Prognostic and diagnostic relevance of *hypermethylated in cancer 1 (HIC1)* CpG island methylation in renal cell carcinoma

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Abstract. The tumour suppressor gene *hypermethylated in cancer 1 (HIC1)* is a transcriptional repressor, which functionally cooperates with *p53*. Loss of *HIC1* function is associated with the development of various tumor entities. The aim of this study was to elucidate the relevance of CpG island (CGI) methylation of *HIC1* in renal cell carcinoma (RCC). DNA methylation of *HIC1* was analysed in a total of 98 tumor and 70 tumor adjacent normal specimens. After conducting bisulfite conversion, relative methylation levels were quantitated using pyrosequencing. Relative methylation values were compared for paired tumor and normal specimen and for correlation with clinico-pathologic and follow-up data of patients. Tumor-specific hypermethylation could not be detected for the subregion of the *HIC1* - CGI analyzed in this study. Comparing the level of methylation in tumors to clinicopathological data solely, patients without lymph node metastases demonstrated a higher level of methylation compared to patients with lymph node metastases (p=0.030). Patients recurrence-free survival (p=0.0074) both in univariate as well as bivariate Cox regression analysis. This study identifies *HIC1* hypermethylation in tumors as an independent predictor of reduced recurrence-free survival, which fits into our current understanding of hypermethylated *HIC1* being a marker for poor prognosis. Therefore, *HIC1* - CGI methylation could be a candidate marker to improve individualized therapy and risk stratification.

Introduction

The incidence of RCC in the US and Europe has increased significantly in the past decades and although RCC only accounts for ~2-3% of all human malignancies, it is the sixth leading cause of cancer related death (1-3). RCC is defined as adenocarcinoma of the renal tubular epithelium and summarizes a heterogeneous group with distinct histologic, genetic and molecular features also used for prediction of clinical outcome and as diagnostic tools (4). Since most of the known gene alterations are found in a minority of cases, sporadic RCC is thought to arise from multiple genetic and epigenetic events (5,6). So far only the *PBRM1* and *VHL* genes show frequent mutation in >30% of RCC (7). Thus, beside PBRM1, showing mutation in 41% of primary clear-cell RCC, analysis of about 3500 other genes demonstrated no other mutation (8).

In contrast, Morris *et al,* were able to show that epigenetic alterations are present in at least a dozen of genes for RCC (9,10). For other genes like *SFRP1* and *RASSF1* an association of CpG island (CGI) methylation with gene-silencing in RCC has already been shown and their possible role as prognostic and/or diagnostic tools was reported (11-16). Though many epigenetic alterations were found to exist in RCC only few of them have been subject of research exploring their functional and clinical relevance.

One of these potential marker genes is *hypermethylated in cancer 1 (HIC1)*, for which CGI-DNA methylation in various human malignancies has been detected such as carcinoma of the prostate, lung, germ cell, breast and lymphoma (17).

HIC1 is a tumor suppressor gene located on chromosome 17p13.3. *HIC1* consists of a DNA-binding C-terminal domain (five Krüppel-like C2H2 zinc fingers), a N-terminal domain (BTB/POZ complex) for protein-protein interactions and a central region while the last two have been described as autonomous transcriptional repression domains (18,19). *HIC1* was shown to interact with *silent mating type information regulation 2 homolog1 (SIRT1)* leading to an inactivation of *p53* when silenced as part of response to stress (20). In addition, very recently an interaction with the SWI/SNF chromatin-remodeling complexes associated with the transcriptional regulation of multiple cell cycle control-related genes was discovered (21,22). The pubished data strongly indicates the crucial role of *HIC1* as a mediator of cancer development and therefore as a possible tool to predict tumor prognosis. To our knowledge there has been up to now only one study investigating the role of *HIC1* in RCC. In 1993, Makos *et al* concluded that *HIC1* hyper-

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methylation precedes the manifestation of genetic alterations in 17p and is involved in development of RCC. For methylation analysis *Not*I and *Not*I/*Bam*HI restriction analysis were used and compared with the frequency of p53 mutation and 17p allelic loss. In comparison early and late stage tumors show higher frequencies of methylation than of 17p allelic loss and even less p53 mutation (23).

However, the small number of 31 tumor samples and the lack of clinic-pathologic data as well as specific quantitative methylation create the need for this study to further investigate the possible prognostic and/or diagnostic value of region-specific *HIC1* CpG island (CGI) methylation in a collective of 98 RCC and corresponding normal kidney tissue samples.

