Overexpression of Functional SLC6A3 in Clear Cell Renal Cell Carcinoma

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Abstract

Purpose: Renal cell carcinoma (RCC) is derived from a tissue with a remarkable capacity for vectorial transport. We therefore performed an unbiased exploration of transporter proteins in normal kidney and kidney cancer to discover novel clinical targets.

Experimental Design: Using The Cancer Genome Atlas (TCGA) database, we investigated differences in membrane transporter expression in clear cell RCC (ccRCC) and normal kidney. We identified the dopamine transporter SLC6A3 as a specific biomarker for ccRCC. To investigate the functionality of SLC6A3, we used a [3H]-dopamine uptake assay on ccRCC cells. We further explored the effect of hypoxia-inducible factor (HIF) proteins on SLC6A3 expression by introducing siRNA in ccRCC cells and by hypoxic treatment of nonmalignant cells.

Results: We show that ccRCC expresses very high transcript levels of SLC6A3 in contrast to normal kidney tissue and other tumor types, which do not express appreciable levels of this transporter. Importantly, we demonstrate that the elevated expression of SLC6A3 in ccRCC cells is associated with specific uptake of dopamine. By targeting the expression of HIF-1α and HIF-2α, we could show that SLC6A3 expression is primarily influenced by HIF-2α and that hypoxia can induce SLC6A3 expression in normal renal cells.

Conclusions: We conclude that the dopamine transporter SLC6A3 constitutes a novel biomarker that is highly specific for ccRCC. We further postulate that the protein can be exploited for diagnostic or therapeutic purposes for detection or treatment of ccRCC.

Introduction

Transporter proteins are an integral part of cellular homeostasis by controlling the import and export of essential substances, like amino acid, ions, vitamins, and drugs, across cellular membranes. The regulation of these proteins is thus fundamental for cell survival and organ function. There are two major transporter families in the human genome: the ATP-binding cassettes (ABCs) and the solute carriers (SLCs). The SLC family comprises more than 400 members organized into 52 subfamilies, predominantly involved in molecule uptake (1). The SLC families vary in substrate specificity; some have a very narrow range of substrates, whereas others, like the SLCO family (OATP), handle a broad spectrum of substrates (2). The ABC transporters consist of 48 members divided into seven subfamilies and use ATP to drive the efflux of molecules through cellular membranes (3). Expression of these transporters is associated with stem and progenitor cells (4) and has been linked to drug resistance in human disease, including chemotherapy resistance in cancer (5). There are connections between several transporters and human malignancies. For example, polymorphisms of the bicarbonate transporter SLC4A7 have been associated with breast cancer (6), and the urea transporter SLC14A1 has been linked to bladder cancer susceptibility (7).

The epithelial cells in the renal tubular system are endowed with a large variety of membrane transporter proteins that control the kidneys’ reabsorptive processes (8). Renal cell carcinomas (RCCs) account for around 2.5% of malignancies worldwide (9). Clear cell RCC (ccRCC) is the most common form of renal cancer constituting around 75% of the cases (10). ccRCC is characterized by loss of function of the von Hippel Lindau (VHL) gene (11). VHL normally acts as a regulator of the hypoxia-inducible factors (HIF) by targeting these proteins for destruction in the presence of oxygen (12). Under hypoxic conditions, stabilized HIFs control the transcription of genes in the cellular adaptive response to hypoxia, such as VEGF, GLUT-1, CAIX, and TGFβ. Collectively, these changes lead to induction of angiogenesis and a metabolic switch to anaerobic glycolysis (11, 12). Generalized, ccRCC does not respond to conventional therapies like chemotherapy and radiation (13). Even though improvement has been accomplished by the introduction of antiangiogenesis therapies and mTOR inhibitors, all patients with metastatic ccRCC eventually succumb to the disease (14).

Herein, we performed an explorative analysis of transporter gene expression, using the TCGA database. This analysis highlighted the dopamine transporter SLC6A3 that displays a unique transcriptional induction in ccRCC. We provide...
Translational Relevance

Despite association with several pathogenic disorders, the family of solute carriers (SLCs) is grossly understudied in cancer. In the current study, we perform an unbiased exploration of transporter proteins in normal kidney and kidney cancer. We show that clear cell renal cell carcinoma (ccRCC) displays a unique expression pattern of transporter genes in comparison to other tumor types. The analysis also showed that the dopamine transporter SLC6A3 was highly overexpressed in ccRCC, whereas minute expression was detected in normal kidney tissue and in other malignancies, including papillary and chromophobe RCC. We further link this overexpression to a tissue-specific hypoxic induction of SLC6A3 via HIF-2α. Importantly, we show the overexpression of SLC6A3 is associated with specific uptake of dopamine, which creates new opportunities for highly specific diagnostic and therapeutic modalities based on modified dopamine analogs. In addition, these findings may be exploited for differential diagnosis of RCC subtypes.

Experimental evidence that the overexpression is associated with a functional uptake of dopamine and that the gene is regulated by HIF-2α in normal renal cells. Collectively, these results provide ample evidence that SLC6A3 is a novel and highly specific biomarker for ccRCC.

