Research

Epigenetic control of the ubiquitin carboxyl terminal hydrolase 1 in renal cell carcinoma

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Abstract

Background: The ubiquitin carboxyl-terminal hydrolase 1 (UCHL1) gene involved in the regulation of cellular ubiquitin levels plays an important role in different cellular processes including cell growth and differentiation. Aberrant expression of UCHL1 has been found in a number of human solid tumors including renal cell carcinoma (RCC). In RCC, UCHL1 overexpression is associated with tumor progression and an altered von Hippel Lindau gene expression.

Methods: To determine the underlying mechanisms for the heterogeneous UCHL1 expression pattern in RCC the UCHL1 promoter DNA methylation status was determined in 17 RCC cell lines as well as in 32 RCC lesions and corresponding tumor adjacent kidney epithelium using combined bisulfite restriction analysis as well as bisulfite DNA sequencing.

Results: UCHL1 expression was found in all 32 tumor adjacent kidney epithelium samples. However, the lack of or reduced UCHL1 mRNA and/or protein expression was detected in 13/32 RCC biopsies and 7/17 RCC cell lines and due to either a total or partial methylation of the UCHL1 promoter DNA. Upon 2'-deoxy-5-azacytidine treatment an induction of UCHL1 mRNA and protein expression was found in 9/17 RCC cell lines, which was linked to the demethylation degree of the UCHL1 promoter DNA.

Conclusion: Promoter hypermethylation represents a mechanism for the silencing of the UCHL1 gene expression in RCC and supports the concept of an epigenetic control for the expression of UCHL1 during disease progression.

Background
The highly conserved ubiquitin-proteasome complex is in addition to its general function in the protein turnover process also associated with the regulation of cell growth, differentiation, the modulation of membrane receptors and cellular stress responses as well as the turnover of different cytoskeletal components. It is comprised of enzymes involved in the protein ubiquitination/deubiquitination as well as of the subunits of the 20S proteasome that degrades ubiquitin-conjugated proteins [1,2]. Ubiqui-
uitation is a reversible biological process consisting of enzymes, that attach single or multiple ubiquitin molecules to protein substrates and deubiquinating enzymes (DUB). e.g. ubiquitin carboxyl-terminal hydrolases (UCH) and ubiquitin-specific proteases (USP) [3,4]. The protein gene product 9.5 (PGP 9.5) also termed ubiquitin carboxyl-terminal hydrolase-1 (UCHL1), a member of the UCH protein family, represents a soluble 25 kD protein with both ubiquitin hydrolase and dimerization-dependent ubiquitin ligase activities [5,6]. As a member of the ubiquitin-proteasome complex UCHL1 is involved in the control of the intracellular proteolysis, protein turnover and regulatory processes, which are important in maintaining normal cellular homeostasis [7]. UCHL1 expression exhibits marked tissue specificity and is mainly expressed in testis and neuronal tissues at various differentiation stages [8,9]. In addition, UCHL1 expression was detected during kidney development, in particular during the differentiation of renal tubules representing the origin of clear cell renal cell carcinoma (RCC) and in the regulation of the cell cycle of parietal epithelial cells of the Bowman’s capsule [10,11]. Since UCHL1 is expressed in pathophysiologial situations of the kidney such as acute ischaemic renal failure, renal hypertrophy, von Hippel Lindau (VHL) disease as well as neoplastic transformation of renal cells it may play a fundamental role in the mechanisms controlling the protein turnover of the kidney. There exists conflicting evidence concerning the role of UCHL1 in tumorigenesis varying from anti-tumor to pro-tumor properties depending on the tumor type analysed [12-14]. Several studies demonstrated aberrant UCHL1 expression in acute lymphoblastic leukaemia, myeloma, melanoma, neuroblastoma, pancreatic, esophageal, lung, thyroid, colon and renal cell carcinoma (RCC). In certain tumor types UCHL1 expression is even associated with tumor progression and decreased survival rates of patients [12,13,15-21]. However there is also evidence that UCHL1 expression might be associated with suppression of tumor growth in RCC [21] DNA methylation at CpG dinucleotides within the promoter region of genes is a common event in the pathogenesis of tumors including urological cancers and has been explored as both mechanism and marker of tumor progression with potential application for diagnosis, classification and prognosis of disease [22-29]. Using different technologies UCHL1 has been identified as a frequently silenced gene in a cancer-specific manner, in particular in pancreatic, gastric, colon, ovarian, head neck squamous cell and hepatocellular carcinoma [14,30-35]. Thus, in order to understand the underlying molecular mechanism of the aberrant UCHL1 expression in RCC lesions [21], microarray analysis of the RCC cell line ACHN either left untreated or treated with the demethylating agent 2’-deoxy-5-azacytidine (DAC) was performed demonstrating an aberrant hypermethylation of the UCHL1 promoter DNA and an association with UCHL1 downregulation in RCC lesions [36]. We here extended these data and determined whether the promoter DNA methylation also contributes to the lack of UCHL1 expression in 32 pairs of primary RCC lesions and corresponding tumor adjacent kidney epithelium as well as 17 RCC cell lines. The given methylation status of the UCHL1 promoter DNA was further correlated with the UCHL1 mRNA and protein expression levels in these samples. Moreover, silenced UCHL1 expression could be restored in RCC cell lines by treatment with the demethylating agent DAC.

