DNA methylation of the SLC16A3 promoter regulates expression of the human lactate transporter MCT4 in renal cancer with consequences for clinical outcome

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**Statement of Translational Relevance:**

Despite implementation of targeted therapies, the overall survival for metastatic clear cell renal cell carcinoma (ccRCC) is poor after 5 year follow-up. Thus, novel tumor-associated markers, which may predict patient outcome and may serve as promising therapeutic targets, are of interest. Monocarboxylate transporter 4 (MCT4) mediates lactate efflux, which is essential for energy metabolism especially in tumor cells and thereby contributes to anti-apoptotic effects in tumor biology. In this study we report that MCT4 protein is overexpressed in >85% of ccRCC and regulated by DNA methylation of the *SLC16A3* promoter resulting in adverse cancer specific outcome. Apart from the prognostic potential for patient outcome, the transcriptional regulation of MCT4 by DNA methylation offers novel and attractive opportunities for therapeutic intervention by specific silencing of MCT4 and subsequent targeting of lactate efflux in ccRCC.
Abstract:

Purpose: The monocarboxylate transporter 4 (MCT4) is a metabolic target in tumor biology since it mediates lactate transport across membranes resulting in anti-apoptotic effects. Cell experiments support the importance of MCT4 in clear cell renal cell carcinoma (ccRCC). In this study, we assessed the prognostic potential of MCT4 expression in ccRCC and its epigenetic regulation by DNA methylation as novel predictive marker for patient outcome using independent ccRCC-cohorts.

Experimental Design: MCT4 protein expression was quantified in 207 ccRCC and corresponding non-tumor tissues. Data of an independent ccRCC-cohort from The Cancer Genome Atlas (TCGA) were analysed on MCT4 mRNA (n=482) and DNA methylation (n=283) level. The findings on MCT4 expression and DNA methylation in the SLC16A3 promoter were validated in a third cohort (n=64). Promoter activity assays were performed in four RCC cell lines.

Results: MCT4 protein expression was upregulated (P<0.0001) in ccRCC and showed significant association with cancer-related death. Upregulation of MCT4 mRNA expression (P<0.00001) was confirmed in the TCGA-cohort. Single CpG-sites correlated inversely with mRNA expression and were associated with overall survival in Kaplan-Meier analyses (HR=0.39; 95%CI=0.24-0.64; P[log-rank]=1.23e-04). Promoter activity studies confirmed MCT4 regulation by DNA methylation. The significant correlation between MCT4 protein and gene expression or DNA methylation at single CpG-sites was validated in a third cohort. Again, higher methylation at individual CpG-sites was associated with prolonged survival (HR=0.05; 95%CI=0.01-0.40; P[log-rank]=6.91e-05).

Conclusion: We identified SLC16A3 promoter DNA methylation as a novel epigenetic mechanism for MCT4 regulation in ccRCC with first evidence of a biological rationale for prognosis and clinical outcome.
Introduction:

Clear cell renal cell carcinoma (ccRCC) accounts for approximately 80% of renal carcinomas (1-3). The only curative treatment for localized ccRCC is partial or radical nephrectomy. However, patients remain at a considerable risk of recurrence and development of metastases after surgery (4), subsequently resulting in an overall survival for metastatic ccRCC of about 20% after 5 year follow-up. This poor survival rate is determined by resistance to radiation and chemotherapy and limited applicability of immunotherapy due to low response rates and severe toxicity (3,5). Despite remarkable progress in the field of targeted therapies, identification of tumor-associated markers, which are able to predict patient outcome and at best may serve as target for ccRCC therapy, is of major interest.

Since several findings suggest that ccRCC is mainly a metabolic disease (6), tumor-specific changes in metabolic enzymes or transport proteins may provide novel therapeutic opportunities. Recent studies identified the monocarboxylate transporter 4 (MCT4) as metabolic target in a variety of tumors (e.g. colon cancer, breast cancer (7,8)). MCT4 (encoded by the SLC16A3 gene) mediates the H⁺-coupled transport of monocarboxylates, preferentially lactate, across the plasma membrane. Especially in highly glycolytic cells, like tumor cells, lactate efflux is essential for energy metabolism in order to ensure the progression of glycolysis and for pH regulation to prevent intracellular acidification (9,10). Genome-wide siRNA screening studies in a ccRCC cell line (11) demonstrated that MCT4 may represent a promising metabolic key protein in ccRCC. Moreover, MCT4 mediated lactate efflux, explored in RCC cells by hyperpolarized 13C-pyruvate magnetic resonance, was significantly higher in metastatic RCC cells (12). However, detailed analyses not only of its expression, but more importantly of the regulation of MCT4 in ccRCC are limited.
Interestingly, in addition to the well-established relationship of HIF deregulation through VHL loss, genetic studies of ccRCC tumors identified mutations in genes encoding epigenetic regulators, like PBRM1 or JARID1C (13). Accordingly, epigenetic modifications, like DNA methylation seem to be important for gene regulation in ccRCC (14,15). While upregulation of MCT4 by hypoxia through a HIF1alpha mediated mechanism has been demonstrated in HeLa cell line (16), the epigenetic regulation of MCT4 in ccRCC has not yet been reported and may offer clue to novel therapeutic strategy.

