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 homolog* (SIR2) 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 published 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-
methylation precedes the manifestation of genetic alterations in 17p and is involved in development of RCC. For methylation analysis *NotI* and *NotI/BamHI* 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 bisulfite-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, M0 76 53; M1 22 19.

**Paired tissue samples**

- **N** 58 46
- **G1** 20 18
- **G2** 63 42
- **G3** 15 12
- **Gender**
  - **Male** 64 44
  - **Female** 34 28
- **Status**
  - **Localized tumor** 52 34
  - **Advanced tumor** 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 -

*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
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 **\textit{HIC1} **CGI methylation analysis.**

Methylation analysis of \textit{HIC1} CpG sites within the CGI (Fig. 1) was conducted using bisulfite-pyrosequencing according to the manufacturers 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\(_2\)O. 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 TTAATTATAT). 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 \textit{HIC1} methylation levels were performed using the paired t-test. Differences between renal tumor sub-groups (clear cell vs. papillary, local-
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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
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).

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.
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.

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

<table>
<thead>
<tr>
<th>HIC1 methylation</th>
<th>Univariate Cox regression analysis</th>
<th>Bivariate Cox regression analysis</th>
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<tr>
<td></td>
<td>p-value</td>
<td>Hazard ratio (HR)</td>
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<td>Methylation</td>
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<td>Methylation vs. dichotomized tumor diameter</td>
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<td>Methylation</td>
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<td>Dichotomized age</td>
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<td>1.0643</td>
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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).

Figure 5. HIC1 methylation levels comparing the histologic entities of clear-cell RCC and papillary RCC.
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 NotI and NotI/BamHI restriction analysis and compared the frequency of methylation with the occurrence 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 suppressor 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 addressed 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 Fleuriet et al (18). Fleuriet 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 Fleuriet 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 instead 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.

References