Methods

Cell lines and tissue culture

The human RCC cell lines employed in this study were established from patients with primary RCC of the clear cell type [21,37,38]. All tumor cell lines were maintained in high glucose Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin/streptomycin, 1 mM non-essential amino acids and 1 mM sodium pyruvate (Gibco/BRL, Life Technologies, Karlsruhe, Germany).

Patients and tumor biopsies

This study used tumor specimens of RCC obtained from patients undergoing nephrectomy at the Department of Urology of the University Hospital in Mainz, Germany. All cases had been reviewed by a pathologist according to the WHO classification criteria. Clinicopathological data obtained from the patients included sex, age, TNM stage and histological subtype. The study design was approved by the Ethical committee of the Johannes Gutenberg University of Mainz and informed consent was obtained from all RCC patients.

DAC treatment

To assess the ability of the DNA methyltransferase inhibitor DAC to induce the expression of UCHL1, RCC cell lines were treated for 5 days with 1, 5 and 10 μM DAC (Sigma-Aldrich GmbH, Taufrkirchen, Germany). Subsequently untreated and DAC treated cells were harvested, lysed and total mRNA and/or total protein extracted. The resulting samples were then subjected to qRT-PCR, Western blot and methylation assays.

Semi-quantitative and real-time reverse transcription polymerase chain reaction ((q)RT-PCR) analysis

Total RNA was extracted from the samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized from 3 μg RNA treated with DNase I (Invitrogen GmbH, Karlsruhe, Germany) using oligo d’ primers (Fermentas, Mannheim, Germany) and Superscript II reverse transcriptase (Invitrogen). Real time PCR was performed with
the UCHL1-specific primer set (sense: 5'-GCCAATGTCGGTGATAGT-3'; anti-sense: 5'-AGCCGACTTCCTCTGTCTTG-3') using an annealing temperature of 62°C. β-actin served as the reference gene (sense: 5'-GAACATTTCCGTTGGACGAT-3'; anti-sense: 5'-TCCTGTGGCATCCACGAAACT-3'). All real time PCR analyses were performed in a thermal cycler (Rotorgene, Corbett Life Science, Australia) using the QuantiTect SYBR-Green PCR Kit (Qiagen). UCHL1 expression levels were normalized against β-actin amplions. The UCHL1 expression after 5-days DAC treatment was calculated as x-fold expression of the respective untreated sample, which was set to 1.