We therefore performed comprehensive analyses of MCT4 expression on protein and mRNA level in independent cohorts of ccRCC patients and demonstrated significant associations with clinicopathological features and patient outcome. We elucidated DNA methylation in the SLC16A3 promoter region as underlying mechanism for expression differences of MCT4 in ccRCC, which was confirmed by in vitro cell culture experiments. Based on an independent ccRCC validation cohort we provided first evidence for single CpG sites in the SLC16A3 promoter as novel epigenetic markers for clinical outcome in ccRCC.
Material and Methods:

Supplementary Materials and Methods

A detailed description of the materials and methods used in the present study is given in the Supplementary Data.

Patient cohorts

Two independent retrospective cohorts (TuCP1 and TuCP2) of ccRCC patients treated at the Department of Urology, University Hospital Tuebingen, Germany, were investigated. The TuCP1-cohort consists of 207 ccRCC patients of Caucasian origin, who underwent partial or radical nephrectomy between 1993 and 2007 with respective paraffin-embedded ccRCC and corresponding non-tumor tissues. For the TuCP2-cohort of 64 consecutive ccRCC patients, who underwent partial or radical nephrectomy between 2007 and 2010, in addition to paraffin-embedded tissues, surgically specimens of resected fresh-frozen ccRCC and matching non-tumor tissues were available. Patients’ characteristics, clinicopathological features as well as survival data are given in Table 1. For further details regarding sample collection and definition of clinical end-points, see Supplementary Data. Informed written consent was provided by each subject prior to surgical resection and the use of the tissue was approved by the ethics committee of the University of Tuebingen. Additionally, an independent cohort of ccRCC patients from The Cancer Genome Atlas (TCGA) with publically available, open-access clinical data as well as mRNA expression and genome-wide DNA methylation data was investigated. The cohort consists of 502 patients, for whom DNA methylation data (n=283; Illumina Infinium Human Methylation 450 Bead Chip) and RNA-seq data (n=482) were available for ccRCC or non-tumor tissue samples at the time of our analyses. Details of the
TCGA-cohort are summarized in Table 1. Processed data sets were obtained from the TCGA Data Portal (http://tcga-data.nci.nih.gov/tcga/) using the Data Browser tool.

**MCT4 immunohistochemical staining**

Tissue microarray slides containing ccRCC and corresponding non-tumor tissue sections were processed as described (17) and immunostained using an antibody against MCT4 (Santa Cruz Biotechnology, Santa Cruz, USA). The slides were scanned using a digital Mirax scanner (Carl Zeiss, MicroImaging, Jena, Germany). For the evaluation of immunohistochemical staining, MCT4 immunoreactivity of all tumor and benign renal areas were classified according to a composite IHC score. This semiquantitative grading of membranous MCT4 staining was based on a comparison of all scanned TMA dots using a picture based software platform (Mirax Viewer, Carl Zeiss, Light Microscopy, Goettingen, Germany). Staining intensity was graded into 0, 1, 2 or 3 corresponding to the presence of negative, weak, intermediate, and strong MCT4 staining. In addition, the percentage of MCT4 stained cells was determined (0%-100%). The composite IHC score was obtained by multiplying the staining intensity and the percentage of stained cells (0%-100%), resulting in an immunoreactivity score value of 0 to 300. The score values 0-300 were finally converted to percentage 0-100% (see Supplementary Data for detailed description).

For validation of the MCT4 antibody, MCT4 siRNA knockdown was conducted in the renal carcinoma cell lines 786-O and A-498. The cell lines used were purchased from CLS (Eppelheim, Germany) and passaged for less than six months. Further authentication was performed using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Applied Biosystems, Foster City, USA). Details are provided in Supplementary Data.
RNA isolation and quantification

For details see Supplementary Data.

DNA methylation analyses

For quantitative DNA methylation analyses, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was applied (18). Mass spectra were obtained using the MassARRAY compact system and evaluated using the EpiTYPER 1.0 software. Details see Supplementary Data.

SLC16A3 promoter reporter analysis

The influence of DNA methylation on SLC16A3 promoter activity was analysed in an in vitro cell culture system using a dual-reporter assay. In brief, SLC16A3 promoter reporter constructs, cloned into the pCpGfree basic reporter vector (Invivogen, San Diego, USA), was either methylated by incubation with CpG methylase M. SsSI (Zymo Research, Irvine, USA) in the presence of SAM or mock-methylated through incubation under equal conditions in absence of CpG methylase M. SsSI. Methylated and mock-methylated SLC16A3 promoter reporter constructs were co-transfected with the control vector pGL4.13, constitutively expressing firefly luciferase for normalization, into four different RCC cell lines (Caki1, Caki2, A498 and 786-O) using FuGene HD transfection reagent (Promega, Madison, USA). SEAP and luciferase activity were measured after 24 h with the Phospha-Light™ System (Applied Biosystems, Bedford, USA) and the Dual-Luciferase® Reporter Assay System (Promega, Madison, USA) respectively, according to manufacturer’s instructions. Details see Supplementary Data.
**Statistical analyses**