Materials and methods

Controls and cell lines. 'EpiTect Control DNA, methylated' (QM) and 'EpiTect Control DNA, unmethylated' (QU) were used as commercially purchased positive and negative controls, which consist of completely methylated i.e. unmethylated and bisulfit-converted human DNA (Qiagen, Hilden, Germany). Primary renal proximal tubular epithelial cells (RPTEC) and human prostate epithelial cells (PrEC) were purchased from Lonza (Basel, Switzerland) and malignant cell lines of kidney (ACHN, 786-O, RCC-GS, RCC-HS, RCC-MF), prostate (LNCap, DU-145, PC-3) and bladder (RT-112, CLS-439, HB-CLS-1, HB-CLS-2, EJ-28, 5637, T24) were obtained through cell line services (CLS, Heidelberg, Germany). Histopathological classification status of primary cancer were: pT1, N0, Mx, G1 for RCC-HS; pT2, N1, Mx, G2-3 for RCC-MF and pT3b, N0, M1, G3 for RCC-GS cell lines. Cells were grown according to the recommendations of the supplier, snap-frozen in liquid nitrogen and stored at -80˚C until extraction of nucleic acids.

Tissue specimens. Ninety-eight renal cell carcinomas of patients subjected to kidney surgery between 2001 and 2005 at the Eberhard Karls University Tuebingen, were collected. The study group consisted of 72 clear-cell RCC, 20 papillary RCC and 6 of mixed histologies. Matched paired tissue samples of tumor and corresponding tumor-free tissue were obtained from 58 RCCs. Corresponding tumor-free tissues were isolated ~2-0.5 cm adjacent from the primary tumor. All tumors were freshly obtained from the urological department's operating room. Resected tissues were snap-frozen and stored at -80˚C. The ethics committee of the institution approved the study and informed consent was obtained from the patients. Two pathologists re-evaluated all specimens with respect to tumor stage, grade, and histological subtypes. Tumor stages were assessed according to the UICC 2002 issue of the TNM system and nuclear grading was based on the Fuhrman grading system. Histological subtypes were assessed according to the consensus classification of renal cell neoplasia. Organ-confined RCC was defined as pT≤2, N0/M0 whereas advanced tumors were defined as pT≥3 and/or N^+/M^+. Data were collected by physicians and data managers and subsequently maintained by a relational database. Clinical and histopathological data are summarized in Table I.

Table I. Clinical and histopathological data of patients with renal cell cancer.

HIC1	All RCC[a]	ccRCC[b]
Cases		
N	98	72
TNM-classification		
T1	55	38
T2	6	4
T3	36	30
T4	1	0
Tx	0	0
N0	86	65
N1	12	7
M0	76	53
M1	22	19
Paired tissue samples[c]		
N	58	46
Grading		
G1	20	18
G2	63	42
G3	15	12
Gender		
Male	64	44
Female	34	28
Status		
Localized tumor[d]	52	34
Advanced tumor[d]	46	38
Age		
Min	35	35
Mean	62.8	61.7
Max	91	90
Progression/follow-up data		
N	57	42
Mean age (years)	64.7	65.0
Male	36	-
Female	21	-

[a]Including mixed and unclassified RCC. [b]Including clear-cell, papillary, mixed and unclassified renal cell carcinoma. [c]Tumor (TU) and adjacent benign renal tissue (adjacent normal tissue; adN). [d]Localized is defined as pT≤2, N0, M0 and G1-2; advanced tumors as pT≥3 and/or N^+/M^+ and G2-3 and/or N^+/M^+ and G2-3. ccRCC, clear-cell renal cell carcinoma, subset of all RCC.

Follow-up assessment. For assessment of follow-up data urologists and general practitioners of the patients were contacted. Every new appearance of disease was defined as recurrence. The duration of follow-up was calculated from the date of surgery to the date of either recurrence as defined before or the date of last follow-up in case of recurrence-free survival within the observation period. Follow-up assessment ended in April 2010.

DNA extraction and bisulfite conversion. Total DNA extraction of 20 serial frozen sections (20 μm) from each tumor and

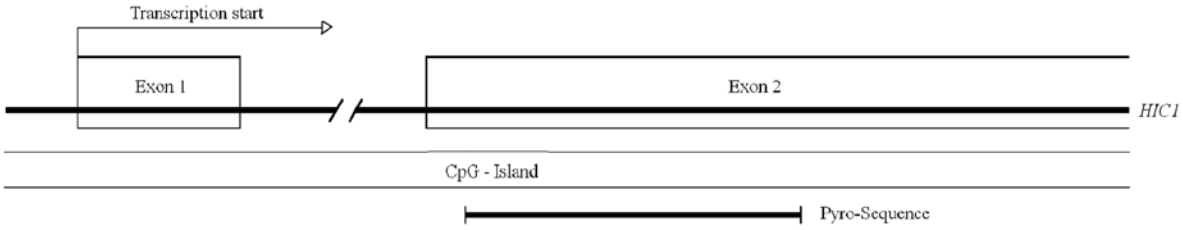

Figure 1. Schematic figure of the *HIC1* transcript used for pyrosequencing in relation to CpG-Island and *HIC1*-Gen.

Table II. Overview of methylation analysis in statistical comparison with clinicopathological parameters.

