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Clonal heterogeneity influences drug responsiveness in renal cancer assessed by ex vivo drug testing of multiple patient-derived cancer cells

Khalid Saeed\textsuperscript{a}, Poojitha Ojamies\textsuperscript{a}, Teijo Pellinen\textsuperscript{a}, Samuli Eldfors\textsuperscript{a}, Riku Turkki\textsuperscript{a}, Johan Lundin\textsuperscript{a}, Petrus Järvinen\textsuperscript{b}, Harry Nisen\textsuperscript{b}, Kimmo Taari\textsuperscript{b}, Taija M. af Hällström\textsuperscript{a,c}, Antti Rannikko\textsuperscript{b}, Tuomas Mirtti\textsuperscript{a,d}, Olli Kallioniemi\textsuperscript{a,e}, Päivi Östling\textsuperscript{a,e}

Affiliations:
\textsuperscript{a}Institute for Molecular Medicine Finland (FIMM), University of Helsinki, Helsinki, Finland
\textsuperscript{b}Department of Urology, Helsinki University Hospital, Helsinki, Finland
\textsuperscript{c}AstraZeneca, Espoo, Finland
\textsuperscript{d}Department of Pathology, HUSLAB, Helsinki University Hospital, Helsinki, Finland
\textsuperscript{e}Science for Life Laboratory, Department of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden

Corresponding authors:
Khalid Saeed, MSc
Institute for Molecular Medicine Finland (FIMM),
University of Helsinki, Helsinki, Finland
Phone: +358 458 798 407
E-mail: khalid.saeed@helsinki.fi

Päivi Östling, PhD
Science for Life Laboratory, Department of Oncology and Pathology,
Karolinska Institutet, Stockholm, Sweden
Phone: +46 73 673 5762
E-mail: paivi.ostling@scilifelab.se

Running title
Subclone-specific therapeutic approach for renal cancer

Key words: renal carcinoma, patient-derived cells, drug sensitivity testing, intratumor heterogeneity, subclones, precision medicine
Novelty and Impact

The comparison of drug responses among multiple variants of patient-derived cells (PDCs) exhibited the impact of intra-tumor genomic heterogeneity, as the individual PDCs from different tumor regions showed distinct drug sensitivity profiles. These data illustrate an approach that could facilitate the design of effective personalized drug combinations needed to target multiple subclones in cancer and for elucidating pharmacogenomic biomarkers.

Although the genotype-phenotype for familial medullary thyroid carcinoma (FMTC) is well studied, only a few low susceptibility risk loci have been identified for familial non-medullary thyroid carcinoma (FNMC). Here, the authors screened and identified a novel susceptibility gene, MAP2K5, as a contributor to FNMC. The data revealed that MAP2K5 variants A321T or M367T can activate the MAP2K5-ERK5 pathway, alter downstream gene expression, and subsequently induce thyroid epithelial cell malignant transformation. The study highlights the potential of MAP2K5 to be used for molecular diagnosis as well as to improve clinical management of deleterious MAP2K5 mutation-carriers.
Abstract
Renal cell cancer (RCC) has become a prototype example of the extensive intra-tumor heterogeneity and clonal evolution of human cancers. However, there is little direct evidence on how the genetic heterogeneity impacts on drug response profiles of the cancer cells. Our goal was to determine how genomic clonal evolution impacts drug responses. Finding from this study could help to define the challenge that clonal evolution poses on cancer therapy. We established multiple patient-derived cells (PDCs) from different tumor regions of four RCC patients, verified their clonal relationship to each other and to the uncultured tumor tissue by genome sequencing. Furthermore, comprehensive drug-sensitivity testing with 460 oncological drugs was performed on all PDC clones. The PDCs retained many cancer-specific copy number alterations and mutations in driver genes such as VHL, PBRM1, PIK3C2A, KMD5C and TSC2 genes. The drug testing highlighted vulnerability in the PDCs towards approved RCC drugs, such as the mTOR-inhibitor temsirolimus, but also novel sensitivities were uncovered. The individual PDC clones from different tumor regions in a patient showed distinct drug response profiles, suggesting that genomic heterogeneity contributes to the variability in drug responses. Studies of multiple PDCs from a cancer patient are informative for elucidating cancer heterogeneity and for the determination on how the genomic evolution is manifested in cancer drug responsiveness. This approach could facilitate tailoring of drugs and drug combinations to individual patients.
Introduction
Genomic and transcriptomic studies have shown significant inter- and intra-tumor heterogeneity (ITH) in clear cell renal cancer (ccRCC) \(^1\)-\(^3\). The discovery of inactivating Von Hippel-Lindau (\(VHL\)) mutations and aberrant mTOR/PI3K pathway regulation has provided key concepts for targeted treatments in ccRCC. The tumor suppressor \(VHL\) is mutated in up to 90% cases and leads to disruption of \(HIF\) levels affecting angiogenesis, genomic integrity, glycolysis and anabolic biosynthesis \(^4\), \(^5\). Deregulation in PI3K/AKT/mTOR pathway is also a consistent feature in ccRCC, influencing tumor progression through facilitating cellular growth and proliferation \(^6\). Genes involved in histone modification and chromatin remodeling such as \(PBRM1\), \(SETD2\) and \(KDM5C\) are also often mutated in ccRCC \(^7\), \(^8\). Despite the presence of common and consistent driver mutations in ccRCC, the variability in the drug responsiveness from one patient to another is still a major challenge \(^9\). This could be attributed to the presence of multiple distinct tumor subclones \(^10\).