Statistical analyses were performed with R-2.15.0 (19) (http://www.r-project.org) as well as GraphPad Prism 5.0 (GraphPad Software, Inc., USA). Statistical significance was defined as $P<0.05$. Where indicated $P$-values were adjusted for multiple testing by Holm’s procedure (20). Detailed description is given in Supplementary Data.
Results:

Study population

Two independent cohorts of ccRCC patients (TuCP1 and TuCP2) from the University Hospital Tuebingen were investigated. Characteristics of these two cohorts TuCP1 (n=207) and TuCP2 (n=64) are summarized in Table 1. Median follow-up times of patients in TuCP1 and TuCP2 were 5.9 and 2.3 years, respectively. Additionally, we investigated a third, independent cohort of ccRCC patients from TCGA (n=502, Table 1). Overall, the cohorts were similar, but some differences did exist, such as grading (G) (TuCP1/TuCP2 vs. TCGA: \( P < 0.001 \), respectively), due to different grading systems in the study cohorts, involvement of regional lymph nodes (N) (TuCP1 vs. TuCP2: \( P < 0.001 \); TuCP2 vs. TCGA: \( P = 0.005 \)) or follow-up times (TuCP1 vs. TuCP2/TCGA: \( P < 0.001 \), respectively; TuCP2 vs. TCGA: \( P = 0.02 \)). Staging of tumors (T) did not differ significantly between the cohorts (Supplementary Table S1).

MCT4 protein expression in ccRCC correlates with clinicopathological parameters and patient survival

MCT4 protein expression in the TuCP1-cohort was investigated by immunohistochemical staining of tissue microarrays. Representative staining is shown in Figure 1A. A scoring system (details see Supplementary Data) was assigned to semiquantitatively determine MCT4 protein expression. Statistical analyses revealed a significantly increased MCT4 protein expression in ccRCC compared to corresponding normal tissue (\( P < 0.0001 \), Fig.1B (left)), confirmed by direct comparison of matched samples (Fig.1B (right)). MCT4 protein expression levels in ccRCC correlated strongly with clinicopathological features, e.g. T, N, M, G or necrosis (Supplementary Table S2). Additionally, patients with higher MCT4 expression in ccRCC had a significantly higher risk for cancer-related death. Kaplan-
Meier analysis based on optimal cutoffs determined by conditional interference tree models revealed that patients with low MCT4 expression in ccRCC had an increased cancer-specific survival time compared to patients with high expression levels (Fig.1C; HR=4.23 (95%CI: 2.29-7.80); \( P[\text{log-rank}]=5.36\times10^{-07} \)). Moreover, non-metastatic patients with higher MCT4 expression in ccRCC tissue (HR=4.94 (95%CI: 2.19-11.13); \( P[\text{log-rank}]=2.05\times10^{-05} \)) had a significantly higher risk for cancer-related death.

**MCT4 mRNA expression is increased in ccRCC, whereas \textit{SLC16A3} promoter DNA methylation at single CpG sites is reduced in ccRCC and correlates with patient survival**

For validation of the expression differences between ccRCC and non-tumor kidney tissue on mRNA level, mRNA expression of MCT4 was evaluated using RNA-seq data from the independent ccRCC TCGA-cohort. As illustrated in Figure 2A MCT4 mRNA expression was significantly increased in ccRCC tissue compared to adjacent non-tumor tissue (\( P<0.00001 \)). Based on the hypothesis, that altered mRNA expression may be caused by aberrant DNA methylation, we investigated DNA methylation in the \textit{SLC16A3} promoter region in the TCGA tissue samples, for which genome-wide DNA methylation data were available. DNA methylation of \textit{SLC16A3} in the promoter region of the TCGA-cohort was significantly decreased in the tumor tissue compared to histologically normal kidney tissue (Fig.2B). DNA methylation of single CpG sites in the promoter correlated significantly inverse with mRNA expression in ccRCC, especially the CpG site cg18345635 (\( r_s=-0.31, \ P<0.00001 \)).

By correlation analysis of DNA methylation of the tumor tissue with clinicopathological parameters, significant associations were again found particularly for the CpG site cg18345635 (Table 2). Moreover, DNA methylation of single CpG
sites correlated with patients’ survival in the TCGA-cohort. Kaplan-Meier analysis based on optimal cutoffs determined by conditional interference tree models showed that patients with high methylation at the CpG site cg18345635 in the SLC16A3 promoter had a significantly prolonged overall survival compared to patients with low methylation (Fig.2C; HR=0.39 (95%CI: 0.24-0.64); $P$[log-rank]=1.23e^{-04}). Again, restriction of analyses to patients without metastatic ccRCC did not substantially change the observed results (data not shown). For the TCGA-cohort only data for overall survival and not for cancer-specific survival were available.