HIC1 methylation	Rel. methylation (mean) (%)	(%)	p-value	Odds ratio (OR)	95% Confidence interval (CI)	Statistical method
All RCC						
Paired tissue samples adN/TU[a]	17.5	19.7	0.35	-	-	Paired t-test
ppRCC/ccRCC	22.3	17.6	0.23	0.98	0.95-1.01	Log regression
Localized/advanced[b]	18.0	18.4	0.89	1.02	0.98-1.03	Log regression
Grading low/high grade[c]	17.4	22.5	0.24	1.02	0.99-1.06	Log regression
Metastases M0/M1	17.5	20.7	0.39	1.01	0.98-1.04	Log regression
Lymph node metastases N0/N1	19.6	7.9	**0.03**	0.91	0.82-0.98	Log regression
Clear cell RCC						
Paired tissue samples adN/TU[a]	15.8	19.5	0.18	-	-	Paired t-test
Localized/advanced[b]	15.1	19.8	0.20	1.02	0.99-1.06	Log regression
Grading low/high grade[c]	16.2	24.5	0.09	1.03	0.99-1.08	Log regression
Metastases M0/M1	16.2	21.5	0.20	1.02	0.99-1.06	Log regression
Lymph node metastases N0/N1	18.9	5.5	0.082	0.85	0.66-0.97	Log regression

[a]Tumor (TU) and adjacent benign renal tissue (adjacent normal tissue; adN). [b]Localized is defined as pT≤2, N0/M0; advanced tumors as pT≥3 and/or N^+/M^+. [c]Low grade tumor (G1-2); high grade tumor (G2-3). Bold text, statistically significant.

normal specimen was performed using proteinase K digestion and DNA extraction via standard phenol/chloroform extraction. Each 1 μg of extracted DNA was then subjected to bisulfite conversion as described previously and aliquots were stored at -80˚C (24). Additionally, two serial cuttings of each tissue sample were stained with hematoxylin-eosin and re-evaluated by pathologists.

Bisulfite-pyrosequencing for HIC1 CGI methylation analysis. Methylation analysis of *HIC1* CpG sites within the CGI (Fig. 1) was conducted using bisulfite-pyrosequencing according to the manufactures protocol (Qiagen, Hilden, Germany). Quantitative methylation analysis of ten CpG-sites was carried out following semi-nested PCR.

For first round amplification 50 ng of converted DNA, 0.4 μM of each primer F1 (5'-TTG GAT TAT GAT ATG GTG AG-3') and Runiv (5'-GGG ACA CCG CTG ATC GTT TAC TTA AAA CA-3'), 1 unit Taq polymerase (Qiagen), 0.2 mM dNTPs, 2 mM $MgCl_2$ and 1.1 μl of the included reaction buffer were mixed and filled up to a final volume of 10 μl with H_2O. PCR was carried out with the following settings: initial denaturation at 95˚C for 180 sec; 45 cycles with 45 sec of denaturation at 95˚C, annealing at 56˚C for 45 sec, primer extension at 73˚C for 45 sec and 180 sec for the last cycle. The second round of amplification was performed at an annealing temperature of 58˚C using 1 μl of the first PCR product and primers F2 (5'-GTT TGG TGT TGG ATT TTA T-3') and the biotinylated universal primer RBio (Biotin-5'-GGG ACA CCG CTG ATC GTT TA-3') according to the universal primer concept previously reported (25).

Following single strand isolation pyrosequencing was done using 0.3 μM of the sequencing primer (GTGTTGGATT TTATTTATAT). Measurements were performed according to the manufacturers protocol using the PyroMark Q24 pyrosequencing system (Qiagen). Probes passing automatic quality control of the manufactures software were subjected to final evaluation of methylation by calculating the mean CpG-site methylation.

Statistical analyses. All analyses were performed exploratory, e.g. p-values are descriptively. Group comparisons between tumor and normal renal tissues according to *HIC1* methylation levels were performed using the paired t-test. Differences between renal tumor sub-groups (clear cell vs. papillary, local-

