

Size-based isolation and detection of renal carcinoma cells from whole blood

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Abstract. Renal cell carcinoma (RCC) is a tumour type with an indolent growth pattern and rather vague symptoms. The present study developed a platform for liquid biopsy of RCC based upon the isolation of circulating tumour cells (CTCs). Founded on the observation that RCC tumour cells are considerably larger than leucocytes, the present study employed a microfluidics-based system for isolation of RCC CTCs from whole blood. Using this system, it was revealed that 66% of spiked-in RCC tumour cells could be retrieved using this approach. Furthermore, it was demonstrated that these cells could be molecularly detected with digital PCR using RCC-specific genes down to one tumour cell, whilst avoiding detection in samples lacking tumour cells. Finally, subtype specific transcripts were identified to distinguish the different subtypes of RCC, which were then validated in patient tumours. The present study established a novel workflow for the isolation of RCC CTCs from whole blood, with the potential to detect these cells irrespective of subtype.

Introduction

Recently, liquid biopsies have garnered attention for their applications in personalised medicine. A liquid biopsy, as opposed to a tissue biopsy, requires that the analytical sample is derived from the bodily fluid of a patient by a minimally invasive method. This includes but is not limited to peripheral-vein blood, saliva, urine and cerebrospinal fluid. When considering blood as a bio-source there are potential

components that can be considered such as circulating tumour cells (CTCs), platelets or plasma as a source of circulating tumour (ct) nucleic acids (1). The choice of component used in a liquid biopsy assay will depend on the disease being queried, the potential for gathering tumour derived information and downstream applications.

Circulating tumour cells shed into systemic circulation by the primary tumour or metastases (2). Tumour cells enter the circulation via intravasation and typically an epithelial-mesenchymal transition as part of the metastatic cascade. However, CTCs may also be found in clusters in the circulation (3). CTCs and/or circulating tumour cell clusters can be isolated from blood in systemic circulation at which point their DNA, RNA, protein expression or functionality can be studied (4). If efficiently isolated, CTCs are rich sources for multi-omic analyses that may not only aid in assessment of disease or disease progression but also in understanding aspects of tumour processes.

The majority (>90%) of solid lesions found in the kidney are renal cell carcinomas (5) and patients with the disease may benefit from the advancement of liquid biopsy based molecular detection (6). More than 60% of RCC patients are asymptomatic, and often radiologic diagnoses are made incidentally (5,7). 20% of these cases are metastatic at diagnosis (8,9). For local disease, surgical resection is performed, however, 30% of these patients will experience recurrence within 5 years after surgery (9,10). Despite these circumstances, currently there are no cost-effective or practical approaches to detecting early metastases or monitoring recurrence (11).

The three most common RCC subtypes are clear cell RCC (ccRCC), papillary RCC (p1RCC & p2RCC) and chromophobe RCC (chRCC) accounting for 75-80%, 15% and 5% of cases, respectively (12,13). These tumour entities arise along the nephron with ccRCC and pRCC originating from cells of the proximal tubules and chRCC from the collecting duct. As a consequence, they are transcriptionally distinct, with ccRCCs and pRCCs retaining much of the HNF-driven transcriptional program found in the proximal tubules and chRCC retaining the FOXI1-driven programme defining collecting duct cells (12). Additionally, almost all ccRCC tumours lack a functional VHL protein, rendering them pseudo-hypoxic due to the

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accumulation of hypoxia inducible factor (HIF) proteins (14). These transcriptional differences between the subtypes set the stage for liquid biopsy approaches that could allow for subtype specific monitoring of RCC tumours.

With regards to blood based liquid biopsies, it has been observed that RCC is a poor shedder of circulating tumour DNA (ctDNA) and hence may be better suited for CTC analyses (15). Importantly, RCC cells are not suitable for the commonly used and FDA approved EpCAM based isolation strategies due to their poor expression of this surface marker, despite being of epithelial origin (16,17). Antigen-dependent enrichment methods, such as the use of the surface marker CA9 in ccRCC, has been previously employed to successfully detect CTCs (18,19). However, this type of enrichment is limited to one subtype or population of RCC CTCs. In order to broadly enrich CTCs from a wider range of RCC subtypes, an antigen-independent approach is desirable such as cell size-based enrichment of CTCs (16,20). We have addressed this need by employing the ClearCell FX platform which uses a size-based, microfluidic enrichment approach. This platform enriches fully intact and viable CTCs directly from whole blood by exploiting the biophysical disparities between CTCs and other cells found in the blood through the Dean Flow Fractionation principle (21). Cells travelling through a spiral microfluidic channel experience counter rotating flow vortices (collectively called a Dean vortex), which channel larger cells towards the inner wall of the channel and smaller cells towards the outer wall. In addition to this, there are cell diameter dependent wall-induced and shear-induced lift forces which further focus the cells along opposing inner wall of the channel. Direct enrichment in buffer is beneficial, compared to the Parsortix system for example, where cells are enriched inside a cassette and washing out these cells may pose a risk of cell loss (22). Enriched, intact CTCs on a limited background of leucocytes within this buffer can then be processed downstream for applications such as gene expression querying. We have evaluated the performance of this platform in relation to enriching RCC cells and then explored and optimized methods for specifically detecting these cells through the use of subtype specific biomarkers.