Materials and Methods

Procurement and dissociation of renal tissue

Renal tissue samples were obtained from nephrectomies performed owing to malignancy. Ethical permission was granted by the ethical committee at Lund University (Lund, Sweden; LU680-08 and LU289-07) and informed consent was obtained from all patients. For normal samples, the cortical tissue farthest from the tumor was selected, and all samples were dissociated as described in Supplementary Methods. All primary tumor samples were pathologically confirmed as ccRCC by an expert kidney pathologist (M. Johansson). Pathologic assessment of the primary tumor samples can be viewed in Supplementary Table S1.

Cell culture and hypoxia

Renal cancer cell lines SKRC7, SKRC10, SKRC17, SKRC21 (kindly provided by E. Oosterwijk, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands), SNU-349, RCB1963 (Korean Cell Line Bank), breast cancer cell line MCF7 (ATCC), and normal prostate cell line PNT-2 (ATCC) were cultured in RPMI-1640 (IMR-32) medium supplemented with 10% FBS. Primary renal samples and RCC cell lines 786-O, SKRC21, SKRC7, and SKRC17 were cultured in DMEM/F12 (Gibco, Life Technologies) supplemented medium for selection. Normal breast cell line MCF10A was cultured in DMEM supplemented with 10% FBS. Primary renal samples and RCC cell lines 786-O (ATCC), and normal prostate cell line PNT-2 (ATCC) were cultured in RPMI-1640 (IMR-32) medium supplemented with 10% FBS. Primary renal samples and RCC cell lines 786-O, SKRC21, SKRC7, and SKRC17 were cultured in DMEM/F12 (Gibco, Life Technologies) supplemented medium for selection. Normal breast cell line MCF10A was cultured in DMEM/F12 (Gibco, Life Technologies) supplemented with 5% horse serum, 100 ng/mL cholera toxin, 20 ng/mL EGF, 0.01 mg/mL insulin, and 500 ng/mL hydrocortisone, all from Sigma Aldrich. Normal endothelial cell line human umbilical vein endothelial cell (HUVEC) was cultured on gelatin-coated plates in endothelial cell growth medium 2 (Promo Cell). All medium was supplemented with antibiotics (PEST 1%). Cell line authentication of SNU-349, SKRC10, KMRC3, 786-O, and PNT2 was performed by DDC Medical. The remaining cell lines were thawed from the authenticated cell stock and used within eight passages and authenticated by examination of morphology and growth characteristics. All cell lines were confirmed to be mycoplasma-free.

Hypoxia was generated in a Whiteley H35 Hypoxystation (Don Whitley Scientific). Cells were placed in the hypoxia chamber at 1% O2 and media were immediately changed to hypoxic preconditioned media. As control, cells were incubated in 21% O2.

RNA extraction and quantitative real-time PCR

Total RNA was extracted and cDNA synthesis was performed as described in Supplementary Methods. Amplifications were run using a GeneAmp 7300 sequence detector (Applied Biosystems). Real-time detection of the PCR product was performed using the SYBR Green Master Mix (Applied Biosystems). All reactions were performed in triplicate and the comparative C_t method was used to quantify relative mRNA. The geometric mean of three endogenous reference genes (UBC, YWHAZ, and SDHA) was used for normalization. Primer sequences are provided in Supplementary Methods.

siRNA transfections

Cells were transfected with siRNA targeting HIF-1α (silencer select s6541 #3490821), HIF-2α (EPAS1 #4390824), SLC6A3_1 (id 12976, silencer select, Ambion), (id 12977, silencer select, Ambion), and siNeg ctrl #2 (silencer select #4390847, Ambion) and siNeg ctrl #1 (silencer select #4390843, Ambion) at 2.5, 5, or 10 nmol/L concentration using Lipofectamine 2000 (Invitrogen; 3 μL/well for 12-well plate) according to manufacturer’s instructions. Transfections were carried out in serum- and penicillin-free OPTI-MEM medium (Gibco). Effect of knockdown was measured 48 hours after transfection.

Western blotting

Cells were lysed in RIPA supplemented with Complete Protease Inhibitor (Roche Diagnostics). Proteins were separated by SDS-PAGE and transferred to 0.2-μm polyvinylidene difluoride (PVDF) membranes (BioRad) using transblot turbo transfer system. Membranes were blocked in 5% milk in PBS-T and incubated with primary antibody anti-DAT1 (Abcam, ab128848), anti-HIF-1α (NB100-479), or anti-HIF-2α (Abcam ab199) overnight and β-actin antibody (MP Medicals, #691001) or SDHA antibody (Abcam, ab14715) as control.