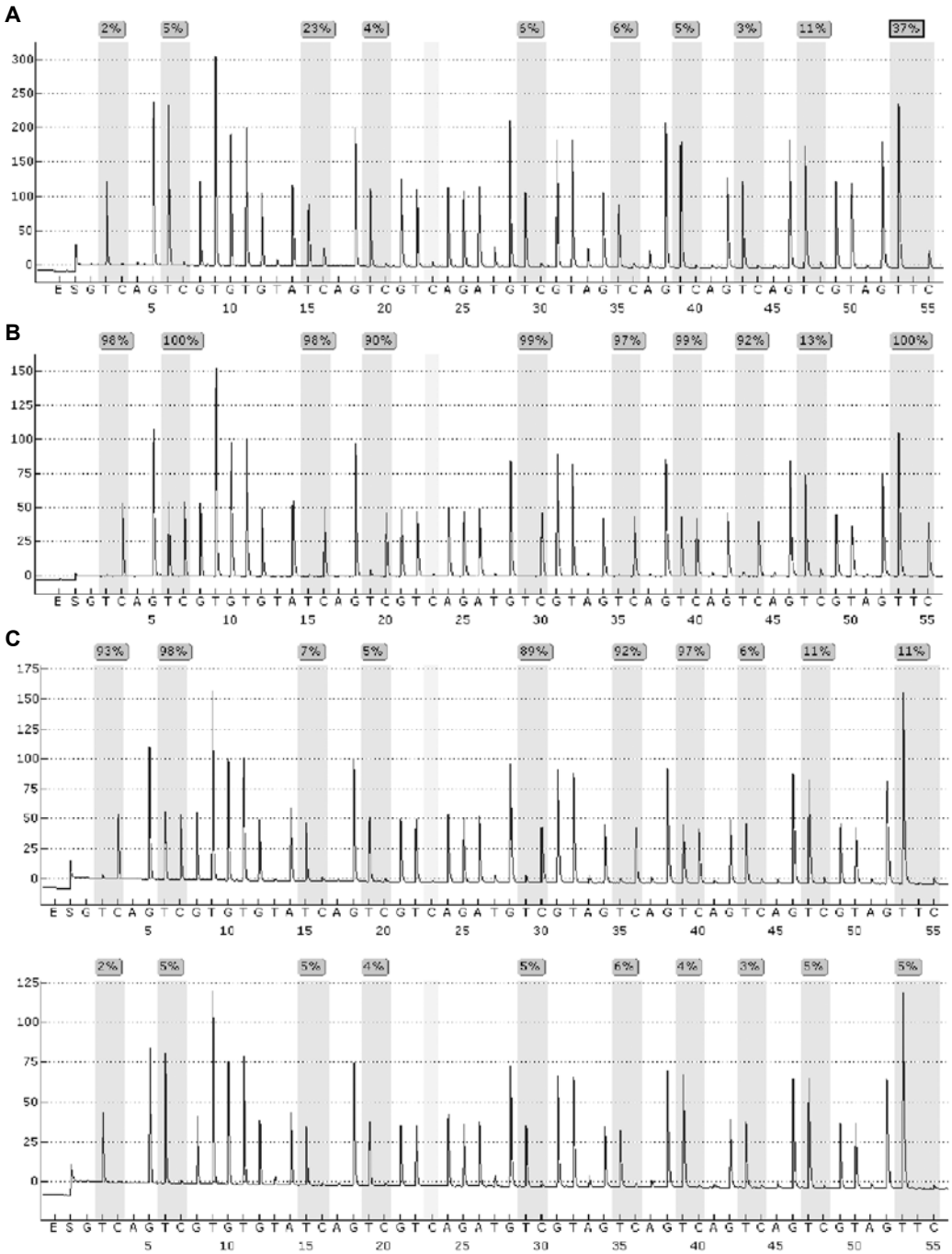

Figure 2. Methylation analysis of ten CpG-sites using pyrosequencing of the *HIC1* transcript. (A) negative QU and (B) positive control QM DNA. Patient samples (C).

ized vs. advanced), lymph node status and distant metastasis were assessed by univariate logistic regression, which was also used to evaluate the association between *HIC1* methylation levels and tumor stage, grade or tumor diameter following dichotomization of values. For visual comparison of the distribution of methylation values obtained for different groups bean plot analyses were applied as an alternative to boxplots. The individual observations are shown as small lines in a one-dimensional scatter plot, the estimated density of the distributions is visible and the mean is shown. Furthermore, uni- and bivariable Cox regression models examined recurrence-free survival. Descriptive hazard ratios (HR), as well as respective 95% confidence intervals (95% CI) and p-values were calculated. In addition, Kaplan-Meier curves were drawn for visualization. All analyses were performed using the R (version 2.12 including libraries 'beanplot', 'lattice' and 'survival') and Java Gui for R 1.7-0. The 'maxRank' package was used to calculate the cutpoint for survival analysis. In all tests, $p<0.05$ was considered to indicate significance.

Results

Controls and cell lines. Converted methylated (QM) and unmethylated (QU) control DNAs and DNA from urological cancer cell lines as well as primary normal cells were analyzed using

