

Original Paper

Limited Association Between Ascorbate Concentrations and Vitamin C Transporters in Renal Cell Carcinoma Cells and Clinical Samples

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Key Words

Vitamin C • ccRCC • pRCC • SVCT1 • SVCT2

Abstract

Background/Aims: Maintenance of whole-body ascorbate levels and distribution is mediated via sodium-dependent vitamin C transporters (SVCTs). The kidney is one of a few organs that express both SVCT1 and SVCT2. Recent evidence suggests that accumulation of ascorbate may be different in tumour compared to normal tissue, but data on SVCT levels in tumours is sparse. **Methods:** The role of the two SVCT isoforms in ascorbate uptake in renal cell carcinoma (RCC) was investigated *in vitro* and in clinical samples. In three human RCC cell lines, we investigated SVCT protein levels and cellular location in response to ascorbate supplementation and withdrawal. In clinical RCC samples (n=114), SVCT patterns of staining and protein levels were analysed and compared to ascorbate levels. **Results:** In cell culture, transporter levels and cellular location were not modified by ascorbate availability at any time up to 8h, although basal SVCT2 levels governed maximal ascorbate accumulation. In clinical samples, SVCT1 protein levels in papillary RCC (pRCC) were similar to matched normal renal cortex, but were increased in clear-cell RCC (ccRCC). Native SVCT2 (72 kDa) was significantly decreased in both pRCC and ccRCC tissues compared to cortex (p<0.01), whereas a modified form of SVCT2 (100 kDa) was significantly increased (p<0.001). There was no association between the transporters (SVCT1, native or modified SVCT2) and ascorbate concentrations in either normal or tumour tissues. SVCT1 and SVCT2 displayed diffuse cytoplasmic staining in both pRCC and ccRCC tumour cells, with cortex showing distinct membrane staining for

SVCT1. **Conclusion:** We observed a re-distribution of ascorbate transporters in tumour tissue compared to normal cortex and a shift from native to modified SVCT2 in cell culture and clinical samples. Data presented here show that SVCT protein levels do not appear to predict intracellular ascorbate accumulation in RCC.

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Introduction

Maintenance of whole-body ascorbate levels and distribution to different compartments is mediated primarily via sodium-dependent vitamin C transporters (SVCTs) [1]. SVCTs are members of the solute carrier gene family 23 (*SLC23*) [2], with three isoforms identified thus far; SVCT1 and SVCT2 that transport ascorbate, and the orphan transporter SVCT3 with still unknown function [3]. SVCT1 and SVCT2 are each comprised of 12 transmembrane domains and exert the cotransport of sodium and ascorbate in a ratio of 2:1 down an electrochemical sodium gradient which is maintained by K/Na⁺ exchange mechanisms [4]. SVCT2 additionally relies on Ca²⁺ and Mg²⁺ for its activity [4]. Expression of the different SVCT transport proteins is tissue and cell type-specific and is controlled by transcriptional regulation of *SLC23* genes [3, 5] and post-translational regulation [6]. The exact control mechanisms are still not fully understood.

SVCT1 (encoded by *SLC23A1*) is expressed in the epithelial tissue of kidney, intestine, liver, lung and skin. SVCT1 is described as a low affinity, high capacity transporter with a Km in the range of 65–237 μM and Vmax around 15 pmol/min/cell, which makes it capable of efficient uptake of ascorbate from the diet [7]. In comparison, SVCT2, encoded by *SLC23A2*, is expressed in almost every tissue and cell in the body and mediates whole body tissue uptake [5]. SVCT2 is characterised as a low capacity, high affinity transporter with a Vmax ~1 pmol/min/cell and Km of 8–69 μM, that is suited to the maintenance of tissue homeostasis [4, 8].

The kidney plays a major role in the maintenance of whole-body ascorbate levels, with kidney epithelial cells expressing both SVCT isoforms [9]. In the renal cortex, SVCT1 is situated in the brush-border membrane of the proximal tubule where it mediates re-uptake of ascorbate from the glomerular filtrate [8]. Expression increases towards the distal regions of the tubules, and is hypothesised to be regulated by a decreasing ascorbate gradient along the tubular system [10]. SVCT2 is expressed in all cells of the kidney, including the proximal tubular epithelial cells, although at lower levels than SVCT1, and is located intracellularly [9]. Reports of SVCTs in cancer are sparse; only two studies have previously measured ascorbate transporter levels in human tumour tissue [11, 12], and SVCTs have never been investigated in renal cell carcinoma (RCC) tumours.

Worldwide, each year over 270,000 individuals are diagnosed with RCC, which is curable at an early stage but has limited treatment options when diagnosed at advanced stage, resulting in a 5-year survival of <10% [13, 14]. Several histological types for RCC are defined, including clear cell RCC (ccRCC), which is the most aggressive and most frequent (~70%) type, and papillary RCC (pRCC) which is less common (~15%) [15].

Recent evidence suggests that accumulation of ascorbate may differ in tumour compared to normal tissue. We have shown that both ccRCCs and pRCCs contained higher ascorbate levels than normal cortex tissue [16], unlike other cancers that showed the reverse [12, 17, 18]. In the analysis of tissue from patients with colorectal, endometrial or breast cancer, no association between ascorbate levels measured in tumour and matched normal tissue was apparent [12, 16-18]. Protein levels of SVCT1 was measured in human colon adenocarcinoma samples with similar levels to normal colon mucosa [11]. SVCT1 and SVCT2 together with ascorbate were measured in clinical breast tumour tissue, showing no clear association between transporter levels and ascorbate content [12]. No other human studies on SVCTs together with ascorbate in tumour tissue have been reported.

In this study we aimed to investigate the role of the two SVCTs in ascorbate uptake in RCC. SVCT protein levels and cellular location in response to ascorbate supplementation and withdrawal were determined in human ccRCC cell lines. SVCT patterns of staining and

protein levels were also analysed in clinical samples of renal cancer and associated normal renal cortex, and compared to measured tissue ascorbate levels.