Materials and methods

Primary patient tissue, primary cells, cell culture and healthy blood controls. A total of 16 primary RCC patient tumour samples (Table I) and 5 primary RCC cell were kindly provided by Dr Helén Nilsson, Department of Translational Medicine, Lund University for validation of selected biomarkers. Patient tumour samples were retrieved from the material collection established after Lund University ethical committee approval (approval no. LU680-08), where informed written consent was obtained before archival. Human renal tissue dissociation and culturing was performed as described in Appendix S1 and previously described in Hansson *et al.* (23). Tumour tissues obtained consisted of ccRCC, p1RCC, p2RCC and chRCC tissues and cells that were predetermined by a trained pathologist. ccRCC cell lines SNU-349 (Korean Cell Line Bank) and 786-O (ATCC) were cultured in RPMI-1640 (Corning, Manassas, USA) and DMEM (Corning, Manassas, USA) respectively, both medium supplemented with 10% foetal

bovine serum (Gibco, MA, USA). All cell lines were incubated in a humidified chamber in 5% CO₂ at 37°C, were STR authenticated, had a passage number of not more than 20 and were mycoplasma free. Healthy blood for controls were obtained from the Blood donation centre in Lund with informed written consent from patients and ethical permission obtained by the regional health care provider (Region Skåne, Lund, Sweden; approval no. 2018:19).

Identification of RCC subtype specific marker genes. CA9 and SLC6A3 were selected as ccRCC subtype specific markers based on a literature search and previously published data from Hansson *et al.* (23). The subtype specific expression of these markers was confirmed in the TCGA data set as described below. The same approach was also used to identify subtype specific markers for the other two subtypes, pRCC and chRCC. Level 3 RNA-sequencing data processed using the 'UNC V2 RNA-Seq' workflow was downloaded from The Cancer Genome Atlas (TCGA) data portal (<https://portal.gdc.cancer.gov/>), as described in Lindgren *et al.* (12). Statistical analyses were performed using the R software (<http://www.r-project.org>). Differential gene expression between clear cell, papillary and chromophobe RCC (KIRC, KIRP and KICH, respectively) tumours was determined using the Limma Bioconductor package (24). For the analysis of taxonomy groups, samples from all 3 TCGA kidney cancer projects [chromophobe RCC (KICH), clear cell (KIRC), papillary kidney carcinoma (KIRP)] were merged into one single dataset (25). Molecular RCC taxonomy groups as well as clinical and histopathologic parameters were used as presented in the article by Chen *et al.* (26). The selection criteria for sub-type specific expression were minimal expression in human blood (based on the blood datasets found in the Human Protein Atlas, available from <https://www.proteinatlas.org>) and distinct RCC sub-type specific expression. For the two types of papillary RCC defined by Chen *et al.* (26) (p.e1 and p.e2) optimal markers for the more common p.e1 subtype (but also displaying elevated expression in p.e2 tumours) were selected (Table SI). These selection criteria resulted in a panel of 7 genes (Table II).

Verification of subtype specific gene expression. cDNA synthesis for cell lines and primary tumours was performed using the High-Capacity RNA-to-cDNA kit. Quantitative PCR (qPCR) was performed on a QuantStudio 7 Flex Real-Time PCR system using TaqMan Gene Expression Master Mix and TaqMan Probes (Table III) (all from Applied Biosystems, Vilnius, Lithuania). Relative gene expression from qPCR data was calculated using the double delta C_q method and normalised to β -actin levels (27).

Cell size measurements. Cell diameter of RCC cell lines and primary cell lines were measured on a Nucleocounter NC-3000 (ChemoMetec, Allerød, Denmark) using NC-Slide A8 with the Cell Viability and Cell Count Assay, according to manufacturer's instructions.

Establishment of ClearCell FX system performance for RCC cells. Whole blood (7.5 ml) was processed for each run on the ClearCell FX system (Biolidics, Singapore). Firstly, red blood cells (RBCs) were lysed by a 10-minute

Table I. Patient tumour characteristics for biomarker panel validation.

Patient ID	RCC subtype	Fuhrman grade	Stage
R294T	ccRCC	F2	pT1a
R320T	ccRCC	F1	pT1b
R375T	ccRCC	F3(I)	pT3a
R363T	ccRCC	F3(I)	pT3a
R256T	chRCC	F3	pT3a
R308T	chRCC	-	pT1a
R377T	chRCC	-	pT1a
R275T	chRCC	F3(I)	F3 (I)
R221T	p1RCC	F3	pT2b
R163T	p1RCC	F2	pT1b
R376T	p1RCC	F2(I)	pT1b
R188T	p1RCC	F1	pT3a
R290T	p2RCC	F4	pT3b
R303T	p2RCC	F2(I)	pT1b
R321T	p2RCC	F2(I)	pT1b
R199T	p2RCC	F4	pT3a

ccRCC, clear cell renal cell carcinoma; chRCC, chromophobe RCC; p1RCC, papillary type 1 RCC; p2RCC, papillary type 2 RCC. (I) denotes tumour grading according to 2016 WHO/ISUP system (39).

incubation with a RBC lysis buffer (G-Bioscience, St. Louis, MO, USA) and discarded via centrifugation and removal of supernatant. The resulting cell pellet containing nucleated cells was resuspended in 4 ml of ClearCell FX re-suspension buffer prior to being loaded onto the ClearCell FX system and running 'Protocol 1'. Renal cancer cell line SNU-349 and primary RCC cells were used to establish the recovery efficiency of the ClearCell FX tumour cell isolation system for RCC cells. Cells were labelled by incubating in serum free media (Corning, Manassas, USA) with a final concentration of 25 μ M CellTracker Green CMFDA (Invitrogen, Carlsbad, USA) dye for 45 min. Cells were then washed with DPBS and diluted to varying numbers before being counted and spiked into 7.5 ml of healthy donor blood. The spiked blood sample was then processed and run through the ClearCell FX system according to manufacturer's instructions. The isolated output cells were then pelleted, resuspended in a lower volume and aliquoted into a 96-well plate. Labelled and isolated cells were counted using an inverted immunofluorescence microscope to calculate the recovery efficiency.