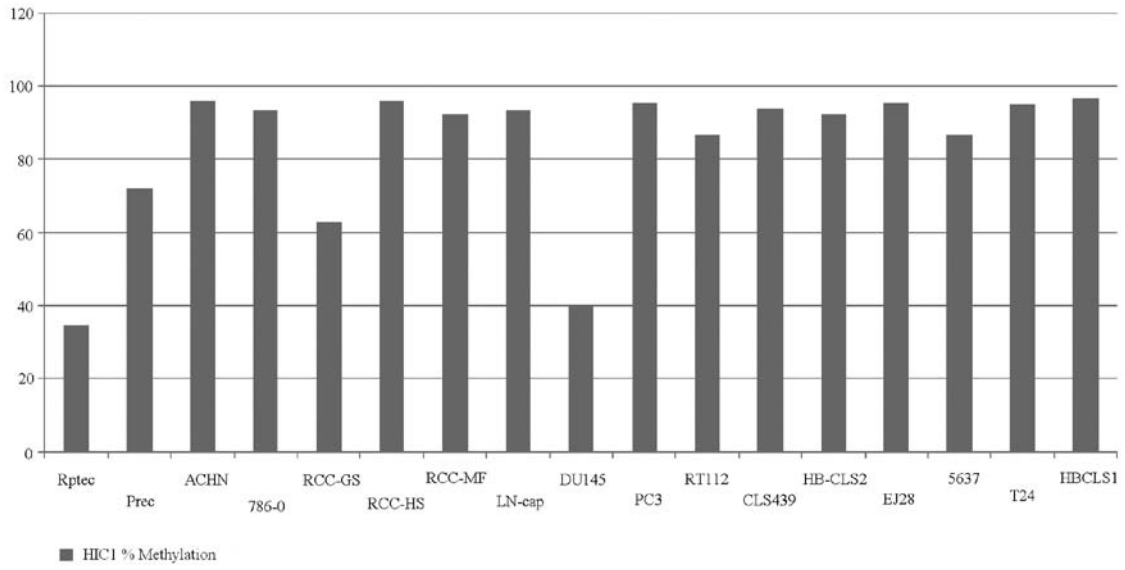

Figure 3. Relative methylation detected by pyrosequencing of 17 cell lines.

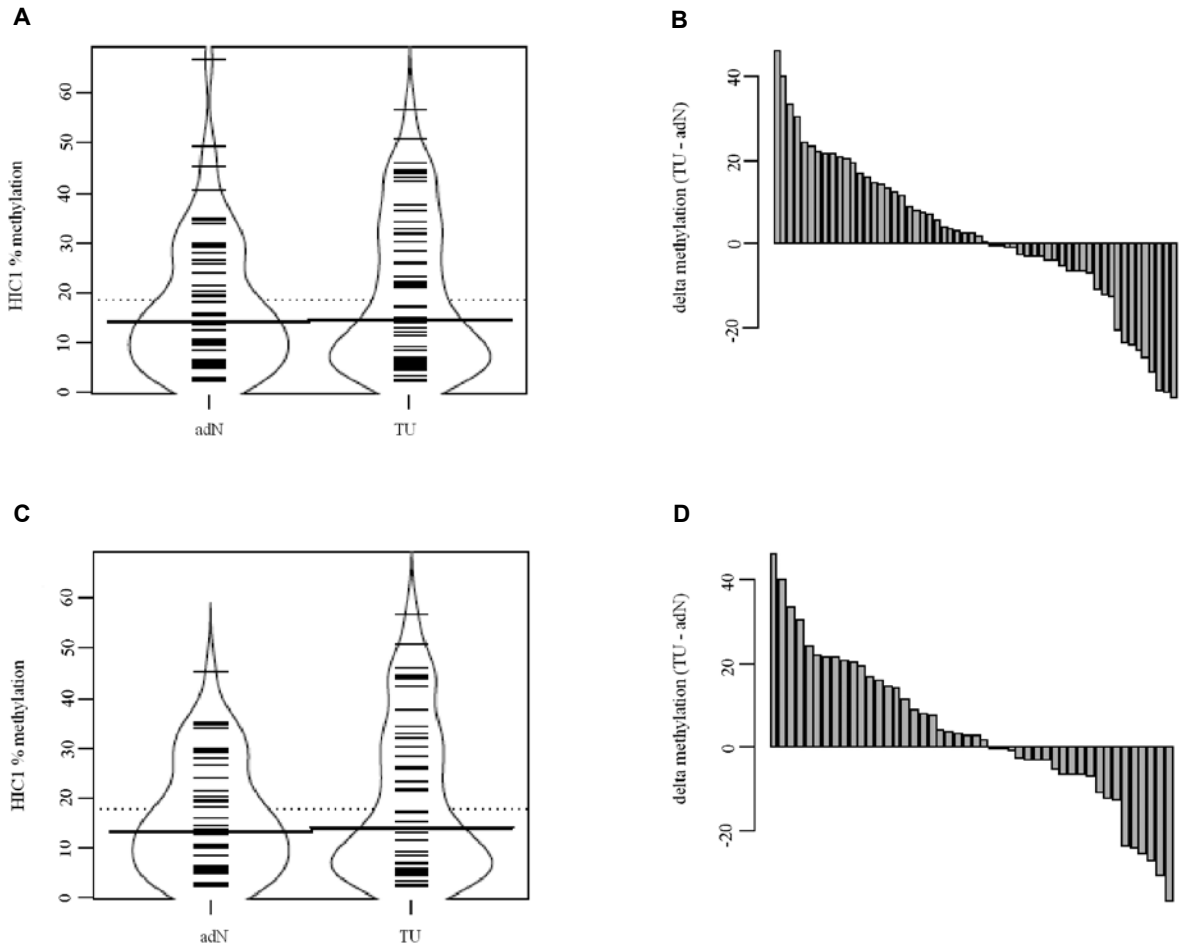

Figure 4. Distribution of relative *HIC1* methylation in paired tumor (TU) and corresponding normal tissue (adN) of (A) all RCC, (B) all RCC paired samples. Sorted methylation difference plots are showed for paired samples (TU - adN) of (C) ccRCC and (D) ccRCC tissues.

pyrosequencing. QM and QU showed mean relative methylation values of 89% and 10% respectively. Both DNAs exhibited heterogeneity in methylation including CpG sites with higher (QU) or lower methylation (QM) (Fig. 2). Malignant kidney cell lines (ACHN, 786-O, RCC-GS, RCC-HS, RCC-MF) show methylation in most cases of above 90% while non malignant renal or prostatic primary cells were measured between 35 and 72% methylation (Rptec, Prec) (Fig. 3).