Functional studies based on model systems to recapitulate genetic and phenotypic characteristics of the patient profile such as patient-derived xenograft (PDX)/tissue grafts (TG), organoids and patient-derived cultures that have recently been established from RCC \(^11\)-\(^15\). TG models were generally well-characterized based on histology, gene expression and genomics, and are known to retain regional ITH \(^13\). TG models only allow for small scale drug testing studies, however, may recapitulate the drug responsiveness in patients \(^12\), \(^15\). A study on a single patient’s PDX models derived from a primary and a lung metastatic sample showed noticeable variation in drug response profiles inferred from the single cell RNA-sequencing (scRNA-seq) of the paired samples, and underlined the impact of ITH in RCC\(^16\). In contrast, 2D primary cell models, provide for material towards more systematic drug tests, but are often challenging due to over-representation of normal cells \(^13\). In addition, these models are often established from single sites not representing the complexity of the disease. Therefore, studies of the inter- and intra-patients’ heterogeneity in drug responses due to subclones present at distinct sites remains an unanswered question at the research bench as well as in the clinic \(^10\), \(^17\).

Here, we developed representative patient-derived cells (PDCs) from normal and (multiple) cancerous RCC tumor regions using conditional-reprogramming-cell-
technology \textsuperscript{18,19}. The genomic profiles and drug testing data were compared with each other and to the original tumor features. We reconstructed phylogenetic trees based on mutations that inferred early- or late-stage events in the cancer progression. Comprehensive drug profiling identified vulnerability of the PDCs against the drugs that hit major axis of action in RCC including established targeted therapies such as temsirolimus. It also showed distinct drug-response profiles in PDCs from the primary, invasive infiltrating \textit{vena cava} and adrenal metastatic sites of the same patient.
Material and Methods

Patients and tissue processing

Renal tissue was obtained from four renal cancer patients that operated with open nephrectomy, at Helsinki University in the urological biobank initiative Hospital (Helsinki Urological Biobank, HUB), with approved informed consent (Dnro 263/13/03/02/2011; 379/13/03/02/2012 and Dnro § 212) (Table 1). Pieces of kidney tissue from the cancerous and normal regions of the kidney was selected by a board-certified pathologist and was processed as illustrated in Fig. 1. The amount of normal and malignant cells (including inflammation level and matrix/stromal content) in the parental tissue was evaluated through hematoxylin and eosin staining by the pathologist from tissue sections adjacent to the section used for patient-derived cells (PDC) establishment (Supplementary Table S1).

Establishment of PDCs

We generated a total of 11 PDCs from four renal cancer patients derived tissues; normal region \((n=4)\), localized/primary-tumor \((n=4)\), invasive/infiltrating vena cava \((n=2)\), adrenal gland metastatic sites \((n=1)\). Cultures were established using conditional-reprogramming-cell-technology (CT) \(^{18, 19}\). Briefly, cells were isolated from the original tissue after chopping into small pieces and with collagenase (40 units/ml) treatment for 2-4 hours. The suspension put into culture dish having 30-50% confluent irradiated Swiss 3T3 fibroblast feeder cells (J2 strain). The co-cultured maintained in in F-medium (3:1 (v/v) constitutes of F-12 nutrient mixture (Ham) - DMEM (Invitrogen), 5% FBS, 8.4 ng/mL cholera toxin, 0.4 µg/mL hydrocortisone, 10 ng/mL EGF, 24 µg/mL adenine), 5 µg/mL insulin, and 10 µM ROCK inhibitor (Y-27632, Enzo Life Sciences, Lausen, Switzerland). Separation of 3T3-cells from the PDCs during passaging was done using differential trypsinization. Drug testing was performed between 2-6 weeks after the initiation of the culture, and at the same time cells were collected for other experiments and characterizations that were used for immunological staining, exome/whole genome sequencing. The passage numbers and duration of culture of each PDCs at the time of these experiments are described in supplementary Table S2.