**Western blot analysis**

20 μg of total protein/lane from untreated or DAC-treated RCC cell lines was subjected to Western blot analysis as previously described [21]. The membranes were incubated either with the anti-UCHL1-specific polyclonal rabbit antibody (PG 9500, BIOMOL, Hamburg, Germany) or with the anti-β-actin-specific monoclonal antibody (mAb) AC15 (ab6276, Abcam Ltd., Cambridge, UK) serving as a loading control. Horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (P0217, DAKO, Hamburg, Germany) or rabbit anti-mouse IgG (P0260, DAKO) were used as secondary antibodies. The immunostaining was visualised using a chemiluminescence detection kit (LumiLight Western Blotting Substrate, ROCHE Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

**DNA extraction and analysis of the methylation status of the UCHL1 promoter**

In order to determine the methylation status of the UCHL1 promoter DNA, a CpG islet within the UCHL1 promoter containing 22 CpG dinucleotides was mapped using the CpGplot tool (EBI Tools, EMBoss CpGPlot; http://www.ebi.ac.uk/emboss/cpgplot). Subsequently, bisulfite-specific primers flanking the transcription start site of the CpG islet in the UCHL1 promoter were designed with the Oligo 4.0 program relying on the reference sequence GI: 16949651 (National Bioscience, MN, USA). Upon isolation of genomic DNA from established RCC cell lines and/or biopsy specimens with the QIAamp DNA Mini Kit (Qiagen), UCHL1 expression levels were normalized against β-actin amplions. The UCHL1 expression after 5-days DAC treatment was calculated as x-fold expression of the respective untreated sample, which was set to 1.

**Results**

**Correlation of the UCHL1 expression level in RCC cell lines of the clear cell type with the promoter DNA methylation status**

We have recently demonstrated a heterogeneous expression pattern of UCHL1 mRNA and/or protein in both RCC cell lines and RCC lesions, which is associated with the RCC subtype, VHL status and with tumor progression [21]. In order to investigate the molecular mechanism(s) involved in this heterogeneous expression pattern, the DNA methylation status of the CpG islet in the UCHL1 promoter was determined in a series of 17 established primary RCC cell lines exhibiting heterogeneous UCHL1 expression levels. As determined by RT-PCR and Western blot analysis, 3/17 RCC cell lines express neither UCHL1 mRNA nor protein, 4/17 RCC cell lines exhibit low UCHL1 transcription, but no UCHL1 protein, whereas 9/17 express high levels of UCHL1 mRNA and protein (Table 1; [21]). Based on this screening we tested whether the lack of UCHL1 expression in RCC cell lines could be attributed to aberrant CpG islet methylation within its promoter region, which represents a common mechanism of gene silencing in various human cancers [31,34]. Therefore, the DNA methylation status of a genomic 265 bp DNA fragment containing 22 CpG dinucleotides next to the transcriptional start site of the UCHL1 gene (Figure 1A) was investigated by both COBRA and direct bisulfite sequencing. As representatively shown for 3 RCC cell lines in Figure 1B, the methylation pattern of the UCHL1 promoter DNA was highly heterogeneous varying from total to partial to lack of methylation. In MZ1851RC cells for example the UCHL1 promoter DNA was not methylated,
whereas the COBRA-based analysis indicated a partial methylation of the UCHL1 promoter DNA in the RCC cell line MZ2862RC, characterized by methylation of some of the CpG dinucleotides within the core region of the UCHL1 promoter while other CpG sites remain unmethylated. In addition strong methylation of the promoter DNA core region, as defined by either methylation of all CpG sites or only few unmethylated CpG sites within the core region of the CpG islet, was found in the RCC cell line MZ1851LN. The status of the methylation pattern was directly associated with the response to DAC treatment: RCC cell lines with a strongly methylated UCHL1 promoter DNA responded to low concentrations of DAC (1 μM, MZ1851RC), whereas higher DAC doses were required to efficiently demethylate partially methylated promoters (10 μM, MZ2862RC). Based on the methylation status RCC cell lines could be classified into 3 different subgroups. The first category consists of RCC cell lines with a high to complete UCHL1 promoter DNA methylation predominantly lacking both UCHL1 mRNA and protein expression. The second exhibits a partially methylated promoter, which corresponds to low to moderate UCHL1 expression levels, whereas the third category is represented by RCC cell lines with unmethylated promoters expressing high levels of UCHL1 (Table 1). In order to verify the COBRA results and to determine the