Table III. Overview of univariate and bivariate methylation analysis for recurrence-free survival.

HIC1 methylation	Univariate Cox regression analysis			Bivariate Cox regression analysis		
	p-value	Hazard ratio (HR)	95% Confidence interval (CI)	p-value	Hazard ratio (HR)	95% Confidence interval (CI)
Methylation vs. distant metastases (M0/M1)						
Methylation	**0.0074**	3.9998	1.45-11.03	**0.0284**	3.1649	1.13-8.88
Distant metastases (M0/M1)	**0.000049**	7.7541	2.89-20.84	**0.0003**	6.4491	2.36-17.59
Methylation vs. LN-metastases (N0/N1)						
Methylation				**0.0065**	4.0945	1.48-11.30
LN-metastases	0.0641	3.3052	0.93-11.72	0.0520	3.5189	0.99-12.52
Methylation vs. state of disease (loc/adv)[a]						
Methylation				**0.0013**	5.4381	1.94-15.27
State of disease (loc/adv)[a]	**0.0016**	7.6299	2.17-26.85	**0.0004**	9.7658	2.73-34.88
Methylation vs. Gender						
Methylation				**0.0075**	3.9943	1.45-11.02
Gender	0.2192	2.0334	0.66-6.31	0.2232	2.0237	0.65-6.29
Methylation vs. dichotomized tumor diameter						
Methylation				**0.0232**	4.1774	1.22-14.35
Dichotomized tumor diameter	0.5046	1.4981	0.46-4.91	0.4123	1.6459	0.50 -5.42
Methylation vs. dichotomized patient's age						
Methylation				**0.0074**	4.0013	1.45-11.05
Dichotomized age	0.9009	1.0643	0.40-2.84	0.9885	0.9928	0.37-2.65

[a]loc, localised is defined as pT≤2, N0,M0 and G1-2; adv, advanced is defined as pT≥3 and/or N^+/M^+ or G2-3. Bold text, statistically significant.

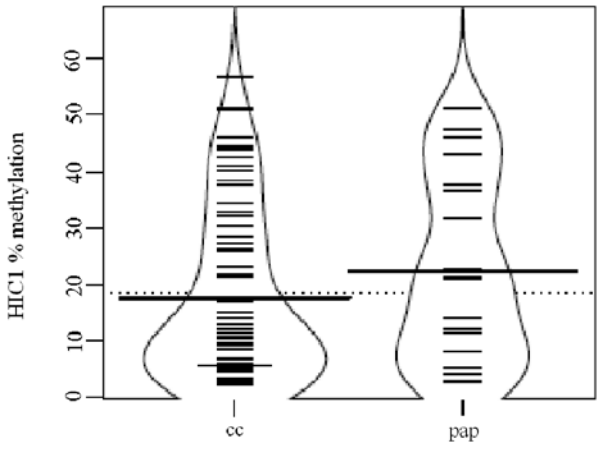

Figure 5. *HIC1* methylation levels comparing the histologic entities of clear-cell RCC and papillary RCC.

HIC1 CGI methylation in paired tumor and corresponding normal tissue samples. We found no difference in mean methylation levels of *HIC1* comparing tumor and corresponding normal renal tissue in paired sample analysis (p=0.35; paired t-test; Fig. 4). A difference was neither found between tumor and corresponding normal tissues in subgroup analysis of ccRCC (p=0.18; paired t-test; Fig. 4).

HIC1 CGI methylation and association with clinical and histopathologic parameters. We investigated whether statistical association can be detected between CGI methylation of *HIC1* and clinicopathological parameters (Table II). The median age of the cohort was 62.8 years, 64 patients were men and 34 patients were women.

Comparing the different histologic entities we found no difference in tissue specific mean *HIC1* methylation between papillary RCC and clear-cell RCC (p=0.23; univariate logistic regression; Fig. 5).

In contrast, a lower level of methylation was detected to be significantly associated with the presence of lymph node metastasis in RCC as well as a trend was found in subgroup analysis of ccRCC (p=0.030; p=0.082; univariate logistic regression; Fig. 6). However, no significant statistical association was observed for histological grading, TNM group stage and the presence of distant metastases (Table II).