Materials and Methods

Materials

All chemicals were obtained from Sigma-Aldrich (St Louis, USA), unless otherwise specified. Cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Bovine serum and antibiotics were from Life Technologies (Carlsbad, CA, USA). The following primary antibodies were used: anti-human SVCT1 (polyclonal rabbit, Aviva Systems, San Diego, CA, USA, OAAB09000), anti-human SVCT2 (polyclonal rabbit, Atlas Antibodies, Stockholm, Sweden, HPA052825) and anti- β -actin (monoclonal mouse, Sigma-Aldrich, A5316).

Renal cell lines

The human ccRCC cell lines Caki-1 (HTB-46), Caki-2 (HTB-47) and 786-0 were used at early passages in ATCC-recommended growth media (modified McCoy's 5A for Caki-1 and Caki-2, Dulbecco's Modified Eagle's Medium (DMEM) for 786-0 cells) with 10% foetal bovine serum and 1% antibiotic-antimycotic solution (Sigma-Aldrich). All cells were regularly tested for mycoplasma by PCR [19].

Ascorbate uptake and measurement

Cells were grown to near confluence in multi-well plates. As growth media contains little or no ascorbate (McCoy's contains 5.4 μ M, DMEM contains 0 μ M), freshly prepared sodium ascorbate was added to a final concentration of 50 or 500 μ M. For measurements of intracellular ascorbate, cells were pelleted at a range of time points and processed for high-performance liquid chromatography with electrochemical detection (HPLC-ECD) analysis, as previously described [20]. Briefly, 0.54 M perchloric acid containing diethylenetriamine penta-acetic acid was added to the cell extract, followed by ascorbate measurements using HPLC-ECD (Thermo Fisher Scientific, Waltham, MA, USA). Ascorbate concentration was assessed relative to standards (freshly prepared ascorbate, 1.25 to 40 μ M).

Patient samples and ethics

Tissue samples, gifted to the Cancer Society Tissue Bank Christchurch (CSTB), were used with ethical approval from the University of Otago Human Ethics committee (reference code H14/020). This cohort of 73 ccRCC and 41 pRCC samples with matched renal cortex has previously been described [16]. In addition, a separate cohort of formalin-fixed, paraffin embedded sections and microarrays (15 pRCC and 63 ccRCC) were received from the CSTB and analysed by immunohistochemistry.

Tissue preparation

Frozen tissue samples were ground to a fine powder in liquid nitrogen, homogenized with RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxyolate, 0.1% SDS, with complete proteinase inhibitor cocktail, Roche, Basel, Switzerland), and DNA content was measured as an indication of the cellular content, as previously described [16].

Antibody blocking

The blocking peptide for SVCT1 (sc-9924 P) resembles amino acids 1 - 30 and the peptide for SVCT2 (sc-31991 P) corresponds to amino acids 183 - 212 of the protein sequence (Santa Cruz, Dallas, TX, USA). For competition assays, antibodies were incubated with 5 times excess of blocking peptides by weight for 1 hour at room temperature before incubation on the membrane.

Western blotting

For cell lines, lysates equivalent to 20 μ g protein, and for tissue, homogenates equivalent to 0.5 μ g DNA, were loaded per well. Proteins were separated on 4 - 12% Bis-Tris Plus SDS gels (Life Technologies, Carlsbad, CA, USA) and transferred to membranes, as described before [16]. Membranes were incubated with the following primary antibodies: anti-SVCT1 (1/1000), SVCT2 (1/500) and β -actin (1/10000),

with horseradish peroxidase labelled secondary goat anti-rabbit/anti-mouse antibodies (Dako, Glostrup, Denmark, P0448 and P0449). Protein bands were detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, USA), captured using the Alliance 4.7 imaging system and quantified using ImageJ.

Immunofluorescence

Cells seeded into 8-well chamber slides (Thermo Fisher Scientific, Waltham, MA, USA) were washed and fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100 and blocked with 1% bovine serum albumin. Transporters were detected using anti-SVCT2 at 1/500 and secondary fluorescent antibody (Donkey anti-rabbit IgG Alexa Fluor 598, 1/1000, Abcam, ab 150076). Cells were co-stained with CytoPainter Phalloiden-iFluor 488 (1/1000, Abcam, ab176753) and 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific). Slides were covered with Vectashield Antifade Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and fluorescence assessed with an Axio Imager 2 using the ApoTome (Zeiss, Oberkochen, Germany).

Immunohistochemistry

Sections cut at 3 μm were baked at 60°C, deparaffinised and pressure heated for antigen retrieval in Tris-EDTA buffer with 0.05% Tween 20. Cell and Tissue Staining kits (R&D Systems, Minneapolis, MN, USA) were used following manufacturer's recommendations to stain for SVCT1 (1/200) and SVCT2 (1/200); negative controls lacked primary antibodies.

Statistical analyses

Data were analysed using GraphPad Prism 5, using the Shapiro-Wilk test for normality. Differences between treatment conditions in cell culture were tested by One-way ANOVA with Dunnett's Multiple Comparison or Bonferroni post-test. Statistical significance between renal cortex and tumour data was tested with the non-parametric Wilcoxon matched-pairs signed rank test. Values of $p < 0.05$ were considered significant.

Results

Ascorbate transporters in renal cell carcinoma cell lines

Specificity of the antibodies against SVCT1 and SVCT2 was determined by pre-absorbing antibodies with blocking peptides that prevent binding to the target epitope [21]. For SVCT1, with a predicted molecular weight of 65 kDa, antibody blocking confirmed that the second immunoreactive band at 80 kDa was non-specific (Fig. 1A). SVCT2 was detected at 72 kDa, its predicted molecular weight, but also at ~ 100 kDa; both bands disappeared in the blocking assay and were therefore considered as specific for SVCT2 (Fig. 1A).

SVCT1 and SVCT2 proteins were confirmed in all three ccRCC cell lines (Fig. 1B). Protein levels of the two transporters varied between cell lines and between individual samples. 786-0 cells appeared to have the highest levels of SVCT1 and lowest levels of both immunoreactive forms of SVCT2 compared to the other two cell lines. Of the three cell lines, Caki-2 cells showed strongest immunoreactivity for SVCT2 (Fig. 1C).