**SLC16A3 promoter DNA methylation at single CpG sites correlates with MCT4 protein expression, clinicopathological features and survival**

Finally, we validated our results in a third independent cohort of 64 matching ccRCC and non-tumor tissues (TuCP2) on mRNA, and protein expression level, as well as on DNA methylation level. Again, MCT4 protein as well as mRNA expression were significantly higher in ccRCC compared with non-tumor kidney (Fig.3A and 3B). MCT4 mRNA expression was normalized to Dipeptidyl-peptidase 4 (DPP4). The highly variable MCT4 mRNA and protein expression correlated significantly in ccRCC tissue ($r_s=0.34$, $P=0.0099$) (Supplementary Fig.S2C). Moreover, the same trends for the associations between MCT4 protein expression and clinicopathological parameters as in the TuCP1-cohort were observed, but correlations did not reach comparable high statistical significance due to the smaller sample size (Supplementary Table S4). With respect to clinical outcome, high MCT4 protein expression in tumor tissue was moderately associated with inferior cancer-specific survival ($P$[log-rank]=0.175, cutoff determined by conditional interference tree, Supplementary Fig.S3).
DNA methylation at the SLC16A3 promoter was assessed in the TuCP2-cohort using MALDI-TOF MS (Fig.3C). The DNA methylation pattern quantified at each analyzed CpG site in normal (red) and ccRCC (blue) tissue showed significantly decreased DNA methylation levels in specific areas of the SLC16A3 promoter in ccRCC compared with non-tumor tissue (Fig.3C). Of note, investigation of DNA methylation by MALDI-TOF MS allowed a more comprehensive coverage and quantification of CpG sites in the SLC16A3 promoter region compared to the genome-wide array analysis performed in the TCGA cohort (see Supplementary Table S3). Again, DNA methylation at individual CpG sites in the validation cohort (TuCP2), quantified by MALDI-TOF MS correlated significantly inverse with MCT4 mRNA and protein expression (Supplementary Fig.S2C and S2D). In particular, the percentage of DNA methylation at four specific CpG sites showed significant inverse correlation with MCT4 protein expression in ccRCC even after adjustment for multiple testing (Supplementary Fig.S2D).

By correlation analysis of the percentage of DNA methylation of the tumor tissue with clinicopathological parameters, the CpG unit 05_CpG_8.9 revealed significant associations (Table 2). This CpG unit is located in close proximity to cg18345635 (Supplementary Table S3), which was associated with overall survival and clinicopathological features in the TCGA-cohort (Table 2). Higher methylation values at 05_CpG_8.9 in tumor tissue were significantly associated with prolonged survival (Fig.3D; HR=0.05 (95%CI: 0.01-0.40); $P_{\text{log-rank}}=6.91\times10^{-5}$). Restriction of analyses to patients without metastatic ccRCC did not substantially change the observed results (data not shown).
Dual-reporter gene assays support regulation of MCT4 by DNA methylation

To determine the influence of DNA methylation on SLC16A3 promoter activity, we used different promoter/reporter fusion plasmids containing either methylated or mock-methylated SLC16A3 promoter fragments for promoter reporter assays (Fig.4). These constructs were then transfected together with the control vector, constitutively expressing firefly luciferase for normalization, into four different RCC cell lines (Caki1, Caki2, A498, 786-O) and subsequently promoter activities were measured. The influence of DNA methylation on SLC16A3 promoter activity by a dual-reporter gene assay confirmed that reporter constructs with a mock-methylated SLC16A3 promoter fragment showed significantly higher promoter activity than constructs with a methylated SLC16A3 promoter, even in the smaller construct and independent of the VHL status of the cells (Fig.4).

To further investigate, if aberrant DNA methylation of SLC16A3 is independent of the VHL genotype, we analysed data of the TCGA-cohort (available for 199 of the 283 samples; 70%). DNA methylation was not significantly dependent on the VHL status (carriers vs. non-carriers of mutations) of the patients (Supplementary Figure S4).

Evaluation of SLC16A3 promoter methylation as predictive marker for ccRCC outcome

Univariate Cox regression analyses were performed and the predictive ability of different models was estimated by calculating Harrell’s c-index (Supplementary Table S5). The pathologic features T, M, and G were able to significantly predict cancer-specific survival in both the TuCP1- and TuCP2-cohort, whereas N and necrosis were only predictive in one of the cohorts (Supplementary Table S5). In line, overall survival could be predicted in the TCGA-cohort by T, M, G, and necrosis. DNA
methylation at specific CpG sites in ccRCC significantly predicted overall survival in the TCGA-cohort (cg18345635: \( P[\text{log-rank}]=0.00065, \text{c-index: 61.3} \)) as well as cancer-related death (which is identical to overall survival) in the TuCP2-cohort (05_CpG_8.9: \( P[\text{log-rank}]=0.0125, \text{c-index: 75.1} \)). Moreover, in the subgroup of non-metastatic patients of the TuCP2-cohort DNA methylation in ccRCC significantly predicted cancer-related death (05_CpG_8.9: \( P[\text{log-rank}]=0.036, \text{c-index: 86.4} \)).