Exome sequencing
The DNA extraction from the PDCs was done at the same passage as performing the drug sensitivity testing, *i.e.*, 3-6 weeks after the establishment of the cultures. The extraction of genomic DNA from original tissues, PDCs and germline control blood cells was done using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Exome capture was performed using the Nimblegen Agilent SureSelect v5 capture kit (Agilent Technologies, Santa Clara, CA, USA). Sequencing was executed using a HiSeq 2500 instrument (Illumina, San Diego, CA, USA). $4 \times 10^7$ and $1 \times 10^8$ 2×100-bp paired-end reads were sequenced per sample, respectively. Lists of somatic point mutations from parental tissues and PDC samples from the same patient were compared to each other to identify any mutations shared between the primary and cultured cells ($p>0.05$). The higher amount of somatic mutations called from the PDCs compared to primary tumor tissue samples (particularly RCC.1 primary cancer/vena cava and RCC.3 primary cancer) was most likely due to mouse DNA contamination from 3T3 cells that were co-culture as feeder cells for PDC.

**Phylogenetic analysis of tumor and PDC**

To construct the phylogenetic cell lineage tree, we adapted a computational method LICHeE (Lineage Inference for Cancer Heterogeneity and Evolution) $^{20}$. These visualizations were reconstructed by utilizing mutational load of all the samples. Excessive nodes with potential mouse contaminating mutations were removed from the final trees. Mutation present in each node are provided in the supplementary file S5. Parameters used for analysis are following.

- **maxVAFAbsent 0**
- **minVAFPresent 0.00001**
- **maxVAFValid 0.9**
- **minClusterSize 2**
- **maxClusterDist 0.2**

**Drug sensitivity testing**

The PDCs established from the patient samples were cultured for 2-6 weeks and used for drug testing with 461-528 approved and investigational oncology drugs (Supplementary Table S6) $^{18, 21}$. The library consists of targeted compounds, such as kinase inhibitors, epigenetic modifiers, differentiating agent, metabolic inhibitors as well as chemotherapeutics. Briefly, PDCs were added to the drug containing plates, in
five different concentrations (normally within 1nM – 100uM concentration range) and let them grow for 72 h. The cell viability was measured with CellTiter-Glo (CTG, Promega, Madison, WI, USA). The raw data was further processed to calculate drug efficacies of individual drugs.

**Statistical analysis**

The percentage inhibition for each drug was calculated by normalizing the CTG raw value of each wells to negative control, dimethyl sulfoxide (DMSO, 0.1%) and positive control, benzethonium chloride (BzCl, 100 μM). The data points obtained from dose-response percent inhibition was fitted into a four-parameter logistic model to calculate IC50, slope, top and lower asymptotes to quantify the drug response. The calculations were executed through a multi-parameter area under the curve sensitivity method named, the drug sensitivity score (DSS) \(^{21,22}\). To focus on the drugs with clear effects or lack thereof, we applied a DSS value of 5 as a cut-off. The combined dose-response curve fits illustrated in Fig 4D-E were made through GraphPad Prism 6. XY-correlation-plots shown in Fig.4 and supplementary Fig.5 were made using Pearson-correlation coefficient.
Results

Phenotypic characterization of patient-derived cells and tumor tissue

Four RCC patients (RCC.1-4), a total of seven malignant and four normal regions were used for establishment of PDCs (Fig.1). Three of the patients had ccRCC (RCC.1, 3-4), while RCC.2, had a poorly differentiated mixed histology of ccRCC and collecting-duct-carcinoma (Table 1). Altogether eleven PDCs were generated with co-culture of primary cells with irradiated 3T3-feeder cells, and Rho/Rock-kinase-inhibitor (Y-27632) supplemented in F-medium \(^{18, 19}\). The histopathological analysis and phenotypic retention of PDCs were assessed by immuno-histochemical staining of carbonic anhydrase IX (CA9/CAIX) expression (Supplementary Fig. 1-2). Several studies have shown that CAIX is constitutively overexpressed in ccRCC \(^{23}\), and has been used to establish the relevance of ex vivo cancer models \(^{13}\). In our analysis, a significantly higher protein expression of CAIX was quantified in most cancerous tissues as compared to the normal (Supplementary Fig. 2C). The cancerous PDCs derived from RCC.1-2 and (metastatic) RCC.3 displayed comparable amounts of CAIX expression to their tissue of origin. An increased CAIX expression may also representing the general hypoxic (ex vivo) environment as PDCs derived from benign tissue were also exhibiting higher CAIX in contrast to their parental tissues.