Ascorbate accumulation and loss over time was measured in the ccRCC cells by HPLC-ECD. Our previous data had shown that all three cell lines reached intracellular ascorbate saturation when incubated with 500 μM ascorbate [20]. Therefore, cells were exposed for 16 h to doses of ascorbate that achieve suboptimal (50 μM) or optimal intracellular levels (500 μM), followed by removal of ascorbate from the culture medium with sampling for up to 24 h. Measurements were compared to cells that did not receive ascorbate (0 μM), which had low/undetectable intracellular ascorbate concentrations, as expected (Fig. 2). Intracellular ascorbate levels in all three cell lines increased significantly over time with exposure to both 50 μM and 500 μM ascorbate, and dropped noticeably once ascorbate was withdrawn (dotted lines in Fig. 2). Supplementation with 50 μM ascorbate resulted in a peak of 0.46 nmol/ 10^6 cells at 8 h in 786-0 cells, 1.85 nmol/ 10^6 cells at 16 h in Caki-1, and

Fig. 1. Protein levels of SVCT1 and SVCT2 in ccRCC cell lines *in vitro*. (A) SVCT antibodies were validated using blocking peptides. Antibodies for SVCT1 and SVCT2 were pre-absorbed with blocking peptides before incubation of the membranes. Western blots show Caki-1 cell lysates probed with the respective antibodies with (+) or without (-) blocking peptides. The specific band for SVCT1 was confirmed at ~65 kDa and SVCT2 was identified as a double band at ~72 and 100 kDa (indicated by arrows). (B) Protein levels of SVCT1 and SVCT2 in 786-O, Caki-1 and Caki-2 cells was analysed by Western blotting. β -actin was used as a loading control. (C) Quantification of relative protein levels of SVCT1 and SVCT2 (both size bands) in ccRCC cell lines. Mean \pm SD, n = 3.

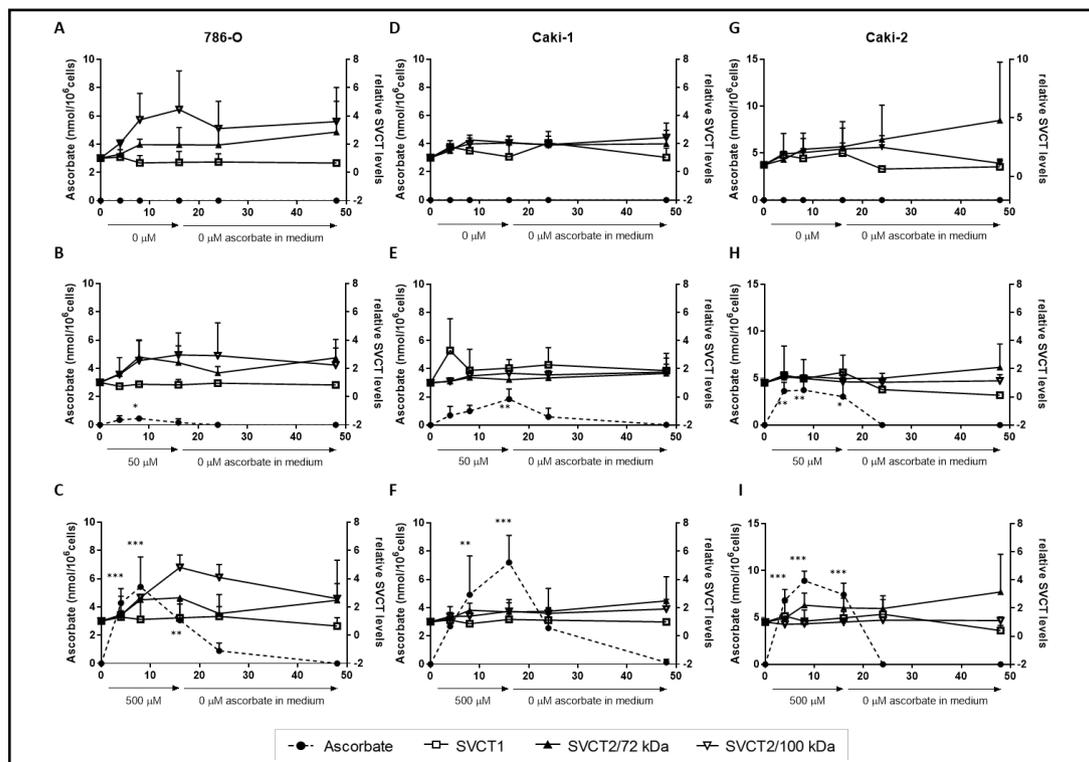
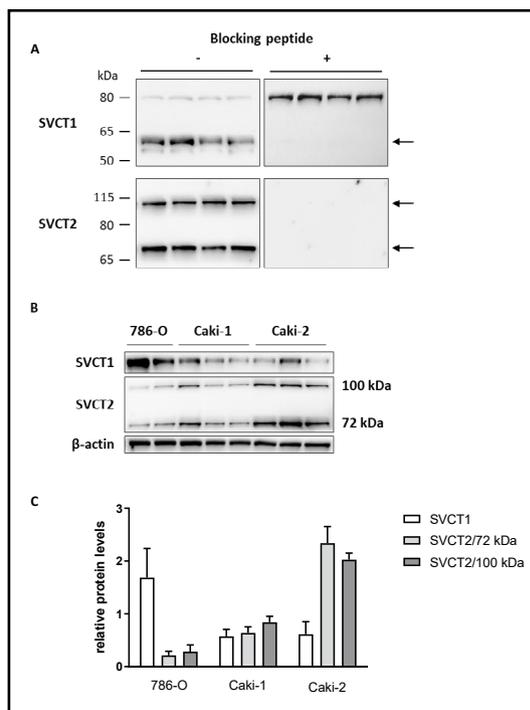


Fig. 2. Levels of ascorbate transporters in response to ascorbate over time in ccRCC cell lines. The ccRCC cells 786-O (A-C), Caki-1 (D-F) and Caki-2 (G-I) were analysed for ascorbate, SVCT1 and SVCT2 (both bands). Cells were treated with 0 μ M (A, D, G), 50 μ M (B, E, H) or 500 μ M (C, F, I) for 16 h and then cultured without ascorbate for 24 h. Ascorbate and protein levels of SVCT1 and SVCT2 was measured at indicated time points. Protein levels are relative to untreated cells at 0 h, and did not change significantly by ascorbate addition or removal. Data are from three independent experiments and are presented as mean \pm SD. Statistical significance was determined by One-way ANOVA with Dunnett's Multiple Comparison Test; * p<0.05, ** p<0.01, *** p<0.001.