RNA extraction, global-preamplification and cDNA clean-up. RNA extraction for ClearCell FX output samples were performed according to manufacturer's instructions using the Norgen Biotek Single Cell RNA Purification kit (Norgen Biotek Corp., Thorold, Canada) and RNA was eluted in 14 μ l of PCR clean water. Global pre-amplification of RNA and cDNA clean-up was performed on RNA extracts from ClearCell FX system outputs using the SMART-Seq v4 Ultra Low Input RNA kit (Takara Bio Inc., Shiga, Japan) according to

Table II. Biomarkers selected to distinguish RCC subtypes.

RCC subtype	Selected markers
Clear Cell RCC	<i>SLC6A3, CA9</i>
Papillary RCC	<i>SOSTDC1, SLC34A2, LRRN4</i>
Chromophobe RCC	<i>SLC26A7, ATP6V0A4</i>

Table III. TaqMan probes used for subtype specific markers.

Gene name	TaqMan probe
<i>CA9</i>	Hs00154208_m1
<i>SLC6A3</i>	Hs00997374_m1
<i>SOSTDC1</i>	Hs00383602_m1
<i>SLC34A2</i>	Hs00197519_m1
<i>LRRN4</i>	Hs00379905_m1
<i>SLC26A7</i>	Hs01104163_m1
<i>ATP6V0A4</i>	Hs00220986_m1

manufacturer instructions. Pre-amplified cDNA was purified using AMPure XP magnetic bead solution (Beckman Coulter, USA) and eluted in 20 μ l TE buffer. RNA concentration, RNA integrity number and pre-amplified cDNA concentration from ClearCell FX system outputs was assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Primary patient tumour tissue (<30 mg) was processed by disruption in a TissueLyser using Trizol (Qiagen, Hilden, Germany). Homogenization was achieved by flushing the disrupted sample through a QIAshredder column. Subsequent RNA extraction was performed using the Qiagen RNeasy Mini (Qiagen, Hilden, Germany) kit according to manufacturer's instructions. RNA concentration and quality from cultured cells and primary tumour material was assessed on a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, USA).

Real-time monitoring of global pre-amplification. Global pre-amplification was monitored in real time to obtain the optimal cycle number by adding 0.1X SYBR Green (Sigma Aldrich, Darmstadt, Germany) and allowing the reaction to run for 40 cycles (28).

Statistical analyses. Mann-Whitney U, mean and SEM calculations were performed on GraphPad Prism software (Graph Pad Software, San Diego, USA). Kruskal-Wallis with Dunn's comparison calculations were performed on R-software (Vienna, Austria) and GraphPad Prism.

Results

Tumour cell isolation strategy. In this study we set out to develop a method for efficient isolation of CTCs from RCC patient blood. Previous studies have indicated that label-free, size-based enrichment is particularly well suited for RCC, which led us to employ the size-based ClearCell FX system for our study (16,29).