Dopamine uptake assay

For uptake experiments, cells were seeded at a density corresponding to confluence at 48 hours. At confluence, assay buffer containing 7 nmol/L dihydroxyphenylethylamine 3,4-[[3H]-Dopamine ([3H]-dopamine; 56.8 Ci/mmol; Perkin Elmer) and 20 μg/ml l-ascorbic acid (Sigma Aldrich) was added for 5 minutes, as detailed in Supplementary Methods. Competitive inhibition of [3H]-dopamine uptake was performed by incubation with either 2 or 20 μmol/L unlabeled dopamine.
hydrochloride (Sigma Aldrich). [3H]-Dopamine uptake experiments were also performed in presence of the specific SLC6A3 inhibitor 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR12909; Sigma Aldrich). Here, cells were pretreated for 60 minutes with varying concentrations (10 nmol/L to 10 μmol/L) of GBR12909 in the growth media, and thereafter uptake was performed in presence of GBR12909 at the same concentrations. The amount of radioactivity was measured with a Tri-carb 2810TR liquid scintillation analyzer (Perkin Elmer). Uptake was assessed by radioactivity in DPM related to pmol of dopamine per assay well or pmol/μg protein from cell lystate from one assay well. Protein concentration of lysates was determined using BCA Protein Assay Kit (Pierce Biotechnology).

Statistical analyses for cell culture experiments
All values are reported as mean ± SEM from at least three independent experiments unless otherwise stated. The two-sided Student unpaired t test was used for statistical analyses, unless otherwise stated. Three levels of significance were used: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Gene expression data
Level 3 RNA-seq data was downloaded from the Cancer Genome Atlas (TCGA) data portal (https://portal.gdc.cancer.gov/). Gene-level RSEM scaled estimates were processed by log2(RSEM × M + 1) to create gene expression levels for 20,531 Entrez gene IDs as annotated in the TCGA hg19 June2011 Generic Annotation Files (GAF). Data were obtained for 25 different types of solid tissue tumors. For 16 of these tumor types, three or more corresponding normal tissue samples were available and included in the analyses. A list of all 7,155 samples used in this study is given in Supplementary Table S2.

For validation purposes, we downloaded the Gene Expression Omnibus (GEO) entry GSE3526 comprising gene expression patterns of 353 normal tissue samples from 20 anatomically distinct sites of the human central nervous system (CNS), as well as 45 non-CNS tissues, measured on the Affymetrix HGU133 plus 2.0 platform (15). We also acquired mRNA expression data from 1,036 different cell lines as provided by the Broad Cancer Cell Line Encyclopedia (CCLE; ref. 16).

Transporter genes
SLC genes were selected from the BioParadigms Genomic Transporter Database (www.bioparadigms.com). In total, 391 unique genes from 52 different SLC transporter families were present in the TCGA data. We further included 51 genes encoding ABC transporters. The complete gene list of 442 analyzed transporter genes is provided in Supplementary Table S3.

Statistical analyses for TCGA data
Statistical analyses were performed using the R software (http://www.r-project.org), Hierarchical Cluster Analyses (HCA), principal component analyses (PCA), and Kaplan–Meier plots were performed using the stats package in R. For HCA, 1-Pearson correlation was used as distance measure and Ward method for agglomeration. For PCA and HCA (Fig. 1A–C), the mean expression value for each transporter gene within the respective TCGA tumor or normal tissue cohorts was used. Differential gene expression between clear cell kidney carcinoma (KIRC) tumor and normal tissues was determined using the Limma Bioconductor package (17). For the analysis of taxonomy groups, samples from all 3 TCGA kidney cancer projects [chromophobe RCC (KICH), KIRC, papillary kidney carcinoma (KIRP)] were merged into one single dataset. Molecular RCC taxonomy groups as well as clinical and histopathologic parameters were used as presented in the article by Chen and colleagues (18).

Results
Expression of the SLC and ABC transporter families in different tumor types
We set out to explore the expression patterns of cellular transporters of the SLC and ABC families in ccRCC and normal kidney in relation to other normal and tumor tissue types. For this purpose, we performed PCA analyses on mRNA sequencing data from TCGA. As illustrated in Fig. 1A, the transcriptional pattern of the 442 SLC and ABC transporter genes stood out in samples collected from normal kidney and liver tissues relative to samples obtained from the remaining 14 tissues analyzed. Likewise, when analyzing data from 25 different tumor types, two kidney carcinomas, KIRC and papillary RCC (pRCC; KIRP), as well as liver hepatocellular carcinomas, clearly differed from remaining tumors; possibly reflecting their cellular origin from cells endowed with considerable transport capacities (Fig. 1B). Also, neuronally derived tumors, such as glioblastoma multiforme, lower-grade glioma, and pheochromocytoma and paraganglioma, displayed large differences in expression patterns compared with other tumors. We further noted that KICH did not deviate as much from remaining tumor types, most likely explained by the notion that this tumor type is derived from a more distal and less transporter-rich region of the nephron (19, 20).

We next performed unsupervised HCA of transporter gene expression in the TCGA data (Fig. 1C and Supplementary Fig. S1). We observed that tumors and their corresponding normal tissues often clustered together, possibly reflecting that tumors often maintain characteristic transporter expression found in their tissue of origin. The HCA further showed a high degree of tissue specificity among groups of transporter genes. We identified five separate gene clusters of transporters with apparent tissue- and/or tumor-specific expression (colored bars, Fig. 1C and Supplementary Fig. S1).