DNA methylation in the tumor was only moderately, but not significantly predictive for cancer-specific survival and overall survival in multivariate analyses in the TuCP2 and TCGA patients (\( P[\text{Wald}]=0.31 \) or \( P[\text{Wald}]=0.31 \)), and in non-metastatic patients only (\( P[\text{Wald}]=0.14 \) or \( P[\text{Wald}]=0.46 \)).

ROC curve analysis further supported our results that DNA methylation in the tumor at a single CpG unit is feasible as novel biomarker for identifying patients at risk for cancer-related death and death (Supplementary Table S6) with AUCs of 76.3% in TuCP2 and 63.9% in TCGA, respectively. In comparison, AUCs for T, N, M, G or necrosis ranged from 56.7% to 83.7% (TuCP2) or 51.6% to 74.7% (TCGA). Of note, the AUCs for the clinicopathological parameters T, N, G, and necrosis were lower than the AUC for DNA methylation at 05_CpG 8.9 in TuCP2. The sensitivity, specificity, and negative and positive predictive values of DNA methylation at 05_CpG_8.9 in TuCP2 for prediction of death were 91.7% (95% CI: 33.1-100%), 68.8% (95% CI: 22.2%-83.3%), 97.1%, and 42.3%, respectively, versus 52.1% (95% CI: 39.4%-62.6%), 69.7% (95% CI: 54.6%-80.6%), 74.4%, and 46.2% for methylation at cg18345635 in the TCGA data set.
Discussion:

Since ccRCC is estimated to be mainly a metabolic disease and a clear example of the Warburg effect in tumor metabolism, we aimed to comprehensively study MCT4 expression as well as regulation by epigenetic mechanisms in ccRCC in order to evaluate its relevance as metabolic target for diagnosis, prognosis and potential therapy. In the present study, we found that MCT4 protein is significantly overexpressed in >85% of all ccRCCs in two independent cohorts. DNA methylation at specific CpGs in the SLC16A3 promoter correlated significantly inverse with MCT4 expression in ccRCC, which was also replicated. In addition, the regulation of MCT4 by DNA methylation was confirmed by reporter gene assays in four different renal carcinoma cell lines, strongly supporting the importance of DNA methylation for MCT4 expression.

Previous results from adenocarcinomas of the lung showed that high MCT4 expression is associated with aggressive tumor behavior (21). Additionally, in mouse xenograft models of human colorectal and breast cancer, release of lactate from tumor cells through MCT4 (and not the isoform MCT1) is sufficient to increase tumor growth (22). Moreover, lactate efflux mediated by MCT4, which can be explored in RCC cells by hyperpolarized 13C-pyruvate magnetic resonance, was significantly higher in a metastatic RCC cell line with increased MCT4 expression (12). In line with these data MCT4 expression in ccRCC of our cohorts as well as DNA methylation at specific CpG units was strongly associated with various clinicopathological features, including sarcomatoid differentiation. Thus, both factors may be valuable parameters for diagnosis of high-grade tumors, and highly aggressive variants e.g. ccRCC with sarcomatoid differentiation and metastatic tumors.
Moreover, our data showed that MCT4 expression and DNA methylation were significantly associated with patient outcome and were able to predict cancer-related death in ccRCC patients. Compared to MCT4 expression, DNA methylation was more strongly associated with cancer-specific survival in TuCP2 indicating that quantification by immunohistochemistry may have some limitations in accuracy (e.g. protein degradation due to fixation or small tissue cores in TMA). The fact that the association of cancer-related death with MCT4 expression was superior in TuCP1 compared to TuCP2 is most likely explained by the larger sample size and longer follow-up times in the TuCP1-cohort. Interestingly, the association of low SLC16A3 DNA methylation at specific CpG sites with worse patient survival could also be detected in the large independent ccRCC-cohort from TCGA with comparable low \( P \)-values, supporting the clinical relevance of SLC16A3 DNA methylation for ccRCC. A similar approach, using the TCGA datasets as an independent confirmatory cohort has recently been successfully performed for the association of BAP1 and PBRM1 mutations with survival in ccRCC (23,24).

MCT4 protein expression and DNA methylation were associated with all important and validated prognostic factors (tumor stage, grade, tumor necrosis or metastasis) known for ccRCC outcome, which further supports the prognostic relevance of MCT4 for cancer-specific survival. Due to the strong relationship of DNA methylation at specific CpG sites with clinicopathological parameters in ccRCC, not surprisingly SLC16A3 DNA methylation at these sites did not independently predict either risk of cancer-related death or overall survival in the TuCP2- and TCGA-cohort after correction for prognostic factors (T, N, M, G, and necrosis) in multivariate Cox regression analysis. However, it showed a similar (TCGA) or even larger (TuCP2) Harrell’s c-index compared to the established pathological risk factors.
Moreover, several reasons exist that the use of quantitative DNA methylation of SLC16A3 in ccRCC as potential novel prognostic predictor is more applicable compared to clinicopathological features. Quantification of DNA methylation by MALDI-TOF MS, but also other technologies (e.g. pyrosequencing), is a highly accurate and reliable method (18), which has already been demonstrated for other biomarkers (25). Potential inaccuracies in the determination of clinicopathological features are inevitable depending on the investigator and/or based on the absence of uniform grading systems for ccRCC resulting in inter- and intraindividual variability even for the most widely applied Fuhrmann grading system (26).