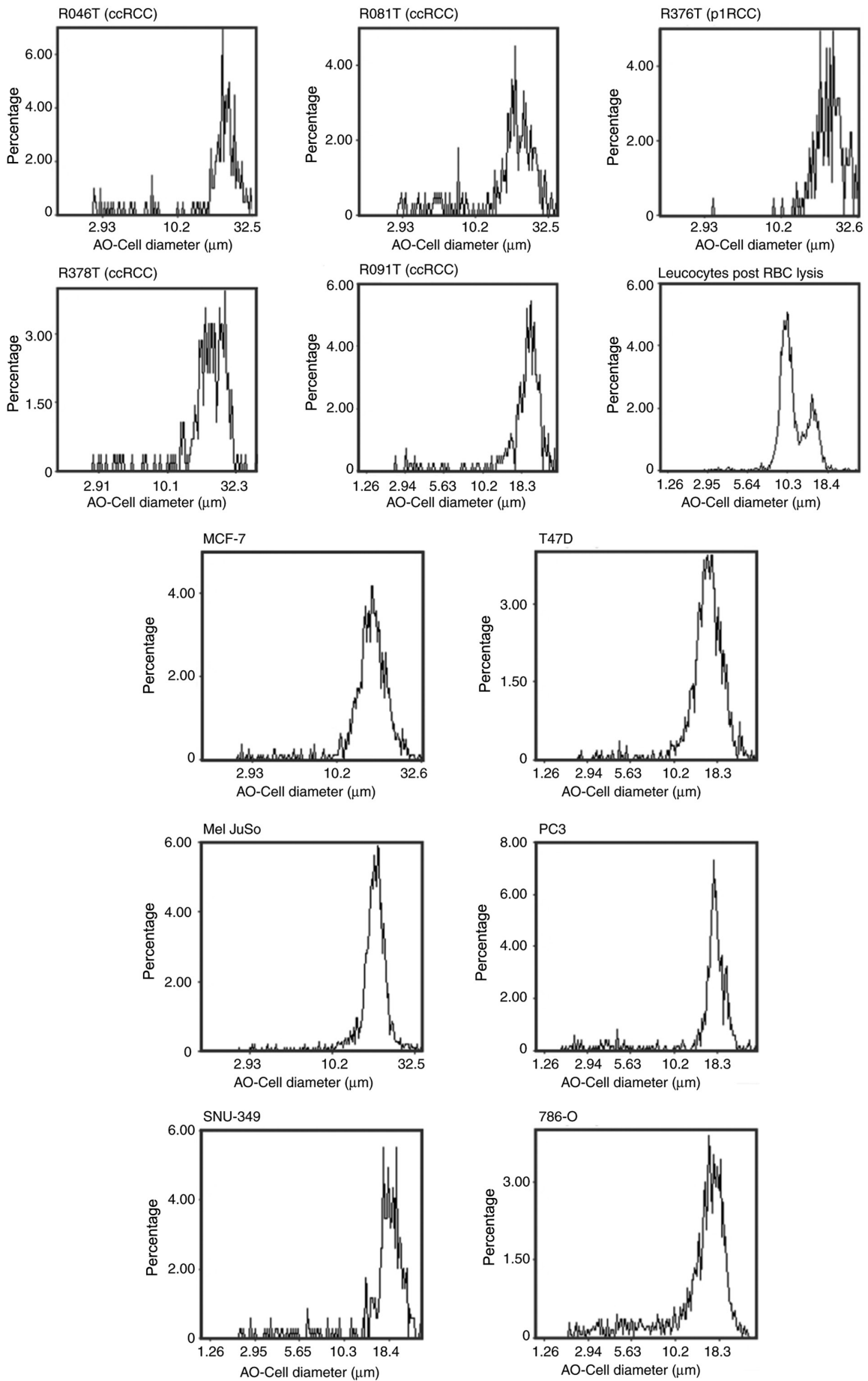


Figure 1. Cell diameters of primary RCC, cultured RCC and non-RCC cells. AO, Acridine Orange; ccRCC, clear cell renal cell carcinoma; p1RCC, papillary type 1 RCC; RBC, red blood cell lysis.

In order to confirm that RCC tumour cells were large enough to be successfully enriched in a size-dependent system, we measured the cell diameters of two ccRCC cell lines along with 5 primary cell lines (ccRCC and pRCC). This data showed that cultured RCC tumour cells SNU-349 and 786-O are approximately 18 μm in diameter whilst cultured primary tumour cells are larger and measure between 20-30 μm (Fig. 1). These values are greater than the lower ClearCell FX isolation size threshold of 14 μm suggesting that our choice of enrichment method is likely suitable for isolation of CTCs from RCC patients. A small fraction of leucocytes from whole blood are larger than 14 μm , these are also presumably enriched and isolated together with the tumour cells. Furthermore, we measured the cell diameter of four non-RCC tumour cell lines (MCF-7, T47D, Mel JuSo, PC3) to confirm that tumour cells in general, and RCC cells in particular, are larger than most leucocytes.

ClearCell FX system performance on RCC cells. To establish the recovery efficiency of the ClearCell FX system with RCC cells, we performed cell spike-in experiments. For these experiments we employed the ccRCC cell line SNU-349 and primary RCC cell lines. The cells were labelled with a fluorescent tracker and the number of fluorescent cells were counted (range of cells spiked-in 10-232) before mixing them with 7.5 ml of healthy donor blood. After lysis of the reticulocytes, the blood sample was subjected to size-based isolation using the ClearCell FX system. The output, containing a background of approximately 10,000 leucocytes and the enriched labelled SNU-349 cells were thereafter analysed using a fluorescent microscope. These experiments show that on average, the system is able to recover 50% of SNU-349 cells and 66% of primary RCC cells that are spiked into whole blood (Fig. 2A). Spiked-in and recovered cell numbers are reported in Table SII. We also performed immunofluorescence staining on ClearCell FX enriched samples originating from whole blood spiked with SNU-349 cells. To distinguish RCC cells from leucocytes we stained cells for CD45 (leucocytes) and CA9 (a well-established marker for ccRCC cells). We were able to clearly distinguish CA9 positive, CD45 negative SNU-349 cells on a background of only CD45 positive leucocytes (Fig. 2B)

Assessment of RNA quality, pre-amplification and assay reproducibility. Next, we wanted to establish and validate a method to detect RCC-specific RNA transcripts from the ClearCell FX enriched CTC fraction. First, RNA was extracted and subjected to quality and quantity assessment (Fig. S1). Once the quality was deemed acceptable (RIN >5), we performed global pre-amplification of reverse transcribed cDNA to yield sufficient sample material and to increase the relatively low transcript copy numbers from the enriched SNU-349 cells present on a background of leucocytes in the sample. Prior to global pre-amplification of cDNA from the CTC enriched samples, the pre-amplification process was monitored with quantitative PCR using SNU-349 cDNA, in order to determine the optimal number of cycles required (Fig. S1B). A successful pre-amplification reaction should yield an increase in fragments in the 400-10,000 base-pair range, which was observed in our samples via fragment analysis. (Fig. S1C).

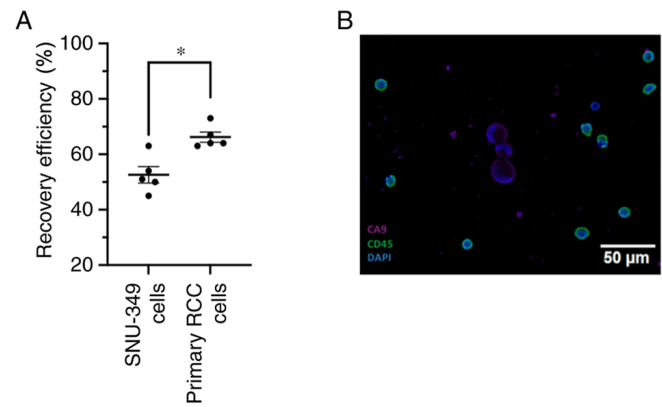


Figure 2. ClearCell FX recovery efficiency and immunofluorescence-based detection of RCC cells. (A) SNU-349 and primary RCC cell line spike-in and recover efficiencies (%). Horizontal lines represent average recovery efficiency and error bars are standard error of mean (SEM). Mann-Whitney U Test, * $P < 0.05$ (two-tailed). (B) Immunofluorescence-based detection of spiked SNU-349 cells enriched from whole-blood using the ClearCell FX system. The output material from 7.5 ml of whole blood spiked with SNU349 cells were immobilized by cytospin centrifugation and labelled with antibodies against CD45 and CA9 for detection of leucocytes and ccRCC cells, respectively. Fluorophore signal is merged. Purple, CA9; Green, CD45; Blue, DAPI. RCC, renal cell carcinoma.