| RCC cell line | mRNA | protein | BstU I | Taq I | sequencing |
|--------------|------|---------|--------|-------|------------|
| MZ1257RC     | +    | +       | U      | U     | U          |
| MZ1774RC     | +    | +       | U      | U     | U          |
| MZ1790RC     | (+)  | -       | M      | M     | P          |
| MZ1851RC     | +    | +       | U      | U     | U          |
| MZ1851LN*    | (+)  | -       | M      | M     | M          |
| MZ1879RC     | -    | -       | M      | M     | M          |
| MZ1940RC     | -    | -       | M      | M     | M          |
| MZ1973RC     | +    | +       | U      | U     | U          |
| MZ2175RC     | -    | -       | P      | P     | P          |
| MZ2733RC     | +    | +       | U      | U     | U          |
| MZ2789RC     | +    | -       | P      | P     | P          |
| MZ2858RC     | +    | +       | U      | U     | U          |
| MZ2861RC     | +    | +       | U      | U     | U          |
| MZ2862RC     | (+)  | -       | P      | M     | P          |
| MZ2885RC     | +    | n.d.    | U      | U     | U          |
| MZ2904RC     | +    | + (pp)  | P      | P     | P          |
| MZ2905RC     | +    | +       | U      | U     | U          |

*Cell line derived from a lymph node metastasis of a patient suffering from RCC. The methylation pattern of the UCHL1 promoter DNA was determined by COBRA and/or sequencing.

(-): no expression detectable; ((+)) weak expression detectable; (+) expression detectable; (U) unmethylated UCHL1 promoter; (P): partially methylated UCHL1 promoter (M): fully methylated UCHL1 promoter; (pp): expression verified by proteomic profiling of the corresponding RCC lesion; n.d. not done
transcriptional silencing of UCHL1 in RCC cell lines. UCHL1 hypermethylation is tightly associated with the DAC treatment varied from the demethylation of 1 to 11 - 13 fold in the RCC cell line MZ1851LN (strong methylated UCHL1 promoter DNA) to 11 - 18 fold in the RCC cell line MZ2862RC (partially methylated UCHL1 promoter DNA).

Restoration of UCHL1 expression in RCC by treatment with DAC
To confirm that UCHL1 promoter DNA hypermethylation is responsible for the silencing of UCHL1, a selected number of UCHL1- and UCHL1+ RCC cell lines were treated with different concentrations of DAC (1, 5, 10 μM) for 5 days. As shown in Figure 2, DAC treatment of RCC cell lines displaying either partially (MZ2862RC) or fully methylated (MZ1851LN) UCHL promoter DNA regions led to the induction of UCHL1 mRNA (Figure 2A) restoring protein expression (Figure 2B). However, as representative shown for MZ1851RC in RCC cell lines lacking UCHL1 promoter DNA methylation DAC treatment did neither alter the mRNA nor the protein expression levels of UCHL1. In contrast, the restored UCHL1 expression was associated with a partial or total demethylation of the UCHL1 promoter DNA as determined by COBRA (Figures 2A and 2B). Based on qRT-PCR analyses the induction at the mRNA level ranges from 1.1 - 1.4 fold in the RCC cell line MZ1851RC (unmethylated UCHL1 promoter DNA) to 11 - 13 fold in the RCC cell line MZ1851LN (strong methylated UCHL1 promoter DNA) to 11 - 18 fold in the RCC cell line MZ2862RC (partially methylated UCHL1 promoter DNA).