HIC1 CGI methylation and association with recurrence-free survival. *HIC1* CGI methylation showed an association with recurrence-free survival (Table III) in univariate analysis using a Cox proportional hazard model and a cutpoint of relative methylation of 21.3%. We found a Hazard Ratio (HR) of 4.0 (p=0.0074; Fig. 7). Using the Cox proportional hazard model for pairwise bivariate analysis of *HIC1* CGI methylation with the clinicopathologic parameters we detected HRs from 3.1 to 5.4

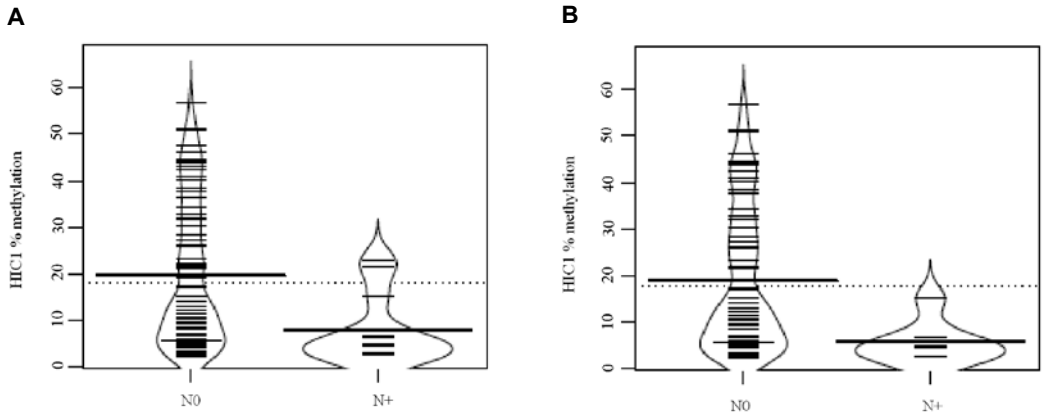

Figure 6. *HIC1* methylation in correlated to tumors with (N^+) and without (N0) lymph node metastases in (A) all RCC and (B) ccRCC.

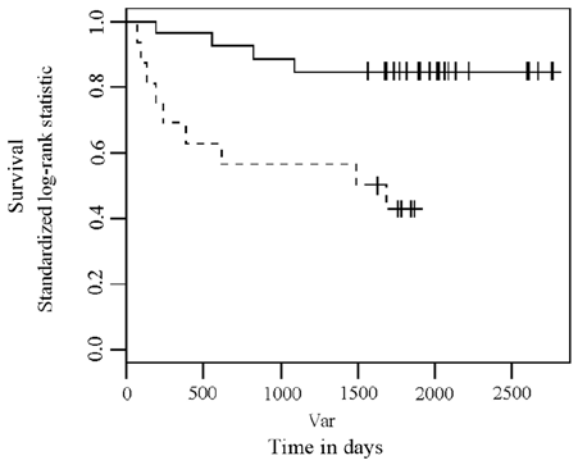

Figure 7. Recurrence-free survival associated to patients with methylation above the calculated cutpoint (dotted line) and to patients with methylation beneath the cutpoint (continuous line).

and p-values between 0.001 and 0.028 for the bivariate regression models (Table III). Only the presence of distant metastases (HR: 3.1649 vs. 6.4491) and state of disease (HR: 5.4381 vs. 9.7658) also exhibited statistically significant HR's methylation levels of the *HIC1* CGI in bivariate comparison.

Discussion

In 1993, Makos *et al* described that the hypermethylation of the genomic locus D17S5, later identified to harbor *HIC1*, precedes the manifestation of chromosome 17p alterations in RCC (23) and is involved in cancer development (18). Makos *et al* measured from methylation via *Not*I and *Not*I/*Bam*HI restriction analysis and compared the frequency of methylation with the occurence of p53 tumor suppressor gene mutation and allelic loss of chromosome 17p. In comparison early and late stage tumors showed higher frequencies of methylation than of 17p allelic loss and even less p53 mutation (23). The microsatellite marker D17S5 subsequently led to the identification of the *HIC1* tumor supressor gene, meanwhile identified to exhibit CGI hypermethylation in many human cancers. To our knowledge this study is the first that carried out a quantitative measurement of *HIC1* CGI methylation. Our study shows that *HIC1* CGI methylation is associated with recurrence-free survival and therefore, might be a possible prognostic marker for RCC prognosis.

This study quantitatively analysed subregion-specific *HIC1* CGI methylation in RCC and corresponding benign kidney tissues. However, we did not find tumor-specific hypermethylation as could have been expected regarding the data of Makos *et al* (23) as well as the frequently shown hypermethylation of *HIC1* in several other tumor entities (18). On the one hand, our analysis might be not comparable to the study of Makos *et al* (23) considering that this study used restriction analysis for detection of methylation and did not provide quantitative data. On the other hand, our study measured only part of the *HIC1* CGI that covers a total of 764 CpG sites according to the annotation given by the UCSC table browser (http://genome.ucsc.edu/) (Fig. 1). Taking into account that to our knowledge, no comprehensive *HIC1* CGI methylation analysis has been reported at yet it is possible that heterogeneity of methylation patterns in this CpG-island is responsible for the divergent findings, a problem that has been adressed recently for the *Gremlin1 (GREM-1)* gene CGI (26). Moreover, a recently published comparison of different methods for methylation detection demonstrated a high concordance between the results obtained by pyrosequencing and bisulfite sequencing, accepted as gold standard of methylation analysis (27).