Digital PCR detection of ccRCC cells after size-based enrichment. In order to identify ccRCC specific marker genes suitable for assessing the presence of ccRCC cells in our spiked-in samples, we analysed data from the publicly available TCGA (The Cancer Genome Atlas) database and the subsequent sub-type classification by Chen *et al* (26), as defined when pooling the three RCC datasets. Using Limma analyses, we extracted a gene list with the most differentially expressed genes, when comparing ccRCC to pRCC and chRCC (Table SI). CA9 and SLC6A3 were amongst the most differentially expressed genes in ccRCC and were selected for further exploration (Fig. 3A). CA9 is a well-documented hypoxia-driven gene and SLC6A3 displays a highly ccRCC specific expression pattern, as described by us and others (23,30). We tested the reproducibility of the digital PCR assays of these marker genes on pre-amplified cDNA (Fig. 3B). Increasing the input cDNA yielded higher copies/ μl in a linear fashion. Next, we assessed the limit of detection of our assays within the context of enriched tumour cell samples. We performed a titration of tumour cells (SNU-349) spiked into 7.5 ml of whole blood resulting in samples with defined numbers of tumour cells after enrichment. We reliably detected transcripts of CA9 at the one cell level with more transcripts being detected with higher cell numbers. Similarly, SLC6A3 transcripts could also be detected from enriched samples containing ≥ 1 to 121 cells (Fig. 3C). However, transcripts were less reliably detected at the one cell level with SLC6A3. Enriched samples from healthy blood controls (4 samples per marker) were also tested, consistently giving readouts of less than 1 copy/ μl .

RCC subtype-specific biomarker panel. With the aim of broadening the applicability of our method to include pRCC and chRCC, we identified genes that are differentially and specifically expressed within these subtypes (Table SI). When selecting markers for p1RCC and p2RCC, markers were