We noted that transcript levels of several transporters were reduced or abolished in some tumor samples, as compared with corresponding normal tissues. This may reflect a dedifferentiation process that takes place during oncogenic transformation or may be caused by differences in cellular composition between tumor and normal tissue such as infiltration of tumor-associated lymphocytes and fibroblasts. Alternatively, the absence of gene expression in a tumor could be explained by cellular heterogeneity within the corresponding tissue of origin, that is, the transporter may be expressed in cell types different from which the cancer stems from. This was particularly obvious in case of the kidney-specific gene cluster. Here, expression of numerous transporters was absent or low in pRCC and ccRCC than in normal renal samples (light green, Fig. 1C). Both these tumor entities are believed to arise from proximal tubule cells. Normal kidney tissue, however, is composed of a large number of cell types, each with its own specialized setup of transporter proteins. Genes
Tumor-specific SLC6A3 overexpression in ccRCC

We next sought to identify transporter genes that were differentially expressed between ccRCC and normal kidney tissue (Fig. 1D and Supplementary Table S4). Surprisingly, the most upregulated gene was identified as the dopamine reuptake transporter SLC6A3, which increased almost 25-fold in ccRCCs relative to normal kidney tissue. The human kidney is an important target organ for dopamine action and the entire nephron expresses various receptors for dopamine. Both circulating and renally produced dopamine affect the kidney in several ways, including vasodilation, control of diuresis, and sodium homeostasis. However, there are no reports indicating a functional role of SLC6A3 in the kidney. We therefore scrutinized SLC6A3 expression in the panel of normal and tumor tissues obtained from TCGA. The high expression observed in ccRCC was not reflected in any other tissue or tumor group; in fact, the vast majority of samples, including the two other kidney tumor types, display near-absent levels of SLC6A3 expression (Fig. 1E). Interestingly, lung adenocarcinoma and mesothelioma, within which a small fraction of samples displayed elevated SLC6A3 levels, are known to harbor focal genomic gains of the TERT gene located close to SLC6A3 at chromosome band 5p15.33 (24–26), raising the possibility that SLC6A3 overexpression in ccRCC is driven by increased expression of SLC6A3 in these samples is a passenger event to increased TERT expression.

We next analyzed SLC6A3 expression in relation to the molecular RCC taxonomy groups described by Chen and colleagues (18). Increased expression was almost exclusively found in samples of the three ccRCC taxonomy subtypes (CC-e1, -e2, and -e3), thus substantiating the possible connection between pseudohypoxia and SLC6A3 overexpression (Supplementary Fig. S2A). The highest expression was noted in the CC-e2 subtype, which is the subtype enriched for ccRCCs of lower stage. SLC6A3 expression was found slightly higher in VHL- and PBRM1-mutated ccRCC samples, which is in line with the notion that the CC-e2 subtype is enriched for these two mutations (Supplementary Fig. S2B and S2C). Decreased expression of SLC6A3 was noted in stage IV tumors (Supplementary Fig. S2F) and was also significantly associated with adverse outcome (Supplementary Fig. S2G). Importantly, among the majority of patients who succumbed to the disease, SLC6A3 expression in the primary tumor was readily detectable and higher than what is seen in other tumor types and normal tissues (Supplementary Fig. S2A and S2H).

The SLC6A3 dopamine transporter, also known as DAT1, is normally expressed in dopaminergic neurons of the CNS. We therefore downloaded microarray expression data of 20 different anatomically distinct sites of the human CNS and 45 non-CNS tissues (15). As expected, SLC6A3 was only found expressed at appreciable levels in substantia nigra and the ventral segmentum, where dopaminergic cells reside (Fig. 1F). Thus, we conclude that SLC6A3 expression in normal tissues is restricted to dopaminergic cell types of the brain and that ccRCC represents the only tumor entity, within the 25 different types of malignancies investigated here, that consistently display high levels of this transporter.