3.73 nmol/10⁶ cells at 8 h in Caki-2 cells. Higher supplementation (500 μM ascorbate) resulted in 2-11-fold higher intracellular concentrations at the same time points (5.43, 7.20 and 8.92 nmol/10⁶ cells in 786-0, Caki-1 and Caki-2 cells, respectively).

Changes in transporter levels in response to varying ascorbate supply over time was monitored by Western blot (Fig. 2). Levels of SVCT1 and both immunoreactive forms of SVCT2 were variable over time in culture in the presence and absence of ascorbate. Neither transporter was significantly affected by the addition or removal of ascorbate (Fig. 2), with SVCT1 remaining particularly stable. However, in 786-0 cells, SVCT2 (100 kDa) levels tended to increase during exposure to 500 μM ascorbate and to reduce during ascorbate withdrawal ($p = 0.081$; Fig. 2C).

When comparing SVCT protein levels from Fig. 1C and maximal ascorbate uptake in Fig. 2, it is noteworthy that the cell line (Caki-2) with the highest levels of SVCT2 proteins (72 and 100 kDa) also showed the highest maximal ascorbate accumulation following supplementation with 50 μM and 500 μM ascorbate. The cell line with the lowest levels SVCT2 proteins (786-0) also showed the lowest ascorbate accumulation, with Caki-1 showing intermediate protein and uptake characteristics. No such association was seen for SVCT1.

As there were no clear changes in overall protein levels of ascorbate transporters, possible differences in intracellular SVCT2 distribution were investigated. Immunofluorescence staining of SVCT2 was carried out in the three cell lines over a time period of either 5-60 min (short-term, Fig. 3) or 2-8 h (longer-term, Fig. 4) exposure to 500 μM ascorbate. Immunofluorescence showed immunoreactivity of SVCT2 that appeared to be concentrated in cytoplasmic 'spots' (Fig. 3, Fig. 4). During early time-points (up to 1 h) there appeared to be translocation to the nucleus or nuclear membrane in some cells (eg Caki-1 at 60 min, Fig. 3, Fig. 4). However, there was no clear translocation to or from the plasma membrane at any time over 8h, despite the clear increase in intracellular ascorbate accumulation over this time period (Fig. 2 C, F, I). Co-staining of actin filaments with fluorescence labelled Phalloidin was used to evaluate cell shape. There was no apparent co-localisation of SVCT2 with Phalloidin, indicating a relative lack of SVCT2 at the plasma membrane.

Ascorbate transporters in clinical samples of renal cell carcinoma

Ascorbate transporter levels and location in clinical samples of renal cell carcinoma were investigated, and compared to ascorbate concentrations. SVCT1 protein was detected in both normal and tumour tissue (Fig. 5, Supplementary Fig. 1 – for all supplementary material see www.cellphysiolbiochem.com). Compared to matched normal renal cortex, protein levels of SVCT1 were similar in pRCC tumours, but were marginally increased in ccRCC tumours ($p < 0.05$; Fig. 5A and C).

In contrast, there appeared to be clear differences between SVCT2 levels in normal cortex and RCCs. Similar to *in vitro*, SVCT2 protein was detected as two specific bands; a non-specific band of intermediate size was ignored. The 100 kDa band was considered as a modified form of SVCT2 and the second band at 72 kDa as the native protein (Fig. 5B). Compared to normal cortex tissue there was a significant decrease of native SVCT2 (72 kDa) in pRCC and ccRCC tumour tissues ($p < 0.01$ and $p < 0.001$, respectively; Fig. 5D). However, the modified form of SVCT2 (100 kDa) showed a significant increase in pRCC and ccRCC tumour samples compared to normal tissue ($p < 0.001$, Fig. 5E).

Our previous data had shown that renal tumours had significantly higher ascorbate levels compared to matched cortex tissue (pRCC tumour 12.48 ± 1.13 mg/100 g tissue (1.33 - 31.76 mg/100 g) vs cortex 6.99 ± 0.46 mg/100 g tissue (2.31 - 15.81 mg/100 g); ccRCC tumour 13.15 ± 1.67 mg/100 g tissue (0.9 - 89.1 mg/100 g) vs cortex 7.97 ± 0.47 mg/100 g tissue (1.7 - 18.2 mg/100 g) (mean \pm SE (min-max range)) [16]). However, there was no association between any of the SVCTs (SVCT1, native and modified SVCT2 protein levels) and ascorbate concentrations in either normal or tumour tissues (Table 1). Specifically, despite detecting higher levels of modified SVCT2 in both tumour types compared to normal tissue, there was no association between protein levels of modified SVCT2 and ascorbate levels in tumour tissue in individual patients (Table 1).