The PDCs retained copy number alterations and somatic mutations present in the original tumor tissue

Comparison of parental tumor tissue and corresponding PDCs showed several shared cancer-specific deletions and amplifications at multiple chromosomal regions (Fig. 2A-C, Supplementary Fig. 3). Some of these shared exact break-points highlighting the clonal relation between the PDCs and their originating tissue. Patients in this study carried recurrent alterations in RCC reported by The Cancer Genome Atlas (TCGA) \(^2\). For example, the 14(del) and 3p(del) that are the most frequent site of non-random aberrations (Supplementary Fig. 3A) with the 3p harboring frequently mutated tumor suppressor genes VHL, PBRM1, SETD2, and BAP1 \(^{24}\). Additionally, we observed Copy Number Alterations (CNAs) in several genes of known relevance to RCC including EGFR amplification and CDKN2A/B deletion (Fig. 2C) \(^2, 6\). In a recent study, deletion at 9p and 14q have been shown as putative biomarker of metastasis in ccRCC \(^{25}\). Interestingly, these CNAs are retained by both cancerous
PDCs of RCC.1 and subclonally in metastatic RCC.3 tissue/PDCs (Fig. 2A-C, Supplementary Fig. 3).

Next, we compared somatic mutations in the tumor tissue and the PDCs to recurrent RCC mutations in the TCGA data (Fig. 2D, Supplementary Table S4). All malignant tissue samples carried somatic mutation in VHL. The same non-synonymous mutation in VHL was also observed in PDCs from RCC.1, RCC.2 and metastatic RCC.3 suggesting that these models retain VHL as a truncal driver mutation (Fig. 2D-E). Additional RCC.1 specific somatic mutations included PBRM1, (the second most frequently mutated gene in RCC), KDM5C, TSC2 and PIK3C2A (Fig. 2D). PDCs from RCC.1, 2 and 3 shared several recurrent somatic founder mutations with the parental tumor tissue suggesting that these models, with the exception of RCC.4, show representation of the original tissue both based on shared CNA and mutations.

Clonal relationship between tumor tissues and PDCs based on the somatic mutation patterns
We inferred cell lineage and clonal relationships between the different tumor sites and their derivative PDCs by reconstructing cancer phylogenetic trees for each RCC patient (Fig. 3, Supplementary Fig. 4). The data indicated a branched evolution, which is consistent with the previously reported studies. The RCC.1 demonstrated a common ancestral clone, harbored 36 truncal mutations that gave rise to all subsequent clones present in both primary and vena cava cancer tissue and PDCs thereof. Subsequently, four additional mutations were acquired by the tumor in the vena cava tissue resulting in a branch away from the primary tumor. The PDC models from vena cava tumor and the primary tumor retained the first and second ancestral subclones thereby recapitulating clonal evolution of the originating tumor tissue (Fig. 3A). Similarly, the common ancestor clone in RCC.3 had nine truncal mutations, and additional events resulted in subsequent branching out in a chronological order from the primary tumor, the tumor infiltrating vena cava, and the metastatic tumor tissue (Fig. 3B). The phylogenetic tree also suggested that the metastatic PDCs and tumor tissue accumulated most of these somatic mutations and retained a close clonal relationship with each other. The RCC.2 cancer PDCs share four mutations including VHL with their originating tumor tissue (Supplementary Fig. 4A). In contrast, RCC.4
was classified as a poorly representative cancer model since the tissue and derived PDCs did not carry any common mutation (Supplementary Fig. 4B).