Assessment of SLC6A3 expression in ccRCC cell lines

To confirm the data obtained from TCGA, we assessed mRNA expression of SLC6A3 using quantitative real-time PCR (qPCR) in ccRCC cell lines. As control, we used the breast cancer cell line MCF7. Quite surprisingly, and in stark contrast to tumor sample expression, conventional ccRCC cell lines, such as 786-O, RCC4, and the SKRCs, showed very low or nondetectable mRNA expression of SLC6A3 (Fig. 2A). We therefore examined SLC6A3 expression patterns of SLC and ABC transporters in normal and tumor tissues. The mRNA gene expression patterns of 442 SLC and ABC transporters were analyzed using mRNA-sequencing data from the TCGA database. A, PCA of 613 samples from 16 different noncancerous tissues and the 442 transporter genes. Each point in the plot represents one sample and is color-coded according to TCGA tumor type. Kidney (green colors) and liver (tan color) samples cluster separately when the first two principal component scores (PC1 and PC2) are visualized. This indicates that most of the variation in the transporter gene expression data is observed between these two tissues and the remaining samples. The panel legend lists the different TCGA tumor types included, their respective color used for plotting, and the number of samples included within parenthesis. B, Similar analysis as in A but using 6542 solid tumors of 25 different histological types. Here, samples representing ccRCC (KIRC) and pRCC (KIRP), and to a lesser extent liver cancer (LIHC), deviate from remaining samples with respect to the first principal component. For the second principal component, glioblastoma samples show most separation from other samples. C, Heatmap visualizing transporter mRNA expression in normal and tumor tissues. Each row represents one of the 442 transporter genes and columns represent the mean expression of these genes within each of the 41 TCGA groups of samples (red, high expression; blue, low expression). Sample groups are colored (top bar; T, tumor; N, normal) using same colors as displayed in A and B legends. The rightmost bars indicate clusters of transporters that were found elevated in specific sample groups. A high-resolution image of the heatmap, annotated with gene symbols, is provided as Supplementary Fig. SI. D, Differential expression of transporter genes comparing 530 ccRCC with 70 normal kidney samples using Limma. Each point represents one of the 442 transporter genes and is color-coded according to fold change (red, high relative expression in ccRCC; blue, high relative expression in normal kidney). Axes represent the mean fold change in ccRCC relative normal kidney (x-axis; log; scale) and the corresponding adjusted P value (y-axis). The gene with highest mean fold change in ccRCC was SLC6A3. E, Boxplot summarizing SLC6A3 gene expression in the TCGA data. The TCGA sample groups are ordered on the basis of the HCA above. F, Boxplot of SLC6A3 gene expression in 353 post mortem samples collected from 20 anatomically distinct sites of the human CNS and 45 non-CNS tissues (GSE3526; Roth and colleagues; ref. 15). The different CNS tissues are colored in pink and kidney tissues (cortex and medulla) are colored in green. ACC, adenocortical carcinoma; BLCA, urothelial bladder cancer; BRCA, breast cancer; CESC, cervical cancer; COAD, colon adenocarcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; LGG, lower-grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic ductal adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; READ, rectal adenocarcinoma; SARC, sarcoma; SKCM, cutaneous melanoma; THCA, papillary thyroid carcinoma; UTEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma.
tionally high levels of kidney cancer cell lines, KMRC3 and SNU-349, expressed exceptionally high levels of SLC6A3 (Supplementary Fig. S3A). Importantly, both these cell lines are derived from pathologically confirmed ccRCC tumors and display DNA copy number profiles that are highly characteristic for primary ccRCC tumors, including chromosome 3p deletion and gain of 5q (Supplementary Fig. S3B). A subsequent qPCR confirmed overexpression of SLC6A3 in these two cell lines (Fig. 2A).

Western blot analyses confirmed that cell lines with high (KMRC3 and SNU-349) or moderate (SKRC10) SLC6A3 mRNA expression displayed appreciable levels of SLC6A3, whereas the commonly used 786-O cell line expressed very low levels of the protein. No protein expression could be detected in extracts from MCF-7 cells (Fig. 2B). KMRC3 and SNU-349 are not widely used and grow very slow than the more conventional ccRCC cell lines. For example, KMRC3 display a doubling time of approximately 7 days compared with 24 hours for 786-O (27). The growth pattern of SNU-349 and KMRC3 is more in line with that of primary ccRCC cultures.

Short-term cell cultures of primary ccRCC displayed high expression of SLC6A3 in contrast to cultures from matched normal renal cortical cells that showed very low levels (Fig. 2C). Primary tumor samples further displayed a higher protein level of SLC6A3 than cells from normal kidney (Fig. 2D). On the basis of these results, we conclude that SLC6A3 is expressed at very high levels in primary ccRCC and in cell lines with growth properties similar to that of primary ccRCC cell cultures. In contrast, the expression is lost in most established ccRCC cell lines.

Hypoxic induction of SLC6A3 in renal cells
ccRCC is characterized by a state of pseudohypoxia due to its functional loss of VHL. We therefore wanted to clarify whether SLC6A3 display properties of an HIF target gene. For this purpose, we first compared the expression of SLC6A3 with that of the well-established bona fide HIF target gene, CAIX. As shown in Fig. 3A, CAIX is induced in all solid tumors comprised in the TCGA data in comparison to their corresponding normal tissue. This most likely reflects the well-documented varying state of hypoxia that characterizes solid tumors. This stands in stark contrast to the ccRCC-specific induction of SLC6A3 (Fig. 1E). Thus, SLC6A3 is not a conventional HIF target gene.