Methylation of UCHL1 in human primary RCC lesions, but not of corresponding normal kidney epithelium
Since an impaired UCHL1 expression was not only found in RCC cell lines, but also at a high frequency in primary extent of methylation bisulfite DNA sequencing of the respective UCHL1 promoter region was performed in representative RCC cell lines [see Additional file 1]. As summarized in Table 1, the bisulfite DNA sequencing data confirmed the heterogeneous methylation pattern of the UCHL1 promoter detected by COBRA in RCC cell lines, but also stressed the point that there exists no strict homogeneity in regard to the methylation status of CpG oligonucleotides. Even within a given cell line the efficacy of the DAC treatment varied from the demethylation of 1 to 18 CpG dinucleotides within the UCHL1 promoter DNA (data not shown). Nevertheless, the data suggest that UCHL1 hypermethylation is tightly associated with the transcriptional silencing of UCHL1 in RCC cell lines.
RCC lesions [21], the methylation status of the UCHL1 promoter DNA in 32 biopsy systems each comprised of a primary RCC lesions as well as corresponding non-neoplastic tumor adjacent kidney epithelium tissues was determined. As representatively shown in Figure 3A, COBRA analysis revealed partial UCHL1 promoter DNA methylation in the RCC lesions 2874 and 2876, whereas the lesion 2878 represented a tumor with a largely demethylated UCHL1 promoter DNA region. In contrast to the COBRA pattern characteristic for partial or rare promoter DNA methylation MZ1940RC cells represent a COBRA pattern characteristic for total promoter DNA methylation. Overall, the COBRA analyses revealed that 12/32 primary RCC lesions could be classified as partially methylated in regard to their UCHL1 promoter, whereas no methylation was found in the tumor adjacent kidney epithelium. The methylation status of RCC lesions was comparable to that of RCC cell lines, in which 9/17 RCC cell lines lack methylation, 3/17 exhibit a partial and 5/17 a total UCHL1 promoter DNA methylation (Table 1). In addition the sequencing of bisulfite-treated DNA confirmed the distinct methylation status of the UCHL1 promoter in the RCC lesions (data not shown). Thus, the epigenetic inactivation of UCHL1 is a common event in both primary RCC cell lines and RCC lesions and may represent a mechanism for its functional loss observed in the early phase of this disease.

Discussion
Promoter DNA methylation has been associated with the regulation of the expression pattern of tumor markers defined in both primary tumor specimen as well as in body fluids [40-42]. In RCC aberrant DNA methylation of the tumor suppressor gene VHL is found at a high frequency, whereas the frequencies of DNA promoter methylation of other tumor suppressor genes vary in this malignancy [22,43].

UCHL1, an essential member of the proteasome targeting ubiquitin-dependent protein degradation pathway plays an important role in distinct cellular processes such as cell proliferation, cell cycle, apoptosis and intracellular signalling [8], which are often disturbed in cancers [44,45]. UCHL1 has been demonstrated to be either overexpressed or silenced in both tumor lesions and/or tumor cell lines of distinct origin [12-14,21,46]. UCHL1 overexpression as found in colorectal cancer, non-small cell lung carcinoma and RCC was associated with a more aggressive potential and/or metastatic phenotype as well as in some cases with a poor prognosis of the respective patient collectives [17,19,21,47]. In contrast, UCHL1 expression has been also shown to be associated with increased apoptosis in breast cancer cells [48]. However, these studies did not analyse the underlying molecular mechanism of the heterogeneity of UCHL1 expression levels. The silencing of UCHL1 was discovered by cDNA microarrays and chemical genomic screening of head and neck squamous cell carcinoma [49] as well as pancreatic carcinoma lesions and pancreatic carcinoma cells either left untreated or treated with demethylating agents [32,35]. In addition, the silencing or downregulation of UCHL1 mediated by hypermethylation in esophageal squamous cell, hepatocellular and gallbladder carcinoma was correlated in these diseases with a poor prognosis of patients [14,31,50]. However, there exist some discrepancies in terms of the existing UCHL1 promoter methylation status, which might at least partially explained by the different methods employed for determination of the promoter DNA methylation status. In our hands, direct bisulfite sequencing is
the most sensitive method when compared to methyla-
tion-specific PCR and/or COBRA analyses and has the fur-
ther advantage of allowing the quantification of the methylation/demethylation ratio.

Beside DNA methylation there exist other gene silencing
mechanisms, such as the modification of the histone
structure by inappropriate deacetylation, or the presence
of the recently discovered microRNAs, which can either
act as selective destructors of targeted mRNA transcripts or
block the translation of mRNAs.