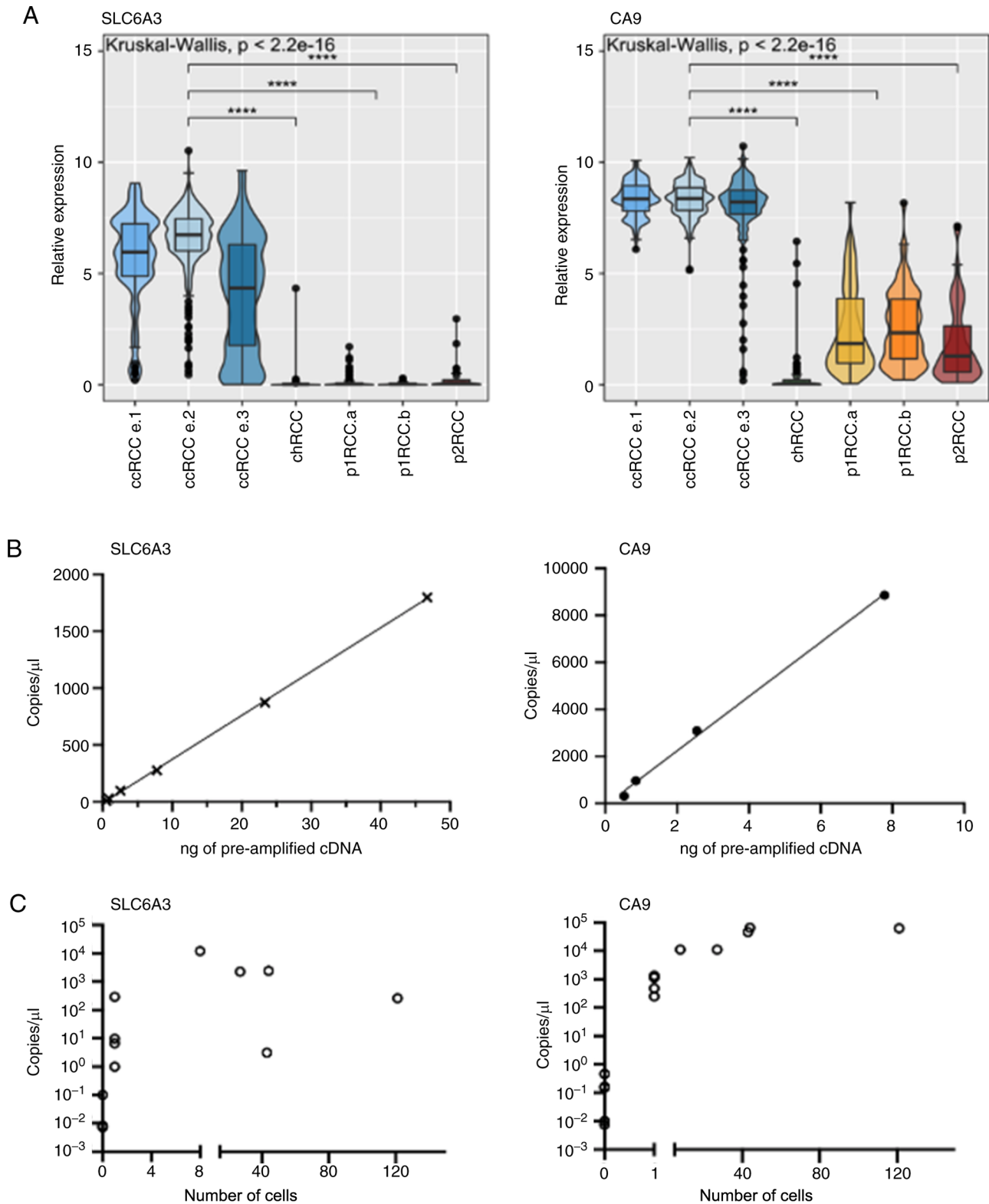


Figure 3. Specificity, reproducibility and detection of ccRCC cells with *SLC6A3* and *CA9*. (A) Specificity of *SLC6A3* and *CA9* expression in RCC tumours within TCGA database. (n=103 ccRCC.e1, 255 ccRCC e.2, 137 ccRCC e.3, 77 chRCC, 134 p1RCCa, 71 p1RCCb, 52 p2RCC). **** $P < 0.0001$. (B) Digital PCR assay efficiency of *SLC6A3* and *CA9* over increasing pre-amplified cDNA inputs with line of best fit. (C) Digital PCR based copies/ μ l measurement of SNU-349 spiked blood samples after ClearCell FX enrichment and downstream processing. Copies/ μ l also shown for 4 enriched blood samples without spiked-in SNU-349 cells. ccRCC, clear cell renal cell carcinoma; chRCC, chromophobe RCC; p1RCC, papillary type 1 RCC; p2RCC, papillary type 2 RCC; cDNA, complementary DNA.

chosen so they indicated a papillary subtype and not based on a papillary type 1 or 2 subtype. The selection criteria for a marker included minimal or absent expression in human blood,

based on analyses of blood datasets within the Human Protein Atlas (31,32) and high RCC subtype specific expression based on Limma analyses of TCGA expression data, which is based

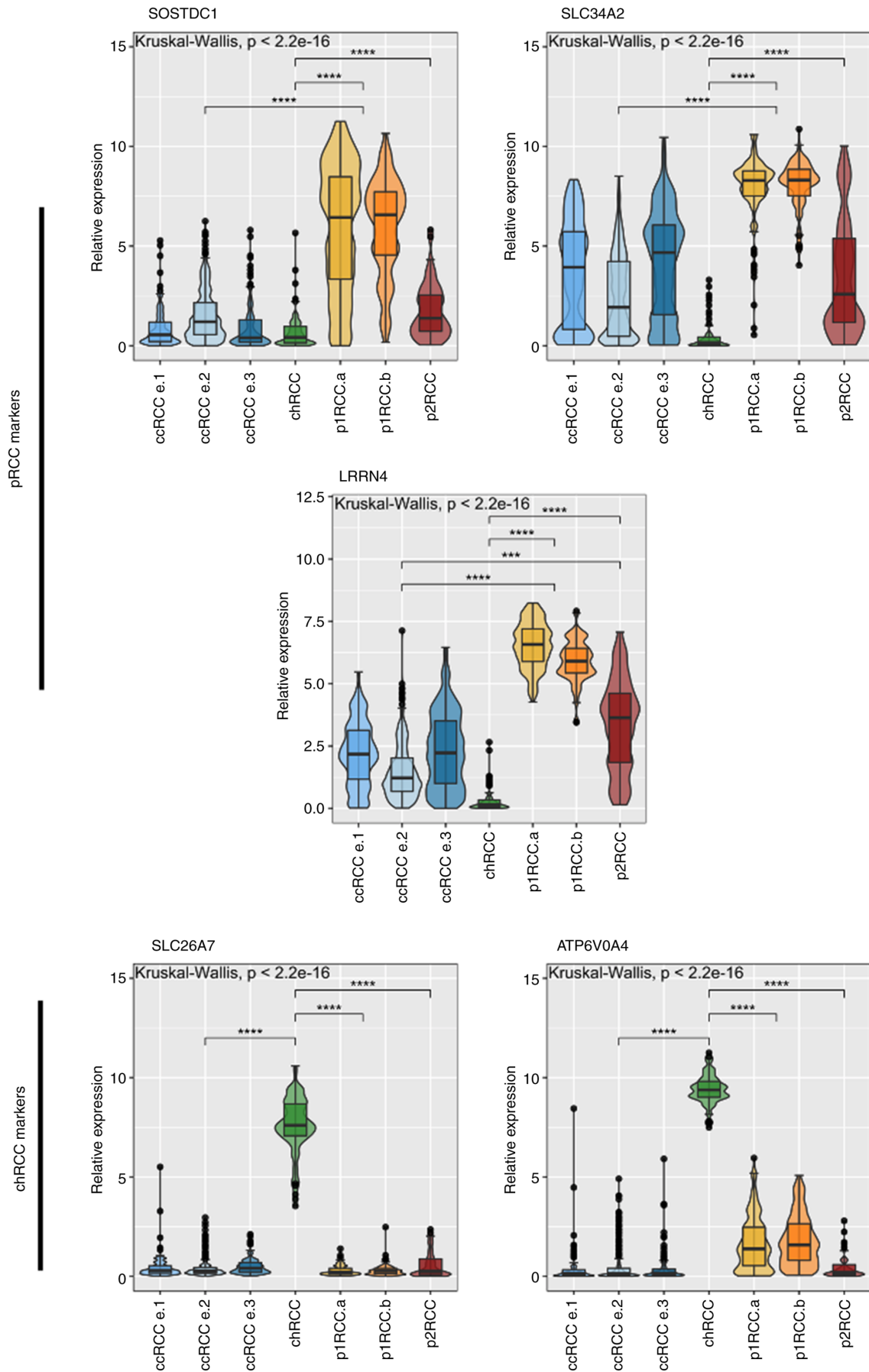


Figure 4. Relative expression of non-ccRCC markers derived from Limma analysis of TCGA expression data. Relevant subtypes are shown according to Chen *et al* (26) taxonomy. ccRCC cohorts and p1RCC cohorts grouped for Dunn's comparison test (n=103 ccRCC.e.1, 255 ccRCC.e.2, 136 ccRCC.e.3, 77 chRCC, 134 p1RCC.a, 71 p1RCC.b, 52 p2RCC). *** $P < 0.001$, **** $P < 0.0001$. ccRCC, clear cell renal cell carcinoma; chRCC, chromophobe RCC; p1RCC, papillary type 1 RCC; p2RCC, papillary type 2 RCC.