We reasoned that the high levels of SLC6A3 in ccRCC could be related to cell-of-origin specific hypoxic induction. We therefore exposed primary normal human renal cells to hypoxia and noticed a consistent upregulation of the SLC6A3 transcript (Fig. 3B). In an attempt to compare this with other

Figure 2.
SLC6A3 expression present in primary ccRCC, KMRC3, and SNU-349 but lost in conventional cell lines. A, Relative mRNA expression of SLC6A3 in ccRCC cell lines and breast cancer cell line MCF7 as control. B, Western blot analysis; protein expression of SLC6A3 and actin loading control in lysates of ccRCC cell lines SNU, KMRC3, SKRC10, and MCF7 as control. C, Relative mRNA expression of SLC6A3 in short-term culture of primary renal cells from matched tumor and normal display upregulated SLC6A3 in tumor samples (P = 0.025; paired two-tailed Student t test). D, Western blot analysis; protein expression of SLC6A3 and SDHA in lysates of short-term cultured primary renal cells tumor (n = 5) and normal (n = 3), to the right; quantification of mean SLC6A3 band intensity relative to SDHA loading control for tumor and normal, respectively (P = 0.040; two-tailed Student t test). Data presented as mean ± SEM, and mRNA levels were related to housekeeping genes SDHA, UBC, and YWHAZ.

Expression in the CCLE database comprising more than 1,000 established cancer cell lines (16). We observed that two of 36 kidney cancer cell lines, KMRC3 and SNU-349, expressed exceptionally high levels of SLC6A3 (Supplementary Fig. S3A). Importantly, both these cell lines are derived from pathologically confirmed ccRCC tumors and display DNA copy number profiles that are highly characteristic for primary ccRCC tumors, including chromosome 3p deletion and gain of 5q (Supplementary Fig. S3B). A subsequent qPCR confirmed overexpression of SLC6A3 in these two cell lines (Fig. 2A).

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Figure 3.
Hypoxic induction of SLC6A3 is specific for renal cells. **A**, Boxplot summarizing CAIX expression from TCGA data of normal (n = 16) and tumor samples (n = 25). The TCGA sample groups are ordered on the basis of the HCA in Fig. 1. **B**, Relative mRNA expression of SLC6A3 in short-term culture of normal primary renal cells subjected to 1% pO2 (hypoxia) or 21% pO2 (normoxia) for different time points: 24 hours (n = 7), 48 hours (n = 6), 72 hours (n = 6). Statistical significance was calculated using a paired Student t test. **C**, Relative mRNA expression of SLC6A3 in normal endothelial cells (HUVEC) and normal epithelial cells from breast (MCF10A) or prostate (PNT-2) subjected to 1% pO2 (hypoxia) or 21% pO2 (normoxia) for different time points: 24 hours (n = 3), 48 hours (n = 3), 72 hours (n = 3). **D**, Relative expression of SLC6A3, HIF-1α, and HIF-2α mRNA after downregulation of HIF-1α, HIF-2α, or both, using siRNA. Expression was normalized against siNeg ctrl in each experiment, and statistical significance was calculated compared with siNeg ctrl. B–D, Data; mean ± SEM. mRNA levels were related to housekeeping genes SDHA, UBC, and YWHAZ. Statistical significance was calculated using two-tailed Student t test. *P < 0.05; **P < 0.01; ***P < 0.001.
nonmalignant cells, we cultured endothelial cells (HUVECs), mammary epithelial cells (MCF10A), and prostate epithelial cells (PNT-2) under hypoxic conditions. Neither HUVEC nor MCF10A displayed hypoxic induction of SLC6A3, whereas PNT-2 displayed a modest increase (Fig. 3C). Primary ccRCC cells that display high levels of SLC6A3 (Fig. 2C) showed no further induction when exposed to hypoxia (Supplementary Fig. S4A). Likewise, commonly known hypoxia targets were not further induced in ccRCC tumor samples treated with hypoxia (Supplementary Fig. S4B). Interestingly, both RCC4 and 786-O cells reconstituted with VHL displayed significantly lower SLC6A3 levels than the corresponding VHL-negative cells (Supplementary Fig. S4C), indicating that long-term normalization of VHL status in ccRCC cells is associated with downregulation of SLC6A3. To further dissect the role of HIF-1α and HIF-2α, the two main effectors of hypoxic signaling, we performed knockdown experiments using siRNA in KMRC3, SNU-349, and SKRC10 (Fig. 3D). Western blot analyses showed that all knockdown experiments using siRNA in KMRC3, SNU-349, and SKRC10 displayed a modest increase (Fig. 3D). Primarily, SLC6A3 transcripts were decreased by siHIF-2α and dual siHIF-1α and siHIF-2α but not with siHIF-1α alone in all three cell lines (Fig. 3D). In KMRC3 and SNU-349, the HIF-1α knockdown displays a corresponding decrease of the HIF-1α-associated target gene CAIX, whereas GLUT1 displays a less HIF subtype-specific regulatory pattern in response to the respective siRNA (Supplementary Fig. S5B). Altogether, these results points toward a link between VHL loss and dysregulation of SLC6A3 in renal cells and ccRCC and that HIF-2α might be an important mediator of this response.