**Comprehensive drug testing showed distinct response profiles among PDCs derived from different regions of a patient’s cancer tissue**

The PDCs were subjected to drug testing with at least 460 approved and investigational oncology-drugs to evaluate their sensitivity profiles (Supplementary Table S6). RCC.1 and RCC.3 PDCs allowed for intra-patient comparison of drug profiles and distinct sensitivities for several key oncology drugs were observed for PDCs from the primary-tumor, vena cava tumor and for RCC.3 in the metastatic-site (Fig. 4, Supplementary Fig. 5). Specifically, in RCC.1 primary cancer PDCs showed efficacy for ZSTK474, a PI3K-inhibitor as well as JQ1 and OTX015 that are both bromodomain and extra-terminal (BET) protein inhibitors (Fig. 4A, D). The RCC.1 vena cava PDCs were sensitive to an anti-mitotic microtubule-inhibitor, ABT-751 and topotecan, a topoisomerase-inhibitor. Both drugs have undergone phase I/II clinical trials in unselected RCC patients with no clinically relevant benefits observed (Fig. 4A, D, Supplementary Table S6)²⁶. Interestingly, pazopanib a multi-kinase-inhibitor approved for RCC treatment showed sensitivity in RCC.1 vena cava PDCs ²⁷. The effective drugs for RCC.3 primary cancer PDCs included docetaxel whereas, the vena cava PDCs showed specific responses to VEGFR-inhibitors, tivozanib and regorafenib (Fig. 4E). The former has recently been approved by the European Medicines Agency for the treatment of RCC and the latter has been approved for treatment of colorectal cancer (Supplementary Table S6, ²⁷. Interestingly, RCC.3 metastatic PDCs showed increased sensitivities to three topoisomerase-inhibitors camptothecin, SN-38 and topotecan. Other drugs showing efficacy were BMS-754807 an IGF1R-inhibitor and gemcitabine, a nucleoside analog that is approved for the treatment of several cancers (Fig. 4B-C, E)²⁷. In summary, our results showed both shared drugs sensitivities for actionable drugs within different subclonal PDC models, as well as a distinct response profiles that need to be taken into consideration for treatment strategies.

**Genomic intra-tumor heterogeneity linked to the drug response profiles**

To explore potential associations between drug sensitivities and genomic vulnerabilities, we highlighted the genetic alterations (GAs) including CNA and non-
synonymous somatic mutations in the phylogenetic trees along with the drug responses of the PDCs (Fig. 5). The two PDCs derived from RCC.1 malignant tissue harbored truncal GAs (Fig. 5A), and exhibited shared sensitivity towards investigational drugs predominantly from mTOR/PI3K pathways (Fig. 5B) of which omipalisib, AZD2014 and dactinomycin have been tested in the clinical trials for their efficacies in RCC patients (Supplementary Table S6) \(^{28}\). The primary cancer PDCs displayed additional mutations in \textit{bromodomain-containing-1-gene (BRD1)} as well as \textit{PBRM1}. BRD-containing-proteins that regulate the transcriptional activities are frequently dysregulated in cancers, and can be targeted with BET-inhibitors \(^{29}\). Interestingly, we observed specific sensitivity of the primary tumor PDCs to the three BET-inhibitors, IBET-151, JQ1 and OTX015 (Fig. 5D). In addition, EGFR-inhibitors (gefitinib, erlotinib, afatinib) and other kinase inhibitors (rigosertib and dasatinib) that exhibited selective responses in these PDCs are approved oncology drugs for other malignancies and have also been investigated in RCC patients (Fig. 5D, Supplementary Table S6). The RCC.1 \textit{vena cava} PDCs were sensitized to tivozanib and pazopanib in addition to investigational drugs (Fig. 5E).

All PDCs from RCC.3 displayed truncal mutations on \textit{BRD4} and \textit{IGFN1} and showed shared sensitivity to mTOR/PI3K, BET and topoisomerase-inhibitors (Fig. 5F-G). BRD4 is a major target for BET-inhibitors and therefore this results suggests a potential link with the vulnerabilities to the observed GA \(^{29}\). The RCC.3 PDCs separate into two sub-branches harboring distinct sets of mutations (Fig. 5H-I) where the branch containing the primary cancer and adrenal metastatic PDCs show shared sensitivity to temsirolimus, gemcitabine and cabazitaxel (Fig. 5J) \(^{27}\). This branch showed further division into distinct clones by accumulation of mutations and interestingly the drug sensitivities are mostly novel, not found in previous or ongoing investigational trials for RCC patients, except for atorvastatin (Fig. 5L-M). In contrast, in the \textit{vena cava} branch the PDCs were mostly sensitive to two VEGFR-inhibitors, tivozanib and regorafenib, approved for other malignancies (Fig. 5N, Supplementary Table S6). In conclusion, our analyses reveal the complexity of genomic ITH in RCC and a spectrum of drug responses among the different subclones of PDCs obtained from multiple regions of cancer patient.
Discussion

The heterogeneous drug responsiveness and resistance mechanisms of targeted drugs in RCC patients are poorly understood and required novel therapeutic targets and drugs. Our study on PDCs generated from advanced RCC tumors highlights how curative treatments and drug combinations preferably would need to target all subclones having unique drug response patterns. It also shows the limitation of single site sampling from each cancer that may underestimate the mutational landscape and variation in drug responses.

