CANCER

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Comment on "Epigenetic activation of the drug transporter OCT2 sensitizes renal cell carcinoma to oxaliplatin"

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Although the combination of decitabine and platinum drugs may be promising for therapy of renal cell carcinoma, the role of OCT2 needs further investigations.

In a recent article, Liu and colleagues (*1*) presented data that epigenetic activation of the organic cation transporter OCT2 by decitabine sensitizes renal cell carcinoma (RCC) cells to oxaliplatin both in vitro and in xenografts. Their conclusion is based on the finding that OCT2, which is encoded by the *SLC22A2* gene, is down-regulated on the mRNA and protein levels in RCC. Especially on the protein level, the authors could not detect any OCT2 expression in 31 RCC cases, including 10 cases of the clear cell RCC (ccRCC) subtype, which is the most aggressive RCC subtype. The authors concluded that, as a consequence of OCT2 absence in these tumors, the uptake of the OCT2 substrate oxaliplatin is inhibited in RCC cells.

We recently published a study (*2*) on the expression and epigenetic regulation of pharmacogenes in ccRCC, metastases, and RCC cell lines, including in-depth analyses of the epigenetic regulation of OCT2. We came up with the same finding as that of Liu *et al.*, that OCT2 is not expressed in any of the studied RCC cell lines because of high DNA methylation in the promoter region (including one CpG site important for transcriptional regulation in the E-box element) and that treatment with decitabine restores its expression in these cell lines. Yet, our conclusions regarding mRNA and protein levels in primary ccRCC and ccRCC-derived metastases are discrepant. Considering mRNA expression data (based on RNA sequencing) from The Cancer Genome Atlas (TCGA), there is a slight decrease of *SLC22A2* mRNA in ccRCC tissue compared to adjacent nontumor tissue. However, *SLC22A2* is among the top 7% expressed genes in 463 ccRCC cases (Fig. 1A). We could confirm these data in our own ccRCC cohort (*2*) based on microarray data (Fig. 1A), where *SLC22A2* belongs again to the 7% of genes with the highest expression in ccRCC. Notably, comparing paired nontumor and tumor samples of the TCGA cohort, again no significant (paired t test, $P = 0.22$) expression differences between nontumor and tumor tissues were observed, and only 22.2% of all cases showed reduced OCT2 expression in the tumor tissue (fold change nontumor/tumor, >2). Of course, high mRNA levels do not necessarily mean that the protein is expressed at all. However, in contrast to Liu *et al.* (*1*), we could demonstrate in our study (*2*) that OCT2 is expressed on the protein level in ccRCC and in ccRCC-derived metastases. Because we used a different antibody compared to Liu *et al.*, we now investigated whether this discrepancy was due to the antibodies used. First, we evaluated the expression of OCT2 in nontumor tissue using four different antibodies, including the two antibodies proposed by Liu *et al.* (*1*). It has previously been demonstrated that OCT2 in nontumor kidney tissue is located on the basolateral membrane of the proximal tubules (*3*, *4*), which is important for the proposed mechanism of drug uptake by OCT2 into the cells [see fig. S13 in Liu *et al.* (*1*)]. As shown in Fig. 1B, we could confirm this basolateral localization using our own previously validated OCT2 antibody [KEK, (*3*)] and an antibody (MAB6547, R&D Systems) previously investigated by Gai *et al.* (*5*), which was raised against the full-length OCT2 protein. Notably, the OCT2 antibody KEK [previously described in Nies *et al.* (*3*)] and the antibody MAB6547 detected OCT2 protein in our human embryonic kidney (HEK) 293–OCT2 transfectants in immunoblots (Fig. 1C). However, using both antibodies from Liu *et al.* (AMAb90791 and AMAb90792, Sigma-Aldrich), staining was clearly detected on the apical membrane of tubules (Fig. 1B), which is in contrast to the currently accepted basolateral localization of OCT2 (*3*, *4*). Apical staining using these two antibodies is also visible in the data from the Human Protein Atlas (*6*) and in the representative images of OCT2 staining provided in the supplementary data of Liu *et al.* (*1*). The apical localization of OCT2 is in complete discrepancy with the proposed mechanism of the study by Liu *et al.* (*1*), that OCT2 mediates the uptake of drugs such as oxaliplatin at the basolateral membrane in proximal tubules. We further performed immunohistochemical analyses using a tissue microarray consisting of 64 ccRCC cases and determined expression intensity using a software-assisted system (Definiens) previously validated for the expression of membrane proteins in ccRCC (*7*). Representative images are shown in Fig. 1D. We observed strong staining of OCT2 in ccRCC tissue using our own antibody (KEK) and the antibody used by Gai *et al.* (MAB6547, R&D Systems) (Fig. 1D) (*5*). Using both antibodies proposed by Liu *et al.* (AMAb90791 and AMAb90792, Sigma-Aldrich), the staining intensity was considerably lower compared with our antibodies, but still several ccRCC cases showed medium or high staining as well.