**Functional SLC6A3 in ccRCC**

The normal function of SLC6A3 is to transport dopamine from the synaptic cleft back into the presynaptic neuron. To assess the functionality of SLC6A3 in ccRCC, the [3H]-dopamine uptake was measured in cultured ccRCC cells. The levels of [3H]-dopamine uptake mirrored SLC6A3 mRNA levels in the ccRCC cell lines, with SNU-349 and KMRC3 displaying the highest uptake (Fig. 4A). To determine whether the uptake of [3H]-dopamine was mediated by a dopamine transporter, we first performed the experiments in the presence of unlabeled dopamine. The only cell lines that showed a significant effect of the competition were SNU-349 and KMRC3 (Fig. 4B). In addition, we used varying concentrations of a highly specific inhibitor at the same concentrations.

**Figure 4.**

Functional SLC6A3 in ccRCC cell lines KMRC3 and SNU-349, but not in conventional cell lines. A, Confluent cultured ccRCC cell lines and breast cancer cell line MCF7 as control were incubated with [3H]-dopamine (0.4 μCi/mL, 7 nmo/L/L) for 5 minutes before abortion of uptake by ice-cold wash buffer. Uptake was assessed by analyzing the radioactivity in the cell lysate and expressed as pmol/μg protein in each well. B, Experiment was performed as in A but the incubation performed in the presence of 2 or 20 μmol/L unlabeled dopamine. C, Experiment was performed as in A, but cells were preincubated with specific SLC6A3 inhibitor GBR12909 in varying concentrations (0, 0.1, 1, or 10 μmol/L) for 1 hour, before [3H]-dopamine uptake in the presence of GBR12909 at the same concentrations. D, KMRC3 and SNU-349 were treated as in C, but with lower concentrations of GBR12909 (0, 10, 30, and 90 μmol/L). E, Experiment was performed in technical triplicates and were repeated for 3 independent experiments, unless otherwise stated. Statistical significance was calculated using two-tailed paired Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
inhibitor of SLC6A3, the cocaine analogue GBR12909. Again, the only cell lines responding to GBR12909 treatment with a decreased [3H]-dopamine uptake were SNU-349 and KMRC3 (Fig. 4C). These two cell lines displayed a significant decrease of [3H]-dopamine uptake also at levels down to 10 nmol/L of GBR12909 (Fig. 4D).

To further link SLC6A3 levels and uptake of dopamine, we performed knockdown experiments using siRNA in SNU-349 cells. The SLC6A3 knockdown was efficient using two different siRNAs and resulted in a decreased uptake of [3H]-dopamine (Fig. 5A and B).

Consistent with SLC6A3 mRNA expression, primary ccRCC tumor cells displayed strongly enhanced [3H]-dopamine uptake in comparison to normal renal cells from the same patient (Fig. 6A). Short-term cultures of primary ccRCCs also showed a decrease in [3H]-dopamine uptake in the presence of 2 and 20 μmol/L unlabeled dopamine (Fig. 6B), an effect not seen in normal renal cells (Fig. 6A). When exposing primary normal kidney cells to hypoxia, the cells displayed a trend toward increased [3H]-dopamine uptake (Fig. 6C), in line with the results of the hypoxia-induced SLC6A3 mRNA (Fig. 3B). Furthermore, when targeting the HIF subtypes in KMRC3 and SNU-349, only siHIF-2α led to a significant decrease in [3H]-dopamine uptake (Fig. 6D). These results further substantiate the notion that HIF-2α is the main driver of functional SLC6A3 expression in ccRCC.

Discussion

This study is based on an explorative assessment of transporter gene expression in multiple tumor types and corresponding normal tissues comprised in the TCGA project. As expected, distinct tissue-specific expression patterns were observed in the normal tissues. Many tumors retained expression of transporters that are highly specific for their tissue of origin and may thus represent an important source for understanding tumor biology and development of functional biomarkers that can be applied in, for example, differential diagnosis. Importantly, we could identify tumor-specific expression of transporters that are suppressed in corresponding normal tissues which, based on their functional attributes, could be exploited for diagnostic or therapeutic purposes. One such example is SLC6A3, which is specifically overexpressed in ccRCC compared with normal kidney and other tumor types. We demonstrate that this overexpression is accompanied by active dopamine uptake in ccRCC cells, making SLC6A3 an excellent functional biomarker for ccRCC. In previous gene expression profiling studies of primary ccRCC, a high expression of SLC6A3 has been noted (28, 29). However, to the best of our knowledge, the present report represents the first functional analysis of SLC6A3 in ccRCC. One explanation for this might be that most established cell lines used in experimental studies of ccRCC, such as 786-O, are virtually devoid of SLC6A3 expression. Although established cell lines have provided an essential source for our current understanding of cancer cell behavior, there is a pronounced lack of fidelity to the original tumor in certain aspects of cancer cell biology. This is particularly true with respect to transporter proteins, many of which are lost or downregulated in conjunction with the in vitro establishment procedure (30). We could nevertheless identify two established ccRCC cell lines that retain high expression of the SLC6A3 transporter.

cRCC is characterized by a functional loss of VHL, causing a cellular state of pseudohypoxia. We thus hypothesized that SLC6A3 could be induced by low oxygen tension. However, in our pan-TCGA comparison between the prototypical hypoxia target CAIX and SLC6A3, it was apparent that the links between SLC6A3 and hypoxia are more complex than the one between CAIX and hypoxia (Fig. 1E and 3A). Nevertheless, we show that SLC6A3 can be induced by hypoxia in normal cortical renal cells, albeit not to the same levels as noted in the primary tumors. Furthermore, the observation that VHL-reconstitution in the ccRCC cell line RCC4 led to a significant decrease in SLC6A3 expression lends support to a link between hypoxia and SLC6A3. Future studies of the chromatin landscape surrounding SLC6A3 will possibly provide more direct clues into why such a prominent and cell type-specific induction of this gene is seen.