The PDCs generated in this study were often enriched for driver mutations and CNAs as compared to the uncultured parental tumor tissue. For example, the mutation frequency in VHL identified in RCC.1 increased from 7 to 28% in the localized tumor to the corresponding PDC, and from 33 to 64% in vena cava tumor tissue to PDCs (Fig. 2E). This observation was further supported with the identification of deeper 9p (del) and 14q (del) in primary cancer derived PDCs of RCC.1 as compared to the original tissue. The same aberrations in vena cava derived tissue/PDCs of RCC.1 confirmed a shared clonal ancestry. The deletion at 9p,14q have been recently shown as hallmark driver of metastasis in ccRCC. The same aberrations in venacavaderived tissue/PDCs of RCC.1 confirmed a shared clonal ancestry. The deletion at 9p,14q have been recently shown as hallmark driver of metastasis in ccRCC25. Interestingly, these CNAs were also detected in the metastatic RCC.3 tissue/PDCs, but were not detectible in the primary or venacaval tissue/PDCs.

The drug profiling identified several drugs with known targets and established clinical RCC efficacies e.g. pazopanib, temsirolimus, tivozanib, gemcitabine, demonstrating the potential of cultured cells and ex vivo drug testing in highlighting clinically relevant drug effects (Fig. 4-5). Also, efficacies of off-label approved drugs were observed such as dasatinib, afatinib, gefitinib, erlotinib, regorafenib, BET-inhibitor as well as topoisomerase-inhibitors that could be further explored in future clinical trials for RCC patients. Notably, in the case of BET-inhibitors, genomic evidence was observed from the PDCs established from both RCC.1 and RCC.3 suggesting that epigenetic mechanisms should be further explored in RCC for clinical relevance. The pro-oncogenic role of BRD4 was recently evaluated in RCC through in vitro and in vivo experiments, which also suggested a treatment rationale for RCC patients30.
Recently, Kim et al., emphasized the variation in drug responses due to functional ITH examined through scRNA-seq of tissue/PDX of primary and metastatic samples. The study further revealed the drug combination strategies to overcome the subclonal activation of multiple signaling pathways. Our analysis provides a proof of concept study showing that ITH can be functionally explored through comprehensive drug testing of multiple established PDCs. While shared, early genetic alterations in the trunk of the cancer phylogenetic tree may highlight common drug efficacies, the subsequent subclones with additional genetic alterations may lead to multiple distinct drug response patterns. Thus, the analysis of multiple variants from a single patient provides significant insight for the pharmacogenomics analyses as compared to studies between diverse cancers with a different genetic background.

However, certain limitations are bound to the study that need to be kept in consideration for future studies. For example, subsequent overgrowth of normal cells in different culture system is repeatedly reported as a consistent challenge in the establishment and maintenance of cancer culture. Though the growth advantage of normal over cancer cells in ex vivo condition is not clear, but a rapid induction of apoptosis in cancer cells has been proposed, likely due to their unstable genetic makeup. Assurance of cancer-specific representation of PDC is important since a small populating of contaminating normal clones can also be enriched, as in the case of RCC. Since CR technology is powerful in expanding both normal and cancer cells and should therefore be used with care to validate the nature of established culture. Cancer-specificity of RCC primary cancer and vena cava PDC is also questionable due to absence of truncal VHL mutation in these cultures. However, both of these cells retained few shared mutations with their respective cancer tissues as shown in Fig.2 and Fig. 5. Another limitation of these cultures is the inability of ex vivo drug testing to recapitulate in vivo scenarios for major targeted drugs such as anti-VEGFR in RCC due to absence of microenvironment/endothelial cells. Since drugs acting on VEGFR/PDGFR axis are acting on endothelial cells but not the tumor themselves. Hence, VEGFR inhibitors (axitinib or sunitinib) did not show responses in the PDCs, while pazopanib had little impact (~20% cell inhibition) can be explained due to its additional multi-kinase inhibitory action or off-side effect (supplementary table 5). One missing aspect of the study is the sampling form the distant metastatic sites which is fundamental issue for clinical burden and the major
target for systemic therapies. Though, these studies presented a proof-of-concept to highlight the cancer specific drug responses observed in ex vivo setting could provide inferences for drug repositioning and possibly in the future for individualized treatment options. However, further dedicated studies with higher number of patients with several (distant) metastatic samples and heterogeneous material would still be needed to confirm these interpretations.