Together, we believe that based on our data and data from TCGA, OCT2 expression on the mRNA and protein levels is not generally repressed in ccRCC tissue. As we have demonstrated in our study (*2*), the expression of OCT2 is variable because of alterations in DNA methylation, but >70% of all ccRCC tumors express high levels of OCT2. In contrast, OCT2 was not expressed in any of the studied RCC cell lines because of very high DNA methylation (*2*). Further studies are warranted to evaluate the four different antibodies and confirm our results in independent primary tumor samples of ccRCC patients. Moreover, the proposed new therapeutic concept by Liu *et al.*, recently even highlighted in an editorial (*8*), using a combination

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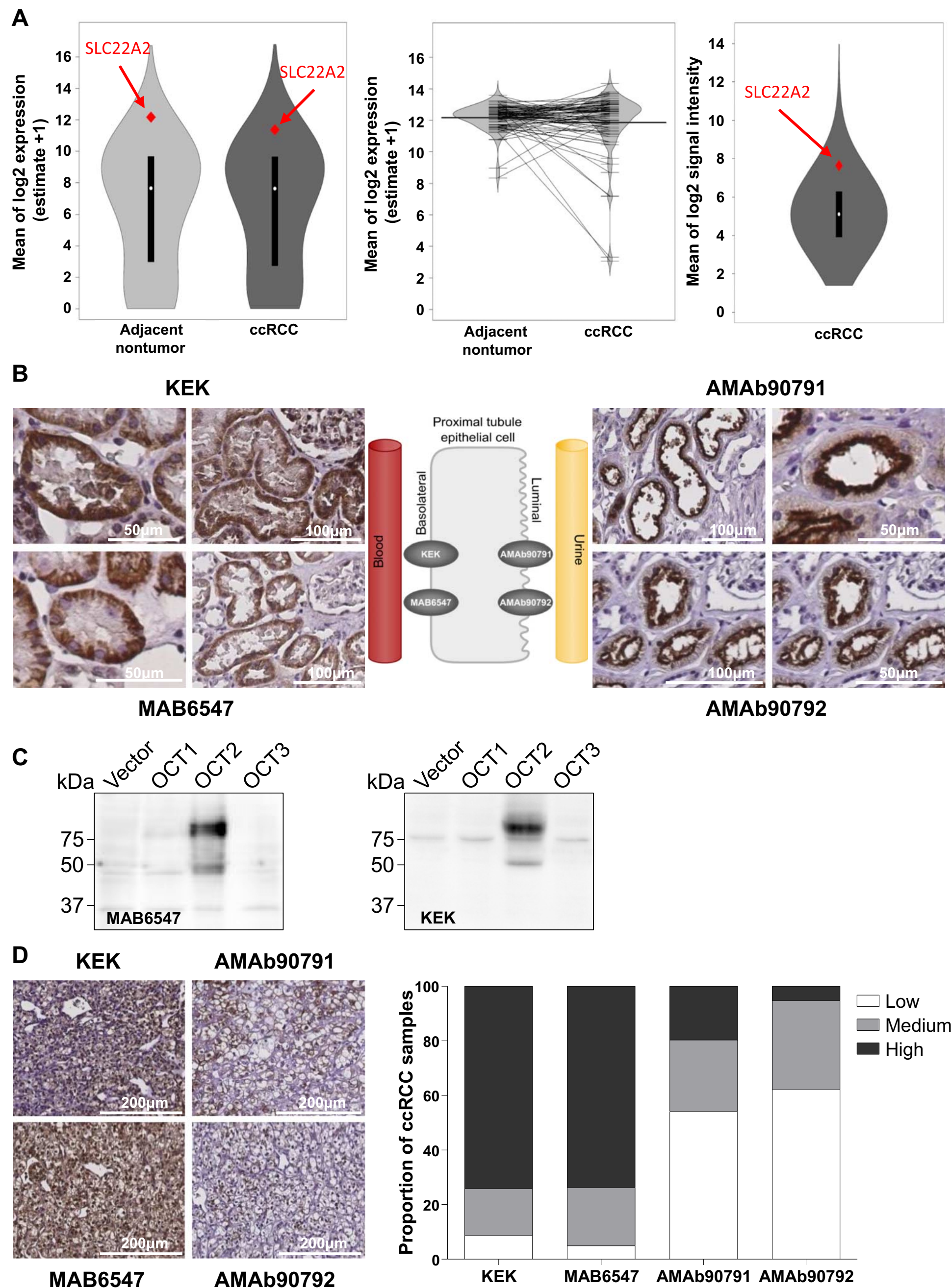

