IP. IP was also involved in the conception of the study. Critical revision of the manuscript for important intellectual content was accomplished by CB, JS and IP. HT, MAK, CB, AS and JH were the most prominent authors in the acquisition of funding, administrative, technical and material support, as well as supervision and major classification and interpretation of the patients. In order to emphasize the participation of all of the mentioned authors in each part of

this manuscript, we would like to define it as the clear active participation of all of them in accuracy and integrity of any part of it.

Ethics approval and consent to participate

The Ethics Committee 'Ethik-Komission an der Medizinischen Fakultät der Eberhard-Karls-Universität am Universitätsklinikum Tübingen (Head Professor Lucht)' and 'Ethikkommission der Medizinischen Hochschule (Head Professor Tröger)' approved the study (ethics votes No. 128/2003V and 1213-2011). Written informed consent was obtained from all participating patients. Informed consent was obtained for publication of patient data without compromising anonymity or confidentiality.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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