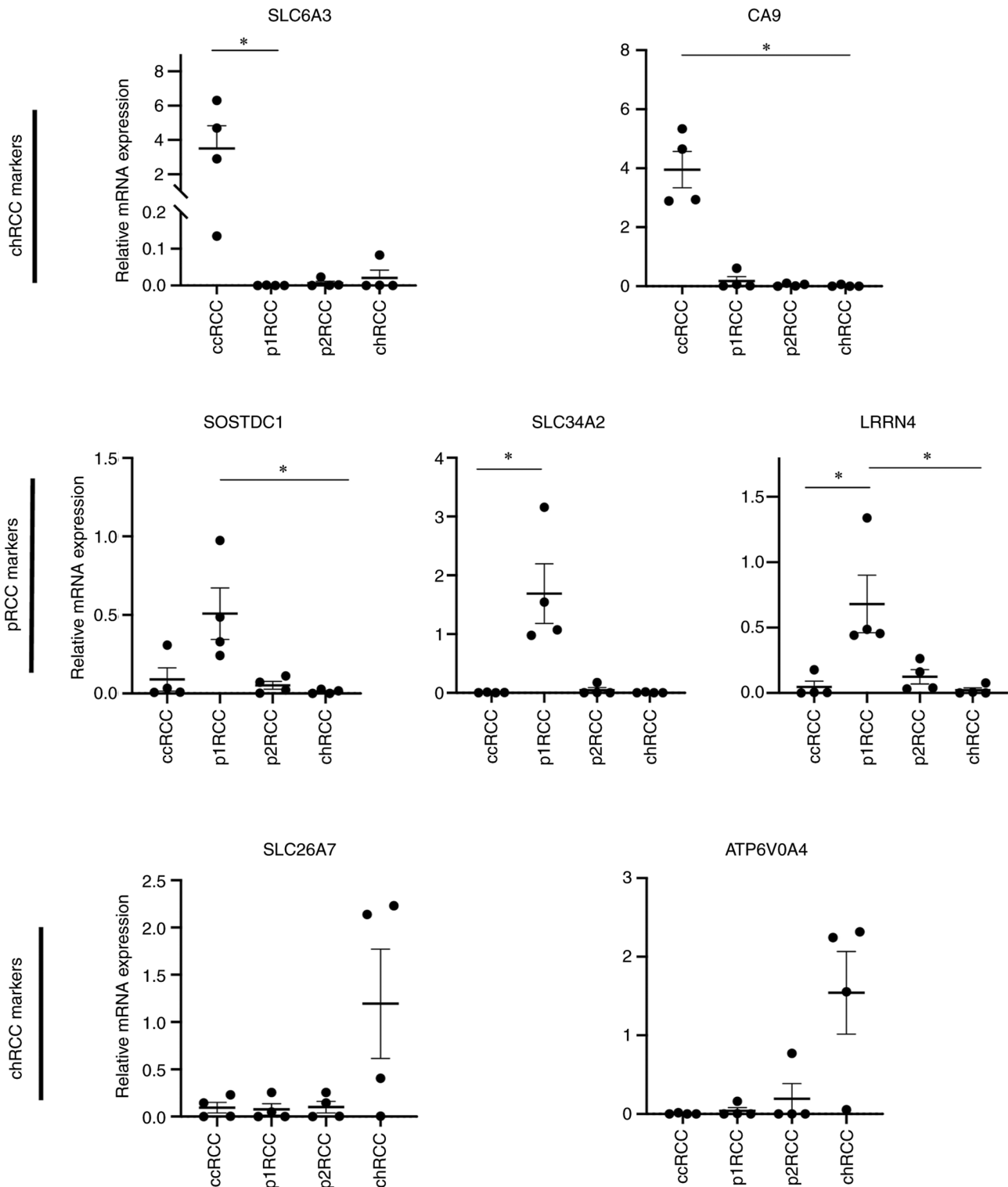


Figure 5. qPCR based mRNA expression relative to beta-actin for all selected markers in patient primary RCC tumour tissue. Horizontal lines represent average mRNA expression and error bars represent SEM (n=4 for each subtype). Data were analysed using Kruskal-Wallis with post-hoc Dunn's test. * $P < 0.05$. Comparisons not reaching significance not indicated. ccRCC, clear cell renal cell carcinoma; chRCC, chromophobe RCC; p1RCC, papillary type 1 RCC; p2RCC, papillary type 2 RCC.

on the Chen *et al* (26) classification of RCC tumours. These selection criteria resulted in a panel of 5 genes in addition to the ccRCC markers *CA9* and *SLC6A3* (Fig. 4).

To validate the sub-type specific expression of the selected markers we analysed RNA extracted from primary tumour samples (Fig. 5). Only RCC subtypes that were confirmed to

be one of the three primary subtypes were included in this patient cohort (Table II). Our expression data was in-line with the pattern seen in the TCGA data set. The two markers for the ccRCC subtype (*SLC6A3*, *CA9*) clearly distinguished this subtype from the rest in the tumour samples tested. Using a joint set of markers to distinguish the papillary RCC subtype

also allowed for clear separation of this subtype from the other two. Finally, the markers for the chRCC subtype distinguished these tumours extremely well, showing elevated RNA expression in a subtype specific manner. We additionally tested all markers on 6 healthy-volunteer blood samples and found them to be negative for our RCC specific markers (Fig. S2).