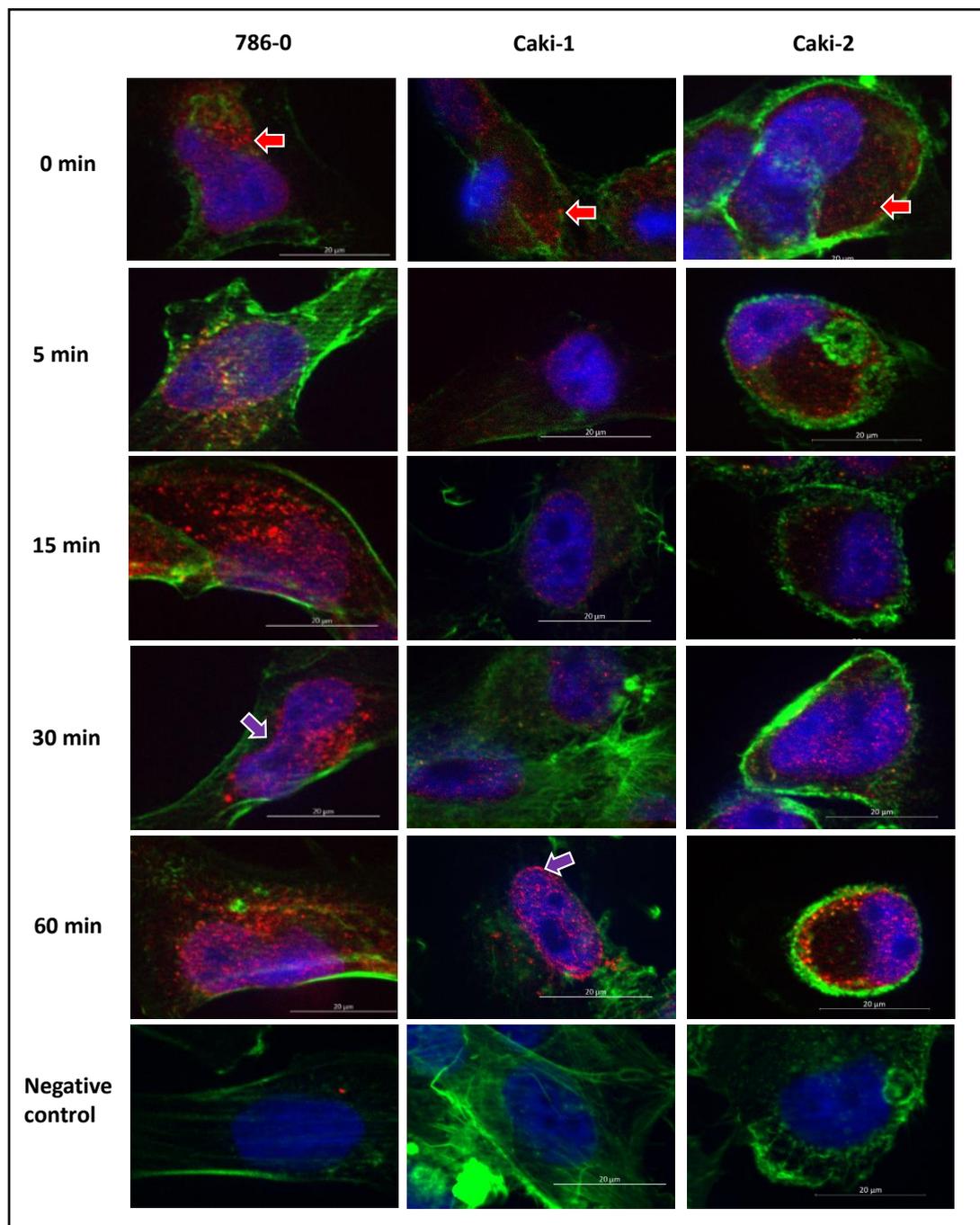


Fig. 3. Localisation of SVCT2 in 786-0, Caki-1 and Caki-2 cells during exposure to ascorbate. Cellular localisation of SVCT2 in response to extracellular ascorbate (500 μ M) was monitored for 5-60 min. Immunofluorescence images for SVCT2 (red) and Phalloidin (green to stain F-actin) of fixed cells were taken at indicated time points and are shown as merged images. Nuclei were visualised with DAPI (blue). Red arrows show examples of cytoplasmic spots, purple arrows illustrate nuclear localisation of SVCT2. 63x objective (oil), scale bar = 20 μ m. Note that photos are enlarged and cropped to show individual cells of interest.

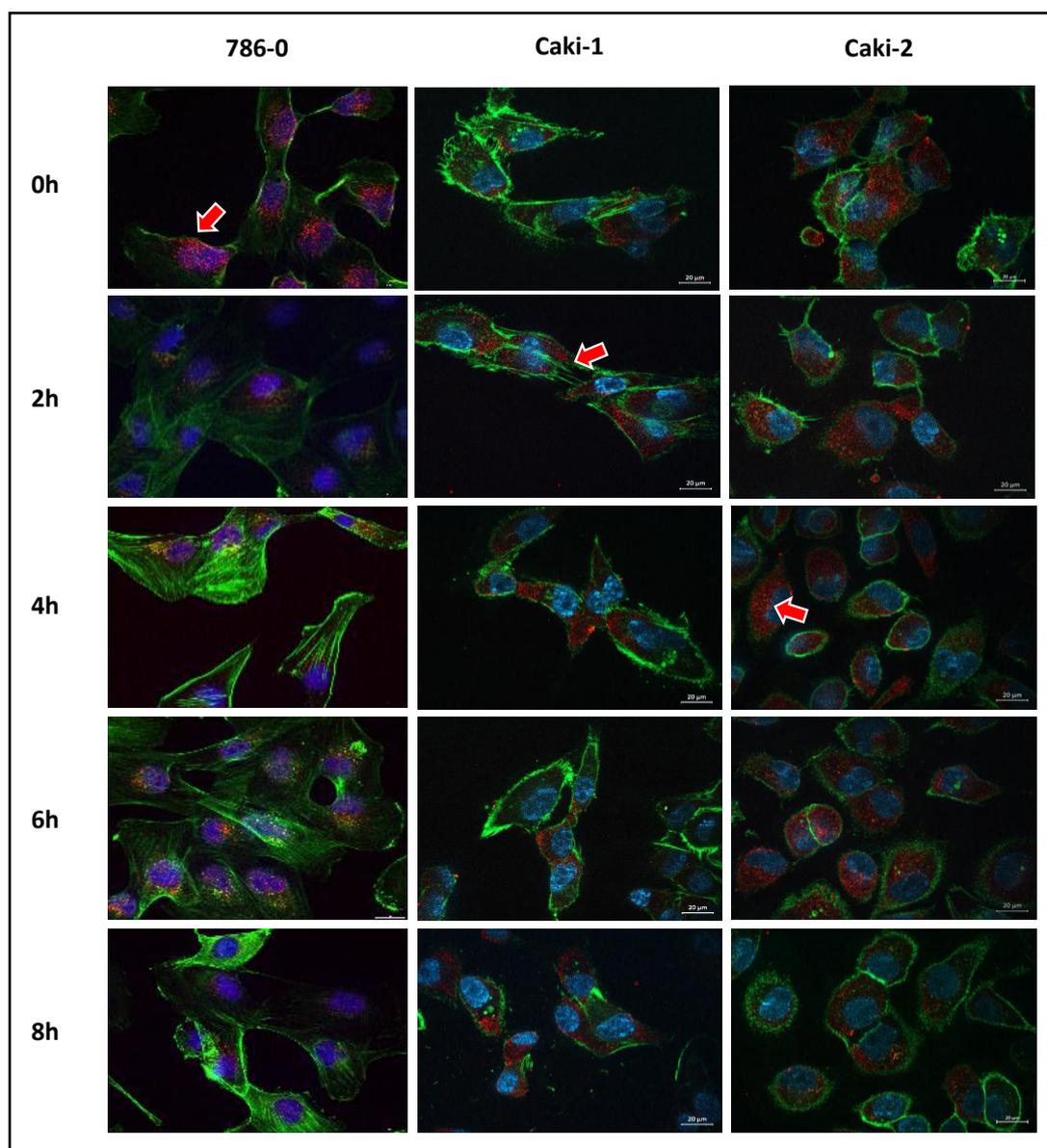
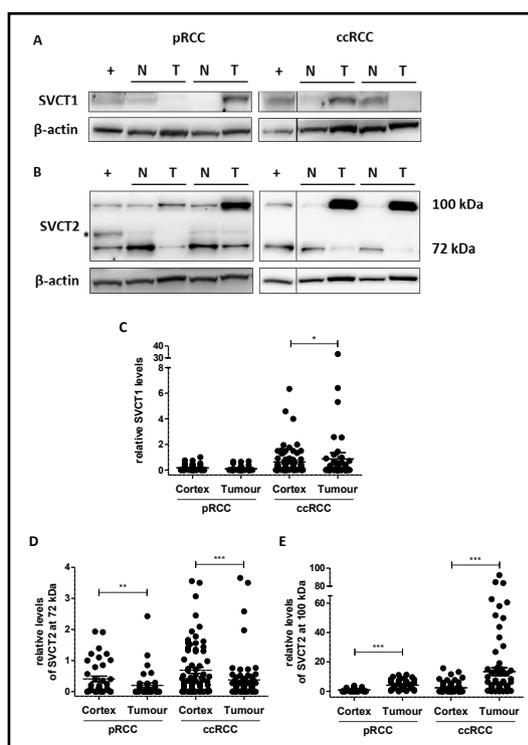