Fig. 1. mRNA and protein expression of SLC22A2/OCT2 in nontumor and ccRCC tumor tissue. (**A**) Gene expression of SLC22A2/OCT2 in adjacent nontumor and tumor tissues of ccRCC patients. Violin plots show distribution of gene expression in adjacent nontumor and tumor tissues of the ccRCC cohort of TCGA (left, n = 463) as well as in our cohort of ccRCC tissues (right, n = 31). SLC22A2 is one of the top 7% expressed genes in 463 ccRCC cases of TCGA (left) and in our own study cohort (right) (*2*). OCT2 expression in paired nontumor and tumor tissues (n = 72) of the TCGA cohort is displayed in the beanplots (middle). No significant (paired t test, P = 0.22) expression differences between nontumor and tumor tissues were observed, and only 22.2% of all cases show reduced OCT2 expression in the tumor tissue (fold change nontumor/tumor, >2). Patients' characteristics for both cohorts are given in Table 1. For the TCGA ccRCC cohort (KIRC), RNA sequencing data (Illumina HiSeq 2000 RNA sequencing platform) were received from the file TCGA_KIRC_exp_HiSeqV2-2015-02-24, downloaded on 20 October 2015 via the Cancer Genomics Browser. Clinical data were downloaded from the Genomic Data Commons Portal (https://gdc-portal.nci.nih.gov/) on 10 October 2016. (**B**) Localization of OCT2 in nontumor kidney tissue. Representative images of immunostaining for OCT2 using four different antibodies in nontumor kidney tissue. Immunostaining was performed using the following antibodies: KEK [polyclonal rabbit, raised against synthetic peptide corresponding to the C-terminal sequence of human OCT2 (*3*)]; MAB6547 (monoclonal mouse, clone #640438, R&D Systems); AMAb90791 (monoclonal mouse, clone CL0628, Sigma-Aldrich); and AMAb90792 (monoclonal mouse, clone CL0631, Sigma-Aldrich). High- (scale bars, 50 μm) and low-magnification (scale bars, 100 μm) images are shown for each antibody. (**C**) Detection of OCT2 by MAB6547 and KEK antibodies. Immunoblotting of OCT2 using HEK-OCT1, HEK-OCT2, HEK-OCT3, and vector control transfectants. MAB6547 and KEK antibodies only detect OCT2 in HEK-OCT2 transfectants. (**D**) OCT2 expression in ccRCC tissue. OCT2 staining in ccRCC tissue (n = 64 ccRCC cases) using four different antibodies. Representative images (scale bars, 200 μm) are depicted, and staining intensity was quantified using Definiens Tissue Studio 4.3. Expression levels were classified for each antibody into tertiles representing low, medium, and high protein expression. Bar plots show proportions of cases with low, medium, and high protein expression.

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Table 1. Patient demographics and clinical characteristics of the TCGA ccRCC cohort (*n* = 463), as well as our cohort (*n* = 31). T, primary tumor; N, regional lymph node; M, distant metastasis present at diagnosis; G, grading; NA, not available.