Epigenetic alterations of *HIC1* have been described to occur in several tissues. In general, normal tissue seems to ubiquitously express *HIC1* (17). Therefore, Wales *et al* assumed that most benign tissues do not demonstrate *HIC1* methylation (17,18,28). At the same time a single methylated allele of *HIC1* has been found in benign tissues including kidney, breast, prostate, brain and cerebellum giving reason for authors to suggest that these tissues are predisposed to inactivation of *HIC1* (28-31). Fleuriel *et al* postulated that these tissues might have a generally higher risk for tumor development (17,18,28). This assumption is supported by the reported hypermethylation of *HIC1* in prostate, breast, gastric, liver, esophageal and human male non-seminomatous germ cell tumors (18). Additionally, hypermethylation and associated loss of *HIC1*-expression have been shown in several tumor cell lines as well as in primary brain and colon cancer (17). Furthermore, *HIC1* hypermethylation was found in almost all recurrent acute lymphocytic

leukemia and blast crisis of chronic myelogenous leukemia proposing a *HIC1* hypermethylation to be a late event in haematopoietic malignancies (18,32).

Therefore, overall data concerning methylation and expression of *HIC1* varies depending on organic system, tissue origin and cancer subtype suggesting a differentiated function of *HIC1*. Of note, we were able to show an association of *HIC1* hypermethylation and shortened recurrence-free survival which severely underlines the importance of *HIC1* CGI-hypermethylation in RCC.

In addition the association of hypermethylation and shortened recurrence-free survival in RCC has only been shown for only a few analyzed genes so far. For *DAPK-1* as well as for *SCUBE3* an association with an increased risk of recurrence has been shown (10,33). Van Vlodrop *et al* were also able to show significantly worse survival to be associated with methylation of one region within the *GREM-1* CGI in univariate analysis and decreased overall survival in correlation with hypermethylation of another *GREM-1* CGI subregion in multi- and univariate analysis (34). By creating a combined methylation score for the Wnt-Antagonists *sFRP-1, sFRP-2, sFRP-4, sFRP-5, Wif-1* and *Dkk-3* Urakami *et al* instead of were able to show statistical association with a shortened overall survival found *SFRP1, BNC1* and *COL14A1* to be related with poor prognosis (9,35). From a clinical point of view at yet only few CGIs show statistical association with clinical parameters. Thus, the independent prognostic information provided by *HIC1* CGI methylation in RCC may be of clinical value. In addition, we found evidence that hypomethylation of *HIC1* could be associated with the presence of lymph node metastases, although low odds ratios were detected and the effect could not be seen in the subgroup of clear cell RCCs. This uncertain result would also partially contradict a study recently published by Fleuriel *et al* (18). Fleuriel *et al* proposing an association of *HIC1* hypermethylation with aggressive tumor behavior. However our finding that quantitatively measured *HIC1* CGI methylation is an independent predictor of recurrence-free survival agrees well with the results of Fleuriel *et al* describing a relationship with poor prognosis of patients (17,18,23,29,36-38).

The most studied functional interaction of *HIC1* is the complex *HIC1*-p53-SIRT regulatory loop. Within this pathway inactivation of *HIC1* is followed by an upregulation of *SIRT* that is able to deactivate p53, which can in turn upregulate *HIC1* expression (18,20). Cells with inactivated p53 will bypass apoptosis and survive DNA damage insted of leaving insert providing a functional explanation how epigenetic silencing of *HIC1* increases risk for cancer development.

Jenal *et al* showed that *HIC1* can be induced by cell cycles and apoptosis regulator E2F1, one out of eight genes of the E2F family (39). However, in a regulatory loop *HIC1* also directly regulates E2F-responsive genes and thereby represses cell growth. The complex interactions of p53 and E2F in vital cellular processes, their cooperation in restriction of tumor development and pro-apoptotic function as well as their well known deregulation in human cancers suggest *HIC1*, being linked to either one, to play an important role in cancer development (40). Noteworthy, a study concerning tumor cell response in breast cancer was also able to show that *HIC1* mediates growth suppression by estrogen antagonists in breast cancer cells. Demonstrating that *HIC1* expression correlates with growth repression in sensitive tumor cells while in antagonist-resistant tumor cells induction of *HIC1* is lost, this study clearly indicates the complexity of *HIC1* (41). Both the potential clinical relevance as well as interactions of *HIC1* epigenetic alterations with cellular regulation are not fully understood at yet therefore underlining the need to take a closer look at *HIC1* function in renal cancer model systems.

In conclusion, *HIC1* hypermethylation is associated with reduced recurrence-free survival in RCC. Therefore, *HIC1* could be seen as a possible marker to improve individualized therapy and risk stratification. Future studies will have to focus on functional analysis to further elucidate the role of *HIC1* in RCC.

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