The concept of simultaneous targeting the bulk tumor representing the trunk with a drug/surgery, and subclones with another specific drug has increased acceptance in clinical research \(37\). Furthermore, novel clinical trial approaches are being instituted under consideration the context of cancer evolution and sub-clonal oriented therapeutic approaches \(38\) \(39\). Future strategies targeting specific subtypes and metastatic tumors are likely to capitalize on the enormous genomic information and molecular understanding of the disease through involving large cohorts and detail integration of comprehensive genetic data with biological response of individual tumor. Recently, Turajlic et al., has elegantly shown metastatic pattern in ccRCC with a sizeable cohort of matched primary metastasis samples, and identified biomarkers (loss of 9p and 14q) of metastatic competent subclones. These efforts could help in patient stratification to identify patients who would potentially benefit from systemic therapies and/or surgical methods\(^{25}\). In additional to the promises of patient-derived-models regarding identification of the most appropriate therapeutic algorithms through integration of molecular profiling with ex vivo drug dependencies of individual patients. In conclusion, we suggest that ex vivo culturing of patient-derived cancer cells can provide highly relevant models for RCC. The availability of multiple PDCs from each cancer enabled us to show that intratumor heterogeneity will impact the drug sensitivity profiles of the cancer cells. This has implications for cancer diagnosis, design of effective drug combinations and precision medicine.
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Financial disclosures and potential conflicts of interest

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Fig. 1 Overview of sample flow and experimental design. A) Schematic diagram of kidney, associated adrenal gland, infiltrating vena cava and the approximate sampling sites indicated. The study includes four patients (RCC.1-4) who underwent unilateral nephrectomy because of kidney tumor. Tissue from two patients with infiltration into vena cava (RCC1, 3) and one with metastatic tissue from the adrenal gland (RCC.3) was included. Patient-derived cells (PDCs) were established from normal and cancer tissue from all patients included in the studies. Every tissue piece was processed the same way, PDCs were generated by co-culturing with feeder cells in F-media. B) The top parts frozen for molecular profiling, while adjacent parts were fixed with PFA for histological characterization of each tissue piece. PDCs were grown and subjected to genomic and histological analysis, compared to the tissue to determine representativity (upper panel), and for the drug profiling with at least 460 oncology compounds (lower panel). C) Clonal relationship of tissue and PDC, and intra-tumor heterogeneity were determined through constructing phylogenetic trees (upper panel). Potential pharmacogenomics associations and impact of clonal heterogeneity were proposed based on results from the molecular profiling and drug testing (lower panel).

Fig. 2: Comparison of genomic alterations and renal specific genetic changes identified clonal relationship between parental tumor tissue and corresponding PDCs: A) somatic CNA similarities between tissue and PDC in RCC.1 (B) RCC.3. (C) selected CNA visualization of RCC specific genes adapted mainly from KEGG pathway. D) Cancer-specific mutations (colored) in patient’s tissue and respective PDC in comparison of recurrent driver (base) mutations reported in TCGA database for ccRCC. Exact non-synonymous mutations in each patient’s tissue/PDCs are displayed with the same color code. The indel/CNV were not included in the % evaluation of these genes from the TCGA databases. E) VHL mutation frequencies in the cancerous tissues and the same base mutation in the PDCs (RCC.1-3). Frequencies were calculated based on number of tumor specific reads in the exome/whole genome sequencing.

Fig. 3: Phylogenetic trees demonstrate clonal relevance and intra-tumor heterogeneity of tissue and derived PDCs. A-B) clonal relationship and intra-tumor heterogeneity of RCC.1,3. similar mutations across different samples in a node, and
samples with different set of mutations placed in distinct nodes within the tree. All of
nodes had arisen form a single hypothetical clone called “Germline”, that is
representing the genetic architecture of the normal tissue/blood of same patient. A) RCC.1 B) RCC.3. The numbers in each node refers to the mutations that were used to
trace the clonal line. The line number indicating similarity score (max 1) and color is
derived from the previous node.