Fig. 4. Localisation of SVCT2 in 786-0, Caki-1 and Caki-2 cells during exposure to ascorbate. Cellular localisation of SVCT2 in response to extracellular ascorbate (500 μ M) was monitored for 2-8 h. Immunofluorescence images for SVCT2 (red) and Phalloidin (green to stain F-actin) of fixed cells were taken at indicated time points and are shown as merged images. Nuclei were visualised with DAPI (blue). Red arrows show examples of cytoplasmic spots, purple arrows illustrate nuclear localisation of SVCT2. 63x objective (oil), scale bar = 20 μ m.

Table 1. Correlations between tissue ascorbate content and ascorbate transporter proteins. RCC renal cell carcinoma, r Spearman correlation, p significance, ascorbate concentrations previously reported [16]

Transporter proteins	Papillary RCC		Clear cell RCC	
	Renal cortex n=38	Tumour n=38	Renal cortex n=71	Tumour n=71
SVCT1	r	-0.028	-0.03	0.051
	p	0.869	0.863	0.671
SVCT2/72 kDa	r	-0.051	-0.213	-0.161
	p	0.761	0.199	0.179
SVCT2/100 kDa	r	-0.002	-0.023	0.089
	p	0.992	0.892	0.458

Fig. 5. Ascorbate transporter levels in RCC tumour tissue compared to renal cortex. Representative Western blots for normal renal cortex (N) and tumour (T) tissue from papillary RCC and clear cell RCC for SVCT1 (A) and SVCT2 (B) are shown together with β -actin as a loading control. Relative protein levels are band densities referenced to a control sample which was loaded on each gel (+, pRCC tumour sample). Non-specific bands are marked with an asterisk. (C) Levels of SVCT1 were unchanged in pRCC and increased in ccRCC tumours compared to normal renal cortex. (D) In both RCC subtypes, levels of the 72 kDa form of SVCT2 were significantly decreased in tumour compared to normal tissue. (E) The 100 kDa form of SVCT2 was increased in both pRCC and ccRCC tumour tissue. Statistical significance was assessed with the Wilcoxon matched-pairs signed rank test; pRCC n = 38; ccRCC n=71; Mean \pm SEM; * p<0.05, ** p<0.01, *** p<0.001.



SVCT location within renal tissue was investigated using tissue microarrays of 15 pRCC and 63 ccRCC cores, as well as full sections of normal renal cortex from two patients without kidney cancer (Fig. 6, Supplementary Fig. 2). In tumour cores, SVCT1 displayed diffuse cytoplasmic staining in both pRCC and ccRCC cells (Fig. 6), with 88% of pRCC and 85% of ccRCC cores staining positive. In contrast, in the normal renal cortex, SVCT1 showed strong apical membrane staining of the proximal tubular epithelial cells but no SVCT1 staining in distal tubules or corpuscles (Fig. 6). As previously described, proximal tubules in normal tissue displayed non-specific staining due to endogenous biotin and peroxidase activity [22, 23], which was not evident in tumour cores. Immunoreactivity of SVCT2 was detected in all tumour cores and throughout the normal cortex, with diffuse cytoplasmic staining (Fig. 6). Both transporters were also detected in infiltrating immune cells (Fig. 6). As ascorbate content was not available for most tumours of this TMA cohort, associations between staining pattern and ascorbate could not be performed.

Discussion

We report levels and localisation of ascorbate transporters and their correlation to ascorbate concentrations in renal cell carcinoma cell lines and clinical samples. Although RCC cell lines with lower basal levels of SVCT2 showed lower ascorbate accumulation and the cell line with higher SVCT2 levels had higher ascorbate accumulation, there was no clear association between changes in ascorbate concentrations and protein levels of SVCT1 and SVCT2 in cell lines *in vitro*. Neither did we observe clear associations between transporter protein levels and ascorbate in clinical tumour samples. SVCT1 protein was marginally higher in ccRCC compared to normal renal cortex, whereas native SVCT2 protein was lower. The modified SVCT2 protein was significantly higher in both pRCC and ccRCC compared to normal cortex tissue.