However, in this study it is demonstrated that the silen-
cing of UCHL1 in both RCC cell lines as well as in primary
RCC lesions mostly of clear cell subtype is rather linked to
the methylation of the UCHL1 promoter DNA. This is fur-
ther supported by the fact that a correlation between the
methylation status of the CpG islet in the UCHL1 pro-
moter DNA and the expression pattern at the transcrip-
tional as well as the translational level is shown. Since
UCHL1 protein expression is more pronounced in meta-
static than in primary RCC lesions [20], one can speculate
that UCHL1 expression is actively silenced during the
early stages of tumorigenesis and that its restored expres-
sion at a later stage may rather represent a reliable marker
for metastatic disease. This is in accordance with a recent
paper demonstrating a high frequency of UCHL1 methyl-
ation in primary RCC when compared to normal kidney
epithelium [36]. Similar results were obtained in colorec-
tal cancer demonstrating a lower frequency of methyl-
ation in metastasis when compared to the primary tumor
[51]. However, the methylation pattern of UCHL1 might
not only serve as a prognostic and/or predictive marker and reflect the metastatic potential of RCC, but might also
modulate the therapy sensitivity thereby influencing the
treatment modalities of RCC patients.

The function of UCHL1 in tumors is still controversially
discussed. In some tumor entities a hypermethylation of
UCHL1 was demonstrated in the primary tumor suggest-
ing a tumor suppressor gene activity, whereas in other
tumor types UCHL1 was highly overexpressed as a cause/
consequence of the transformation process. The initial
downregulation of UCHL1 by DNA promoter methyla-
tion might provide a growth advantage for these tumor
cells and thus represent a tumor escape mechanism since
the antigen cannot be recognized by the immune system
[34]. However, the functional consequences of temporary
UCHL1 inactivation still need to be determined. In the
UCHL1 knock out mice (gad mice) ubiquitin levels were
not induced and did not modulate the apoptosis-sensitive
phenotype [8]. If changes in the methylation pattern are
involved in the development of resistance against che-
motherapy and radiation in cancer cells, the determination of
the given methylation status of the UCHL1 promoter may
contribute to the understanding of the role of a differen-
tial UCHL1 expression during tumorigenesis and progres-
sion of human cancers as well as in the course of
developing therapy resistance. UCHL1 is characterized by
its dual function as a hydrolase in order to generate free
ubiquitins and as a ligase involved in producing multi-
ubiquitinated proteins [52]. The reexpression of UCHL1
in metastatic RCC indicated a tumor stage-specific UCHL1
hypomethylation suggesting that UCHL1 acts as an onco-
gene rather than as a tumor suppressor gene. However, it
still has to be defined, which proteins might be protected
from (UCHL1 deubiquitination activity) or alternatively
directed to undergo (UCHL1 ubiquitin ligation activity)
proteosomal degradation. Possible candidates for its res-
cue activity might be proteins contributing to the chemo-
and radiation resistance of RCC such as multi drug resist-
ance factors, whereas the targeted degradation of apopto-
sis inducing factors might help to evade such elimination
mechanisms.

Since UCHL1 (over)expression frequently occurs during
tumor progression this protein might be beneficial for the
progression and metastases formation process in certain
cancers [12,19,21]. This concept is further strengthened
by an enhanced cell proliferation and migration capacity
observed upon UCHL1 overexpression in UCHL1/RCC
cell lines [21].

In addition it has been shown that UCHL1 interacts with
the jun activating binding protein JAB1 and p27Kip [53].
Due to the interaction with JAB1, p27Kip is degraded in the
cytoplasm leading to reduced p27Kip expression levels.
However, a relationship between UCHL1 and p27Kip
expression in cancers including RCC has also not yet been
determined.

Promoter DNA methylation has been linked to the expres-
sion of tumor markers not only defined in primary
tumors, but also in body fluids [40-42,54]. Indeed, can-
cer-specific DNA methylation pattern can be detected in
circulating tumor cells of the body fluids, such as urine
and blood. If UCHL1 methylation is RCC-related, detec-
tion of UCHL1 DNA promoter methylation in addition to
the existence of UCHL1-specific autoantibodies detected
in sera of tumor patients [46,55,56] may further help to
define patients with poor prognosis. Thus one upcoming
aim that will be addressed in the near future is to deter-
mine the suitability of UCHL1 as a serum marker in order
to distinguish between patients with different clinical out-
come.

List of abbreviations
ab: antibody; COBRA: combined bisulfite restriction anal-
ysis; DAC: 2’-deoxy-5-azacytidine; DMSO: dimethylsul-
foxide; DUB: deubiquitinating enzymes; FCS: fetal calf
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