Taken together, the association of DNA methylation and patient survival was observed not only in our cohort but also in the TCGA cohort, despite patients from different centers were included and some differences in the patient cohorts did exist. Hence the association is apparently not dependent on the site of biospecimen`s recruitment, ethnicity of patients or the method used for quantification of DNA methylation, which further supports that MCT4 DNA methylation may serve as a novel biomarker in clinical practice. However, to definitely prove the importance of DNA methylation as a novel biomarker for prognosis, large-scale prospective studies are needed (27).

Apart from its prognostic potential, therapeutic targeting of MCT4 in ccRCC may be attractive because of the high MCT4 expression only in tumor and not in the corresponding non-tumor tissue. Gerlinger et al. demonstrated that silencing of MCT4 in ccRCC cells has an influence on cell proliferation and induction of apoptosis (11). Interestingly, the effect was independent from the VHL genotype, indicating that...
MCT4 targeting would be a therapeutic option irrespective of patients’ VHL status. Theoretically, MCT4 silencing could be achieved by MCT4 inhibitors. However, so far, only isoform specific MCT1 inhibitors (e.g. AR-C155858 (28)) have been evaluated in Phase I/II trials. Interestingly, treatment of patients with statins, which are known to inhibit MCT4 (29), reduced the risk of RCC (30). Thus, the development of isoform specific MCT4 inhibitors appears to be a promising therapeutic approach beneficial for several cancer types (e.g. breast cancer) (8).

An alternative approach is targeting of MCT4 expression and thus lactate efflux by modulating DNA methylation. In contrast to the approved therapy of the myelodysplastic syndrome using DNA demethylating agents like 5-Azacytidine, specific MCT4 silencing by DNA methylation may be an attractive strategy for ccRCC treatment. It has recently been shown that zinc-finger proteins can target the DNA methyltransferase DNMT3a to specific promoter sites, resulting in stable reprogramming of breast cancer cells (31).

Our hypothesis generating, retrospective study has some limitations. First, we do not have complete data on treatment regimens for the study cohorts. However, when we compared patients with chemotherapy and targeted therapy in a small subgroup of the TCGA-cohort where treatment data were available, DNA methylation of SLC16A3 in ccRCC tissue was not significantly different. Moreover, a substantial proportion of patients in the TuCP1-cohort (86.5%) and TCGA-cohort (43.8%) was diagnosed before 2006, the time when targeted therapies were introduced into clinical practice (3), indicating that confounding of our results by novel therapeutic strategies is most unlikely. Second, genetic variants in SLC16A3 that might also alter variability of MCT4 expression, have not been investigated.
In summary, we identified DNA methylation as a so far unknown underlying epigenetic mechanism for MCT4 regulation in ccRCC. Quantification of DNA methylation of specific CpG sites in the $SLC16A3$ promoter offers considerable clinical potential for diagnosis and prognosis of ccRCC. Large-scale studies are warranted to elucidate its clinical utility.
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Disclosure of Potential Conflicts of Interest:

No potential conflicts of interest were disclosed.

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Development of methodology: Pascale Fisel, Stephan Kruck, Marcus Scharpf, Stefan Winter

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Pascale Fisel, Stephan Kruck, Marcus Scharpf, Jens Bedke, Jörg Hennenlotter, Arnulf Stenzl, Stefan Winter

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Pascale Fisel, Stephan Kruck, Stefan Winter, Jens Bedke, Jörg Hennenlotter, Anne T. Nies, Falko Fend, Arnulf Stenzl, Matthias Schwab, Elke Schaeffeler

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Reference List:


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Table 1: Descriptive data of the three independent patient cohorts TuCP1, TuCP2 and TCGA

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<td>42.2</td>
<td>172</td>
<td>34.3</td>
</tr>
<tr>
<td>age</td>
<td>64 (17-90)</td>
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<td>64 (36-88)</td>
<td></td>
<td>61 (26-90)</td>
<td></td>
</tr>
<tr>
<td>tumor size [cm]</td>
<td>4.5 (0.3-12.5)</td>
<td>5.5 (1.4-16)</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumor necrosis</td>
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<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>sarcomatoid differentiation</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>follow-up time [years]</td>
<td>median (range)</td>
<td>5.9 (0-18)</td>
<td>2.3 (0-4.7)</td>
<td>3 (0-10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>recurrence</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>overall survival</td>
<td>deceased</td>
<td>alive</td>
<td>deceased</td>
<td>alive</td>
<td>deceased</td>
<td>alive</td>
</tr>
<tr>
<td>cancer-specific survival</td>
<td>42</td>
<td>12</td>
<td>18.8</td>
<td>161</td>
<td>77.8</td>
<td>52</td>
</tr>
</tbody>
</table>

1Parameters were not available for the TCGA-cohort. 2Age at surgery for TuCP1 and TuCP2, age at initial pathologic diagnosis for TCGA. 3Of note, all patients in the TuCP2-cohort died due to ccRCC; therefore, cancer-specific survival and overall survival is identical in this cohort. 4Percentages do not sum up to 100% due to rounding. 5Percentages do not sum up to 100% because of missing values.