Reports on the effect of ascorbate supplementation on changes in SVCT expression are conflicting. In the human bronchial epithelium, SVCT2 protein levels inversely correlate

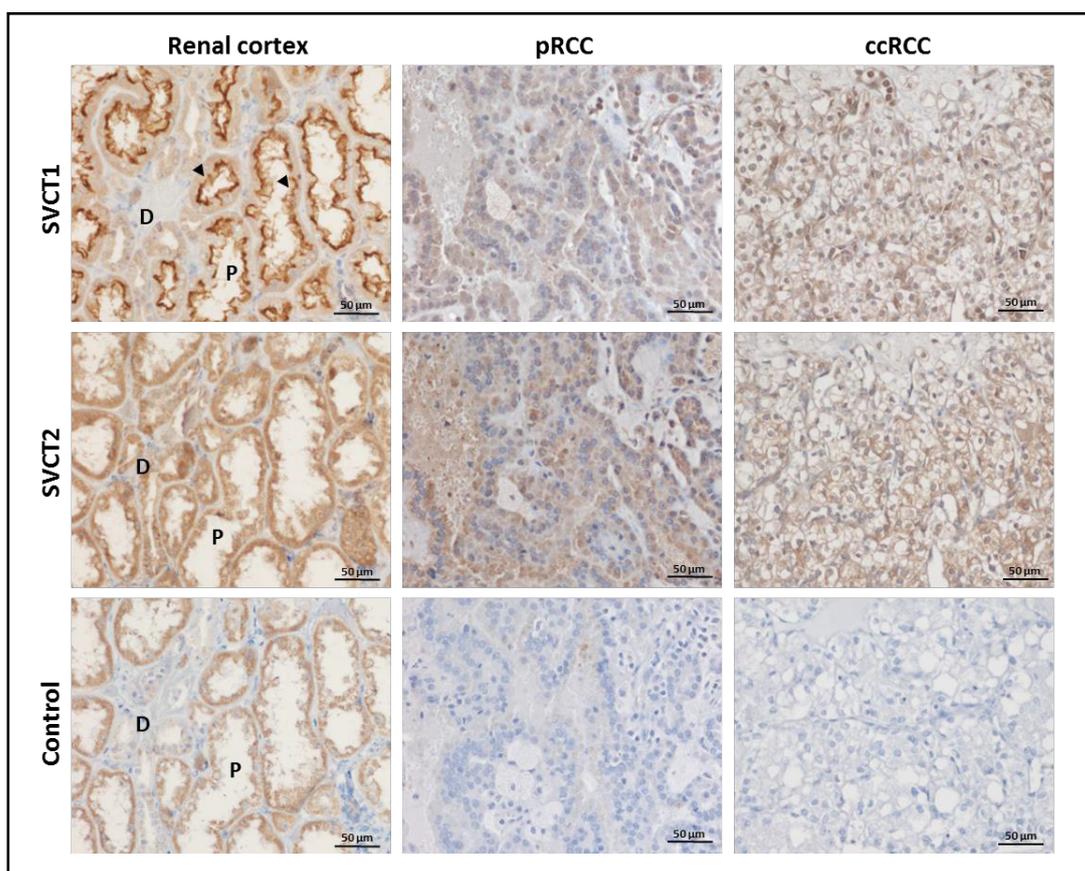


Fig. 6. Immunohistochemical detection of ascorbate transporters in renal cortex and RCC tumour tissue. Tissue sections of human renal cortex, pRCC and ccRCC tumours were analysed for ascorbate transporter distribution by immunohistochemistry. In normal renal cortex tissue, SVCT1 was localised to the apical membrane of proximal tubular epithelial cells (P; indicated with arrowheads) and was absent in distal tubules (D). In pRCC and ccRCC tissue, staining of SVCT1 was cytoplasmic. Immunoreactivity of SVCT2 was seen in the cytoplasm of both cortex and tumour tissue. The negative control (no primary antibody) of renal cortex showed marked unspecific background staining that was especially strong in proximal tubular cells. No staining was seen in the negative controls of tumour tissue. Representative photos for SVCT1, SVCT2 and control are taken from serial sections of the same location; 20x objective, scale bar = 50 µm.

with ascorbate concentration in the respiratory tract lining fluid [24]. Long-term exposure of human skin fibroblasts to ascorbate increased mRNA expression of SVCT2 [25], although protein was not measured. On the other hand, in human colon carcinoma cells, high concentrations of ascorbate down-regulated SVCT1 mRNA expression *in vitro* [26]. A pharmacokinetic study in tumour-bearing Gulo(-/-) mice has shown that in response to a single high dose administration of ascorbate, there was a prolonged presence of ascorbate in the tumour compared to plasma and liver, where ascorbate levels reduced rapidly [27]. In these mouse tumours, protein levels of SVCT1 were low with little variation following ascorbate administration, whereas SVCT2 protein levels varied over time with some evidence of increase with increasing ascorbate over time [27]. Gene expression of SVCT1 and SVCT2 is regulated by transcription factors, such as NF- κ B [28] and suppressed by p53 [29], and via different epigenetic mechanisms (DNA methylation and histone modifications) [30]. In the ccRCC cell lines used in this study, protein levels of both transporter isoforms were highly variable and largely independent of intra- and extracellular ascorbate. Only in 786-0 cells (mutant p53) did the levels of SVCT2 (100 kDa) tend to be elevated during exposure to ascorbate. Ascorbate has been shown to induce activity of the ten-eleven translocase

demethylase enzymes leading to DNA demethylation [31], which could potentially be involved in epigenetic regulation of the expression of transporters in these cells.

Different molecular weights have been reported for both SVCT isoforms, and in our study, blocking peptides confirmed SVCT1 as a 65 kDa protein and SVCT2 as two specific bands of 72 kDa and 100 kDa. This size shift for SVCT2 can potentially be explained by N-glycosylation adding multiple sugar chains to the transporter [32]. Glycosylation is known to be necessary for function and trafficking of the transporter between cellular compartments [32-34]. A shift of more than 20 kDa in molecular weight due to glycosylation is unusual, but a similar band shift for human SVCT2 has been reported before, which could be reversed with a glycosylation inhibitor [33]. Glycosylation of SVCT1 has also been reported [33], but was not observed in our study. Alternatively, ascorbate transporters are also regulated via post-translational phosphorylation [6, 32], but glycosylation is the more likely reason for the observed SVCT2 100 kDa species due to the magnitude of the shift, but this needs to be confirmed.