Discussion

Despite the recent surge in liquid biopsy development and clinical implementation in other cancer types, RCC seems to have largely missed this wave. Potential explanations range from the poor compatibility of RCC cells with EpCAM based isolation methods to the use of cell-free DNA, where RCC tumours have shown to be poor shedders of ctDNA (15,16). Additionally, CTC based liquid biopsy approaches have their inherent pre-analytical challenges (33). The first of these is the sparsity of CTCs in the blood of patients, hence the enrichment procedure requires high recovery efficiency. Secondly, the isolation procedure is often a trade-off between efficiency and specificity; high CTC numbers are desirable but are difficult to obtain with minimal contamination of leucocytes. Finally, sample material is often limited, and methods are required to generate sufficient analytical material for multiple downstream analyses, such as in the case of querying the expression of multiple disease specific transcripts.

Here we develop a method that overcomes the hurdles associated with a CTC based liquid biopsy approach in RCC. The use of the ClearCell FX system successfully isolated spiked-in RCC tumour cells from whole blood with a recovery rate of 66% for primary cells, which is higher than previously reported recovery rates with other isolation systems such as CellSearch[®], RosetteSep[®] or Parsortix[®] (16,29,34). These observations are in line with the characteristics of RCC tumour cells as our data and others' show that tumour cells including RCC cells are typically larger than 15 μm , suiting them for isolation with a size-based approach that enriches cells larger than 14 μm (17,35).

After enrichment, our data show that we are able to successfully detect ccRCC tumour cells present in the enriched sample via dPCR and immunofluorescence. With one of the ccRCC markers, *CA9*, we were able to consistently and reliably detect down to one ccRCC tumour cell via dPCR in the ClearCell FX enriched sample. The other ccRCC marker *SLC6A3* was marginally less consistent at low cell numbers. This is likely due to the differences in transcript numbers of these two genes present in ccRCC cells, where the absolute transcript levels in ccRCCs are higher for *CA9* than for *SLC6A3*, both in cultured ccRCC cells and within the ccRCC cohort of the TCGA. We still reliably detected *SLC6A3* transcripts in enriched samples that contained 8 or more ccRCC cells, demonstrating the relevance of the described workflow in relation to the reported number of CTCs isolated from ccRCC patients using a size-based approach, that may range from 1 to >100 cells per 10 ml whole blood (17,36). Importantly, the pre-amplification step introduced in our workflow generates an excessive amount of cDNA for multiple analyses.

Finally, we curated a 7-marker gene panel to distinguish the three major RCC subtypes. This is facilitated by the fact

that RCC subtypes arise from differing cells of origin and have further defining features based on their subtype specific oncogenetic alterations (37). For example, ccRCC and papillary RCC arise from the proximal segment of the nephron whereas chRCCs arise from the collecting duct. These anatomical distances translate into transcriptomic and functional differences since cells perform distinct functions along segments of the nephron (12,38). As a consequence, chRCC specific markers are unchallenging to define and show a pronounced subtype specific pattern. In stark contrast, ccRCCs and pRCCs arise from the same proximal segment of the nephron, making them far more difficult to molecularly define. However, the virtually universal loss of VHL and the resulting pseudo-hypoxic drive can be leveraged to distinguish ccRCCs from pRCCs, while pRCC specific markers are less precise. This is evident in our primary tumour tissue analysis where overlapping expression of pRCC specific markers with ccRCC markers is observed. Due to this, identification of further pRCC specific markers is warranted. The lack of available cell lines as well as the rarity of these RCC subtypes posed a challenge in validating these markers within this workflow. Ultimately, these markers will require enriched CTCs from pRCC or chRCC patient blood for validation.

Although a few primary ccRCC tumours show raised expression for non-ccRCC specific markers, we predict it would be straightforward to assign them as ccRCC due to the relatively high expression of their respective markers *CA9* and *SLC6A3*. Vice-versa, the expression of the combination of the papillary markers can be leveraged against the low expression of non-papillary markers to designate a papillary subtype. Thus, overlapping expression of subtype specific markers is unlikely to affect the overall sub-type designation of a CTC isolate for these reasons. Regardless, it is warranted and required to explore the use of these markers on CTCs from RCC patient blood.

There were certain limitations to the present study. Firstly, size-based CTC isolation platforms can miss smaller CTCs, as CTC sizes in patients may vary more than observed in this study with cultured and primary cells. Furthermore, the scarce availability of CTCs in patient blood can add to the recovery sufficiency of CTCs.

In conclusion, we established a clear workflow for the isolation and detection of RCC tumour cells from whole blood with obvious implications for use with CTCs in RCC patients. Further work is required to experimentally validate our novel 7-marker gene panel on a larger cohort of patient tumours and to demonstrate its ability in classifying tumour subtype. This should be complemented with a larger cohort of healthy controls as well as RCC patients with other confounding conditions, such as inflammatory or kidney diseases.

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Availability of data and materials

For limma analyses, level 3 RNA-seq data of chromophobe renal cell carcinoma (KICH), clear cell kidney carcinoma (KIRC), papillary kidney carcinoma (KIRP) were downloaded from The Cancer Genome Atlas (TCGA) data portal (<https://portal.gdc.cancer.gov>) as described in (12). The other datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HA, JH and RDA conceived and designed the study. HA, JH, DL and RDA developed and designed the methods. DL implemented computational software for TCGA analysis. RDA verified the reproducibility of the results. HA, RDA and SS synthesised and analysed data. RDA, AT and CM performed experiments and collected data. BS and PE performed surgery, provided clinical advice and contributed to study design. HN and MJ provided primary patient tissue and established primary cell lines. HA and RDA wrote and prepared the original draft; all authors reviewed and edited the manuscript. HA, DL, SS and RDA prepared figures. HA provided supervision, project administration and funding acquisition. HA and RDA confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Regional Healthcare provider (Region Skåne) and Lund University ethical committee (approval nos. LU680-08 and 2018:19). Informed written consent was obtained from all subjects involved in the study.

Patient consent for publication

Written informed consent was obtained from patients for publication of results.

Competing interests

The authors declare that they have no competing interests.

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