Fig.4: Individualized cancer-specific sensitivity profiles of RCC.1, RCC.3
displayed responses towards notable drugs. A) The comparison of original DSS
indicating differences in drug responses in the whole drug library between RCC.1
primary-cancer vs vena cava PDC, B) RCC.3 primary-cancer vs metastatic PDCs, and
C) RCC.3 vena cava vs metastatic PDCs. Some of the drugs with noticeable
differences are indicated with red-colored data points. D) Dose response curve fit for
few example drugs that exhibit specific efficacies in RCC.1 primary cancer vena cava
PDCs and E) RCC.3 primary cancer, vena cava and metastatic PDCs.

Fig.5: Distinct genomic features leads to diverse drug response across PDCs. (A-E)
Clonal hierarchy of RCC.1 PDCs exhibited drug efficacies and retention of
genomic alterations (GA). The grey-lined boxes within the hierarchal lines
represented the recurrent GA: base mutations (black bold fonts), CNA (blue font
deletion, red font amplification). Both primary cancer and vena cava PDCs (A)
harboring GA in VHL, PBRM1, EGFR, PIK3C2A, KDM5C, and CDKN2A/B (B)
exhibited drug efficacies. (C) distinct GA in both vena cava and primary cancer PDCs
including NRAS, RAF, SETD2 and BRD1, ATR, TCF7, TNM1 respectively. (D)
Specific responses in primary cancer (E) vena cava PDCs.
All the RCC.3 PDCs (F) retained GA (G) showed drug efficacies. (H) Vena cava
separated with a sub-clone containing ZNF800 and PIK3CG. (I) while primary cancer
and metastatic PDCs shared some common mutations (e.g. ARID1A, MACF1) (J)
drug efficacies. (K) Distinct GA in primary cancer PDCs: VEGFA, SPEN; Metastatic
PDCs: VHL, EGFR, CDKNA2A/B). (L) Specific drug responses in metastatic (M)
primary cancer (N) vena cava PDCs. The bar graphs plotted with DSS values,
threshold of >5 in atleast one sample.’1’ at the arrow in Fig A and F indicating the
drugs that showed substantial responses in all the PDC derived from the patient. ‘2’
at the arrow in Fig I indicating the drugs that showed responses in primary cancer and
metastatic but not *vena cava* PDCs. All other responses showed in graphs are largely site specific.
Fig. 1

A Patient material and model development
- primary cancer, adrenal metastatic
- benign region, vena cava
- RCC 1, RCC 2, RCC 3, RCC 4
- collected tissues
- Patient-derived cells

B Characterization and functional testing
- Genomics: Mutation/CNA
- Histopathological analysis
- RNA expression
- Drug testing with 460 oncology compounds

C Outcome
- Phylogenetic trees
- Clonal evolution and tumor heterogeneity
- Drug response

Impact
Fig 4

A  

B  

C  

D  

E  

DSS: Primary cancer

DSS: Vena cava

DSS: Metastatic

ZSTK474

BMS-754807

Topotecan

Gemcitabine

JQ1

ABT-751

SN-38

Docetaxel

OTX015

Camptothecin

Tivozanib

Topotecan

Regorafenib

Molar concentration

Molar concentration

Molar concentration

Molar concentration

Molar concentration

% inhibition

% inhibition

% inhibition

% inhibition

% inhibition

% inhibition

% inhibition

% inhibition

% inhibition

% inhibition

% inhibition

% inhibition
<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>WHO grade</th>
<th>cTNM/ pTNM</th>
<th>Tissue source</th>
<th>Pre-operative treatment</th>
<th>Gender/ age</th>
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<tbody>
<tr>
<td>RCC.1</td>
<td>Clear cell carcinoma</td>
<td>G2</td>
<td>T3cN0/ pT3N0</td>
<td>Nephrectomy</td>
<td>none</td>
<td>Male/61</td>
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<tr>
<td>RCC.2</td>
<td>mixed clear cell, collecting duct carcinoma</td>
<td>G4</td>
<td>T4NxM1/ pT4N0</td>
<td>Nephrectomy</td>
<td>N/A</td>
<td>Male/40</td>
</tr>
<tr>
<td>RCC.3</td>
<td>Clear cell carcinoma</td>
<td>G3</td>
<td>T3N0M1/ pT3bNxMx</td>
<td>Nephrectomy</td>
<td>follow-up without treatments</td>
<td>Male/68</td>
</tr>
<tr>
<td>RCC.4</td>
<td>Clear cell carcinoma</td>
<td>G3</td>
<td>T3NxM1/ pT3aNxM1</td>
<td>Nephrectomy</td>
<td>pazopanib, sunitinib</td>
<td>Male/75</td>
</tr>
</tbody>
</table>

Table I:
Patient information and tumor characteristics.