Subcellular localisation of the transporters in non-cancerous cells has been intensely studied. SVCT2 protein is localised in mitochondria, intracellular vesicles, the endoplasmic reticulum and the Golgi apparatus [35-37], and has different kinetic properties depending on its location [35]. Mitochondrial SVCT2 has been proposed to function as a low-affinity transporter due to differences in intracellular sodium and potassium concentrations, enabling transport into intracellular organelles [37]. Transporters may normally reside elsewhere but rapidly traffic to the membrane upon stimulation. In neurons in culture, SVCT2 translocated from the cytoplasm to the plasma membrane as extracellular ascorbate concentration increased [36]. Based on data from cancer (HeLa) and non-cancer cells (HEK293), the same group suggested that there are two responses to ascorbate: an acute response within 5 - 10 minutes, leading to recycling of transporters that are in close proximity to the membrane through endocytosis; and a post-acute response at 30 - 60 minutes, where glycosylated SVCT2 is transported from the endoplasmic reticulum to the plasma membrane [34]. We did not see clear changes in compartmentalisation of SVCT2 in ccRCC cells upon ascorbate addition, even at the early time points, with the exception of a potential shift towards the nucleus or nuclear membrane. The *in vitro* staining patterns observed in our study are largely consistent with predominant localisation of the SVCTs in vesicles or mitochondria, with relatively lower levels of expression at the plasma membrane. These intracellular locations may be associated with known functional requirements, such as the support of transcription factor activity and epigenetic regulation [31, 38].

The kidney has a unique distribution of ascorbate transporters, with SVCT1 situated in the brush-border membrane of proximal tubule cells mediating renal reabsorption, and with SVCT2 expressed basolaterally in all cells of the kidney except the proximal tubule [8, 39, 40]. In our clinical tissues, SVCT1 showed membrane staining of the proximal but not distal tubular epithelial cells in the normal renal cortex, as expected. However, no membrane staining for SVCT1 was seen in either pRCC or ccRCC tumours, where diffuse cytoplasmic staining was evident. We found cytoplasmic localisation of SVCT2 in epithelial cells of normal renal cortex including the proximal tubule, contrasting previous reports [39, 41]. In the tumour samples, SVCT2 was not located at the cell membrane, but rather in cytoplasmic aggregates or diffusely in the cytoplasm. This observation is in line with the above-mentioned *in vitro* studies [34-37]. Overall, these data suggest that ascorbate can be variously compartmentalised within the cell, and SVCT distribution may differ between non-cancerous and cancer cells.

Availability of ascorbate to tumour cells is often limited due to decreased plasma levels in patients with cancer [42-44] and inadequate tissue vascularisation and poor perfusion [45]. The fact that renal tumours had increased ascorbate levels [16] may indicate an increase in active ascorbate transport, and we measured higher levels of the modified SVCT2 (100 kDa). This observed shift from an unmodified to a modified form of SVCT2 (potentially glycosylated and more active), together with re-distribution of ascorbate transporters in tumour tissue compared to normal cortex, and a shift from SVCT1 to SVCT2, could result

in altered transporter function and ascorbate uptake dynamics in RCC. These changes may influence the observed increased ascorbate content in tumour compared to normal tissue. However, at the individual patient level, transporter levels did not correlate with ascorbate levels in tumour or normal tissues. As dietary ascorbate intake in this patient cohort was not known, and neither was ascorbate content of their plasma, this lack of correlation remains to be further explored. We observed that ccRCCs had high numbers of infiltrating immune cells, confirming previous studies [46, 47], which could result in elevated ascorbate measurements of whole tissue lysates of some tumours as immune cells can concentrate up to 100-times more ascorbate compared to plasma levels [48, 49].

Alternative options for ascorbate accumulation have been proposed. The oxidised form of ascorbate, dehydroascorbate (DHA), can be accumulated through facilitated diffusion via glucose transporters in competition with glucose [50, 51]. Once in the cell, DHA gets rapidly reduced to ascorbate by dehydroascorbate reductase. However, under physiological conditions, at neutral pH, DHA rapidly hydrolyses to diketogulonic acid and is subsequently lost [5, 52]. Therefore, DHA levels in plasma are very low or undetectable (<10% of total ascorbate) in correctly handled samples [53]. Our previous studies showed no association between glucose transporter 1 with ascorbate concentrations in either clinical RCC samples or cell lines *in vitro* [16, 20].

Increasingly patients with cancer choose to receive high-dose ascorbate infusions [54], often outside of regular clinical care and oversight. Outcomes are not monitored and there is still no robust evidence to support this practice. However, the newly discovered functions of ascorbate that include a proposed prooxidant activity and enzyme cofactor function have demonstrated potential anticancer activities [38, 55, 56]. Current clinical trials are evaluating high-dose ascorbate in combination with anticancer agents in ccRCC, with no outcomes yet reported [57]. Our data here demonstrates that RCCs accumulate ascorbate at high levels with dietary intake and that SVCT levels were not modified by ascorbate supply *in vitro*, but how high-dose infusion would modify tumour levels is unknown.

Conclusion

In summary, our data indicate that SVCT isoforms and protein modifications may differ between tumour and normal renal tissue. The SVCTs appear to be predominantly located at intracellular sites, and expression levels do not change appreciably in the presence or absence of ascorbate. Hence there is not a simple relationship between tissue ascorbate content and SVCT levels, and our data indicate that SVCT protein levels do not predict intracellular ascorbate accumulation in RCC. Also, ascorbate supply may not modify SVCT protein levels and there may be complex dynamic changes in sub-cellular localisation of the transporter, but any functional impact of such changes in renal cancer cells is unknown.

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Author Contributions

GD conceived the study, CW, EB and MN collected the data, MV and EP helped analyse the data, and CW composed the draft manuscript. MV, BR and GD edited and refined the manuscript. All authors finalized the manuscript. GD, MV and BR obtained funding for the study.

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Statement of Ethics

Human tissue samples were collected by the Cancer Society Tissue Bank (CSTB) Christchurch and used with ethical approval from the University of Otago Human Ethics committee (reference code H14/020). Use of samples for this study was approved by the CSTB board. All CSTB donors gave informed written consent for the use of their samples for research.

Disclosure Statement

The authors have no conflicts of interest to declare.

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