Abbreviations: T, primary tumor; N, regional lymph nodes; M, distant metastasis; G, grading; L, invasion into lymph vessels; V, invasion into veins; R, resection status.
Table 2: Correlation of DNA methylation status with clinicopathological parameters in ccRCC tissue at cg18345635 (in TCGA-cohort) and at 05_CpG_8.9 (in TuCP2)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>cg18345635 (TCGA)</th>
<th>05_CpG_8.9 (TuCP2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median signal</td>
<td>median % DNA</td>
</tr>
<tr>
<td></td>
<td>methylation array</td>
<td>methylation</td>
</tr>
<tr>
<td></td>
<td>(range)</td>
<td>(range)</td>
</tr>
<tr>
<td>age</td>
<td>0.0311 (r_s=-0.13)</td>
<td>0.1760 (r_s=0.18)</td>
</tr>
<tr>
<td>sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>0 (-0.35, 0.26)</td>
<td>56 (20, 90)</td>
</tr>
<tr>
<td>female</td>
<td>0.06 (-0.25, 0.3)</td>
<td>64 (24, 93)</td>
</tr>
<tr>
<td>tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>0.05 (-0.3, 0.3)</td>
<td>58 (25, 93)</td>
</tr>
<tr>
<td>3</td>
<td>-0.04 (-0.35, 0.25)</td>
<td>63 (20, 91)</td>
</tr>
<tr>
<td>N</td>
<td>0.0018</td>
<td>0.0098</td>
</tr>
<tr>
<td>x/0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>-0.16 (-0.23, 0.03)</td>
<td>47 (24, 83)</td>
</tr>
<tr>
<td>1</td>
<td>0.03 (-0.35, 0.3)</td>
<td>30 (90, 180)</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.03 (-0.35, 0.3)</td>
<td>63 (20, 93)</td>
</tr>
<tr>
<td>1</td>
<td>-0.03 (-0.28, 0.24)</td>
<td>49 (24, 83)</td>
</tr>
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<td>G</td>
<td>&lt;0.0001</td>
<td>0.0009</td>
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<td>0.1 (0.04, 0.16)</td>
<td>68.5 (45, 93)</td>
</tr>
<tr>
<td>2</td>
<td>0.06 (-0.24, 0.3)</td>
<td>60 (25, 91)</td>
</tr>
<tr>
<td>3/4</td>
<td>-0.03 (-0.35, 0.25)</td>
<td>41 (20, 65)</td>
</tr>
<tr>
<td>L</td>
<td>-</td>
<td>0.0495</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>59 (20, 93)</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>47 (37, 49)</td>
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<tr>
<td>V</td>
<td>-</td>
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<td>0</td>
<td>-</td>
<td>58.5 (25, 93)</td>
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<td>1/2</td>
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<td>R</td>
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<td>-</td>
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<td>1</td>
<td>-</td>
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<td>yes</td>
<td>-0.01 (-0.35, 0.25)</td>
<td>47.5 (24, 56)</td>
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<td>63 (20, 93)</td>
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<td>59.5 (24, 93)</td>
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<tr>
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<td>-</td>
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<td>recurrence</td>
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<tr>
<td>yes</td>
<td>-</td>
<td>51 (20, 72)</td>
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<tr>
<td>no</td>
<td>-</td>
<td>59 (24, 93)</td>
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<tr>
<td>overall survival^2</td>
<td>0.0001</td>
<td>0.0051</td>
</tr>
<tr>
<td>deceased</td>
<td>-0.04 (-0.35, 0.25)</td>
<td>49.5 (20, 72)</td>
</tr>
<tr>
<td>alive</td>
<td>0.04 (-0.3, 0.3)</td>
<td>63 (24, 93)</td>
</tr>
</tbody>
</table>

^1Unadjusted P-values. ^2Overall survival and cancer-specific survival is identical in the TuCP2-cohort.