		ccRCC TCGA		ccRCC cohort	
		(*n* = 463)		(*n* = 31)	
Variable	**Levels**	***n*, value**	**%**	***n*, value**	**%**
Sex	Male	297	64.1	24	77.4
	Female	166	35.9	7	22.6
Age	Median (range)	61 (26–90)*		62 (44–90)†	
T	T1	226	48.8	9	29
	T2	59	12.7	1	3.2
	T3	168	36.3	21	67.7
	T4	10	2.2	0	0
N	N0	216	46.7	24	77.4
	N1	14	3	4	12.9
	N2	0	0	3	9.7
	NX	233	50.3	0	0
M	M0	374	80.8	21	67.7
	M1	76	16.4	10	32.3
	MX	13	2.8	0	0
G	G1	7	1.5	2	6.5
	G2	200	43.2	18	58
	G3/G4	255	55.1	11	35.5
	GX	1	0.2	0	0
Follow-up time (years)	Median (range)	3.56 (0.005–12.42)		2.25 (0.09–8.56)	
Cancer-specific survival	Alive/ non–cancer-related death	354	76.5	16	51.6
	Cancer-related death	104	22.5	15	48.4
	NA	5	1.1		
Overall survival	Alive	304	65.7	14	45.2
	Deceased	159	34.3	17	54.8

*Age at initial pathologic diagnosis. †Age at surgery.

therapy of decitabine and oxaliplatin based on missing OCT2 expression in ccRCC primary tumors due to *SLC22A2* DNA methylation needs further investigations, including studies with oxaliplatin-sensitive and oxaliplatin-resistant patients.

Notably, we observed in our study (*2*) that several uptake transporters are epigenetically regulated in ccRCC and in RCC cell lines. Moreover, RCC cell lines, including the cell lines Caki-1 and 786-O, that were used to generate the mouse xenografts by Liu *et al.* (*1*), are of limited benefit for the prediction of drug effects because of epigenetic alterations in pharmacogenes, including drug transporters (*2*). Although Liu *et al.* provide comprehensive mechanistic evidence about the epigenetic regulation of OCT2, the demethylating agent decitabine rather induces global demethylation instead of gene-specific demethylation, and we further demonstrated in our study by Winter *et al.* (*2*) that the expression of several of these uptake transporters is increased because of demethylation using decitabine. As a consequence, additional uptake transporters might be responsible for enhanced oxaliplatin uptake after decitabine treatment and need to be considered as well. Moreover, in contrast to Liu *et al.* (*1*), based on our experiments, decitabine also sensitizes the cells to cisplatin, indicating that the effect might depend on the cell lines used. Both cisplatin and oxaliplatin are not exclusively transported by OCT2, but also other transport proteins such as OCT3, or even copper transporter CTR1, are discussed (*9*). Moreover, both drugs are substrates of the multidrug and toxin extrusion protein SLC47A1 (*9*). Therefore, model systems such as patient-derived tumor xenografts (*10*), which reflect more closely the in vivo situation and especially the expression and epigenetic regulation of SLC (solute carrier) transporters, might be more appropriate for designing new therapeutic strategies for ccRCC.

Thus, further studies, including cohorts of oxaliplatin-sensitive and oxaliplatin-resistant patients, are warranted to identify valid biomarkers for optimizing safety and efficacy of a combination therapy of decitabine and platinum drugs in ccRCC.

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Acknowledgments: We gratefully acknowledge U. Waldherr and S. Hübner for the excellent technical assistance. We would like to thank The Cancer Genome Atlas initiative, all tissue donors, and investigators who contributed to the acquisition and analyses of the samples

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used in this study. Information about TCGA and the investigators and institutions that constitute the TCGA research network can be found at http://cancergenome.nih.gov/. **Funding:** This work was supported by the Robert Bosch Foundation. **Author contributions:** P.F., A.T.N., and E.S. acquired, analyzed, and interpreted the data. S.W. and F.B. analyzed the data and performed statistical analyses. A.S., J.B., and M.S. contributed to the interpretation of data and discussion of the present findings. P.F., M.S., and E.S. drafted the manuscript. **Competing interests:** The authors declare that they have no competing interests.

Submitted 20 October 2016
Accepted 7 April 2017
Published 24 May 2017
10.1126/scitranslmed.aal2439

Citation: S. Winter, P. Fisel, F. Büttner, A. T. Nies, A. Stenzl, J. Bedke, M. Schwab, E. Schaeffeler, Comment on "Epigenetic activation of the drug transporter OCT2 sensitizes renal cell carcinoma to oxaliplatin." *Sci. Transl. Med.* **9**, eaal2439 (2017).

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Sci Transl Med **9**, eaal2439.
DOI: 10.1126/scitranslmed.aal2439

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