It is not uncommon that tumor cells harness different transporter proteins for maintaining their growth and survival. For example, the HIF target gene SLC2A1 (GLUT1) is frequently upregulated in tumor cells to ensure sufficient energy supply. Similarly, the high-affinity glucose transporter GLUT3 (SLC2A3) is overexpressed in breast, colorectal, and bladder cancers while normally being expressed primarily in neuronal cells (31–33). LAT1 or SLC7A5 transports neutral amino acids into the cell, which can be used as building blocks and energy. SLC7A5 is upregulated in a variety of cancers (34). Including ccRCC, where it has been found to activate mTORC1 via HIF-2a induction (35). In addition, tumors are often dependent on glutamine for the maintenance of cellular NADPH pools as well as for synthesizing the antioxidant glutathione (36).

Consequently, many SLCs with glutamine-transporting functions are upregulated in cancer cells (37). In above examples, there is an obvious link between the specific function of the transporter and the metabolic need of the tumor cells. In an
effort to assess whether SLC6A3 play any such role in ccRCC, we compared the effect on viability upon SLC6A3 inhibition in two cell lines expressing high levels of SLC6A3 (KMRC3 and SNU-349) versus 786-O which do not express appreciable levels of the transporter. We detected a similar effect of GBR12909 treatment in all cell lines regardless of the levels of SLC6A3 (Supplementary Fig. S6). Thus, any potential functional benefit of the specific upregulation of the SLC6A3 transporter in ccRCC is presently not clear. The observation that the SLC6A3 levels are generally lower in high-stage tumors indicates that SLC6A3 may be important for initial stages of tumorigenesis rather than having a role during later stages of tumor progression.

Irrespective of the reasons for the high expression levels of functional SLC6A3 in ccRCC, we postulate that this finding could be exploited for development of novel therapeutic or diagnostic modalities. For example, Ioflupane I123, a cocaine analogue that binds to SLC6A3 and hence accumulates on dopaminergic neurons, is widely used for diagnosing Parkinson disease. The results presented in this study, showing ccRCC-specific expression of functional SLC6A3, call for a clinical trial of this diagnostic tool for ccRCC. In addition, a plethora of molecules that bind to and/or inhibit SLC6A3 have been developed. Given the biologic properties of SLC6A3, there are also several possibilities of exploiting its transporter function for ccRCC therapy based on radionuclide labeling of such compounds. However, to fully appreciate the potential use of SLC6A3 expression in ccRCC, several key issues have to be clarified, including the underlying molecular mechanisms causing the overexpression, and the potential advantage of SLC6A3 overexpression for the tumor cells.

**Disclosure of Potential Conflicts of Interest**

J Hansson, L Gustavsson, H Axelsson, D Lindgren, and E Johansson are listed as co-inventors on a patent on utilization of SLC6A3 overexpression in ccRCC for diagnosis and treatment of ccRCC that is owned by Akuru Pharma. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: J. Hansson, D. Lindgren, L. Gustavsson, H. Axelsson

Development of methodology: J. Hansson, D. Lindgren, M. Johansson, L. Gustavsson, H. Axelsson

Figure 6.

Active dopamine uptake in primary ccRCC and increased dopamine uptake in primary normal renal cells in hypoxia. A, Confluent short-term cultured primary renal cells from a matched tumor and normal sample were exposed to [3H]-dopamine (0.4 µCi/mL, 7 nmol/L) for 5 minutes before abortion of uptake by ice-cold wash buffer. Uptake was assessed by radioactivity related to pmol of [3H]-dopamine per assay well. B, Short-term cultured primary ccRCC cells were treated as in A. C, Short-term cultured primary normal renal cells were subjected to 1% PO2 (hypoxia) or 21% PO2 (normoxia) for 72 hours prior to [3H]-dopamine uptake assay. Cells were then exposed to [3H]-dopamine (0.4 µCi/mL, 7 nmol/L) for 5 minutes before abortion of uptake by ice-cold wash buffer. Uptake was assessed by radioactivity in DPM related to pmol of dopamine relative to amount of protein in one well. For A to C, statistical analyses were based on technical triplicates for each primary cell culture, presented as mean ± SD. D, [3H]-dopamine uptake of cells transfected with siNeg ctrl, siHIF-1α, or siHIF-2α. Uptake was performed as described in Fig. 4A, 48 hours after transfection and was normalized to siNeg in each experiment (N = 3) and statistical significance was calculated compared with siNeg ctrl. Data presented as mean ± SEM.
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