Abbreviations: T, primary tumor; N, regional lymph nodes; M, distant metastasis; G, grading; L, invasion into lymph vessels; V, invasion into veins; R, resection status
Figure Legends:

Figure 1: MCT4 protein expression in ccRCC and non-tumor kidney tissue of the TuCP1-cohort. (A) Immunohistochemistry of MCT4 exemplarily shown in non-tumor kidney with weak staining and in ccRCC tissue with strong membranous staining. (B) Evaluation and statistical analysis of MCT4 protein expression in ccRCC compared to non-tumor tissue. MCT4 protein expression was significantly increased in ccRCC tissue compared to non-tumor tissue ($P<0.0001$). MCT4 protein expression was highly variable in ccRCCs (mean +/-SEM: 40.39 +/-2.1) in contrast to non-tumor tissues (mean +/-SEM: 8.01 +/-0.51). (C) Kaplan-Meier curves of cancer-specific survival based on percentage of MCT4 protein expression in ccRCC tissue (percentage of MCT4 protein expression $\leq$63% (grey) and $>63%$ (black); cutoff values were determined by conditional inference tree models), hazard Ratio (HR)=4.23 (95% confidence interval (CI): 2.29-7.80).

Figure 2: MCT4 mRNA expression and SLC16A3 promoter DNA methylation in ccRCC and non-tumor kidney tissue of the TCGA-cohort. (A) MCT4 mRNA expression was significantly increased in ccRCC tissue compared to non-tumor kidney tissue ($P<0.00001$). (B) (left) DNA methylation levels of seven CpG sites within the SLC16A3 promoter region in ccRCC and non-tumor tissue are shown in the heatmap. (right) Scheme of the SLC16A3 gene locus and the examined promoter region. SLC16A3 promoter DNA methylation profile in normal kidney (red) and ccRCC (blue) tissue are shown. Diamonds represent median methylation levels at single CpG sites; shaded areas are defined by 25%/75% quantiles. The DNA methylation profile in ccRCC showed strong variability and significantly decreased DNA methylation levels in specific areas of the SLC16A3 promoter compared with non-tumor tissue. (C) SLC16A3 DNA methylation predicts patient survival. The
Kaplan-Meier plots with cutoffs determined by conditional inference tree models show survival probability of the TCGA-cohort based on the DNA methylation status at CpG site cg18345635 in ccRCC tissue of the TCGA-cohort, discriminating between high (black) and low DNA methylation (grey), HR=0.39 (95% CI: 0.24-0.64).

**Figure 3: MCT4 mRNA, protein, and SLC16A3 promoter DNA methylation in ccRCC and non-tumor kidney tissue of the TuCP2-cohort.** (A) MCT4 mRNA expression was significantly increased in ccRCC tissue compared to non-tumor tissue. Relative MCT4 mRNA expression was determined by quantitative real-time PCR. (B) Evaluation and statistical analysis of MCT4 protein expression in ccRCC compared to non-tumor tissue samples. MCT4 mRNA expression was significantly increased in ccRCC tissue compared to non-tumor tissue. Protein expression was investigated in TMAs by semiquantitative immunohistochemistry. Immunohistochemical staining of MCT4 is shown exemplarily in one non-tumor and one ccRCC tissue sample. (C) Scheme of the SLC16A3 gene locus and the examined promoter region and SLC16A3 promoter DNA methylation profile in non-tumor renal tissue (red) and ccRCC tissue (blue). Diamonds represent median methylation levels at single CpG sites; shaded areas are defined by 25%/75% quantiles. The DNA methylation profile in ccRCC showed strong variability and significantly decreased DNA methylation levels in specific areas of the SLC16A3 promoter compared with non-tumor tissue. Significantly different methylated CpG sites are marked with stars (Holm-adjusted \( P \)-values; \*\( P<0.05 \), \**\( P<0.01 \), \***\( P<0.001 \), \****\( P<0.0001 \)). (D) SLC16A3 DNA methylation predicts patient survival. The Kaplan-Meier plots with cutoffs determined by conditional inference tree models show survival probability of the TuCP2-cohort based on the DNA methylation status at CpG.
site 05_CpG_8.9 in ccRCC tissue, discriminating between high (>56%, black) and low DNA methylation (≤56%, grey), HR=0.05 (95% CI: 0.01-0.40).

**Figure 4:** (A) Scheme of the SLC16A3 gene locus and the examined promoter region. Two promoter fragments, from -1049 to +287 (promoter fragment 1) and from -621 to +287 (promoter fragment 2), respectively, were cloned into the CpG-free reporter vector pCpGfree basic. (B) Reporter activity depending on methylation status of the promoter fragments in Caki1 (VHL wildtype (11)), Caki2 (VHL deficient (11)), A-498 (VHL deficient) and 786-O (VHL deficient) cells. Reporter constructs with a mock-methylated SLC16A3 promoter fragment showed significantly higher relative promoter activity than constructs with a methylated SLC16A3 promoter. Relative SEAP activities of the methylated fragments are shown relative to the mock-methylated fragments, whose activities were set to 100%. Experiments were performed in triplicates.
Figure 2
Figure 4

A

B

♀ = methylated
♀ = unmethylated

Caki1

Caki2

A498

786-O

% relative SEAP activity

% relative SEAP activity

% relative SEAP activity

% relative SEAP activity

0 50 100 150

0 50 100 150
DNA methylation of the SLC16A3 promoter regulates expression of the human lactate transporter MCT4 in renal cancer with consequences for clinical outcome

Pascale Fisel, Stephan Kruck, Stefan Winter, et al.

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