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

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Renal cell cancer (RCC) has become a prototype example of the extensive intratumor 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 our 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, KDM5C and TSC2 genes. The drug testing highlighted vulnerability in the PDCs toward 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 patient with cancer 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.

Genomic and transcriptomic studies have shown significant inter- and intratumor heterogeneity (ITH) in clear cell renal cancer (ccRCC).1–3 The discovery of inactivating Von Hippel–Lindau (VHL) mutations and aberrant mTOR/PITK 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 remains 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.\textsuperscript{16} In contrast, two-dimensional primary cell models, provide for material toward more systematic drug tests, but are often challenging due to overrepresentation of normal cells.\textsuperscript{13} In addition, these models are often established from single sites not representing the complexity of the disease. Therefore, studies of the inter- and intrapatients’ 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.\textsuperscript{10,17}

Here, we developed representative patient-derived cells (PDCs) from normal and (multiple) cancerous RCC tumor regions using conditional-reprogramming-cell-(CR) technology.\textsuperscript{18,19} The genomic profiles and drug testing data were compared to 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.

\section*{Material and Methods}

\subsection*{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 were selected by a board-certified pathologist and was processed as illustrated in Figure 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 PDCs establishment (Supporting Information Table S1).

\subsection*{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 \textit{vena cava} \((n = 2)\), adrenal gland metastatic sites \((n = 1)\). Cultures were established using CR technology.\textsuperscript{18,19} Briefly, cells were isolated from the original tissue after chopping into small pieces and with collagenase \((40 \text{ units/ml})\) treatment for 2–4 h. The suspension put into culture dish having 30–50% confluent irradiated Swiss 3T3 fibroblast feeder cells \((J2\text{ strain})\). The co-cultured maintained in an 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 \((Y-27632,\text{ Enzo Life Sciences, Lausen, Switzerland})\). Separation of 3T3 cells from the PDCs during passaging was done using differential trypsinization. Drug testing was performed between 2 and 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 Supporting Information Table S2.

\subsection*{Exome sequencing}

The DNA extraction from the PDCs was done at the same passage as performing the drug sensitivity testing, that is,
3–6 weeks after the establishment of the cultures. The extraction of genomic DNA from original tissues, PDCs and germ-line 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). Sequencing was executed using a HiSeq 2500 instrument (Illumina, San Diego, CA). 4 × 10⁷ and 1 × 10⁸ 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) Supplementary Table S3. 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 Table S4. 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 S5).¹⁸,¹⁹ 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 1 nM–100 μM concentration range) and let them grow for 72 h. The cell viability

Figure 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 intratumor 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). [Color figure can be viewed at wileyonlinelibrary.com]
was measured with CellTiter-Glo (CTG, Promega, Madison, WI). 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 multiparameter area under the curve sensitivity method named, the drug sensitivity score (DSS). To focus on the drugs with clear effects or lack thereof, we applied a DSS value of 5 as a cut-off. The obtained from dose–dose response curve fits illustrated in Figures 4d and 4e were made through GraphPad Prism 6. XY-correlation plots shown in Figure 4 and Supporting Information Figure 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 11 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. The histopathological analysis and phenotypic retention of PDCs were assessed by immuno-histochemical staining of carbonic anhydrase IX (CA9/CAIX) expression (Supporting Information Figs. 1 and 2). Several studies have shown that CAIX is constitutively overexpressed in ccRCC, and has been used to establish the relevance of ex vivo cancer models. In our analysis, a significantly higher protein expression of CAIX was quantified in most cancerous tissues as compared to the normal (Supporting Information 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 (Figs. 2a–2c, Supporting Information Fig. 3). Some of these shared exact break-points highlighting the clonal relation between the PDCs and their originating tissue. Patients in our study carried recurrent alterations in RCC reported by The Cancer Genome Atlas (TCGA). For example, the 14(del) and 3p(del) that are the most frequent site of nonrandom aberrations (Supporting Information Fig. 3a) with the 3p harboring frequently mutated tumor suppressor genes VHL, PBRM1, SETD2, and BAP1. Additionally, we observed Copy Number Alterations (CNAs) in several genes of known relevance to RCC including EGFR amplification and CDKN2A/B deletion (Fig. 2c). In a recent study, deletion at 9p and 14q have been shown as putative biomarker of metastasis in ccRCC. Interestingly, these CNAs are retained by both cancerous PDCs of RCC.1 and subclonally in metastatic RCC.3 tissue/PDCs (Figs. 2a–2c, Supporting Information 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 S3). 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 (Figs. 2d and 2e). Additional RCC.1 specific somatic mutations included PBRM1, (the second most frequently mutated gene in RCC), KDMS5C, 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, Supporting Information 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 (Supporting Information 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 (Supporting Information 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 S5). RCC.1 and RCC.3 PDCs allowed for intrapatient comparison of drug profiles and distinct sensitivities for several key oncology drugs.

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**Figure 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 nonsynonymous 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. [Color figure can be viewed at wileyonlinelibrary.com]
were observed for PDCs from the primary tumor, vena cava tumor and for RCC.3 in the metastatic-site (Fig. 4, Supporting Information Table S6). 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 (Figs. 4a and 4d). The RCC.1 vena cava PDCs were sensitive to an antimitotic 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 (Figs. 4a and 4d, Supporting Information Table S6).26 Interestingly, pazopanib a multikinase-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 (Supporting Information 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 (Figs. 4b, 4c, and 4e).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 intratumor 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 nonsynonymous 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 toward 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 (Supporting Information Table S6).28 The primary cancer PDCs displayed additional mutations in bromodomain-containing-1 gene (BRD1) as well as 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, Supporting Information Table S6). The RCC.1 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 BRD4 and IGFN1 and showed shared sensitivity to
mTOR/PI3K, BET and topoisomerase-inhibitors (Figs. 5f and 5g). BRD4 is a major target for BET inhibitors and therefore this results suggests a potential link with the vulnerabilities to the observed GA. The RCC.3 PDCs separate into two sub-branches harboring distinct sets of mutations (Figs. 5h and 5i) where the branch containing the primary cancer and adrenal

Figure 4. Individualized cancer-specific sensitivity profiles of RCC.1, RCC.3 displayed responses toward notable drugs. (a) The comparison of original DSS indicating differences in drug responses in the whole drug library between RCC.1 primary-cancer versus vena cava PDC. (b) RCC.3 primary-cancer versus metastatic PDCs, and (c) RCC.3 vena cava versus 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. [Color figure can be viewed at wileyonlinelibrary.com]
Figure 5. Distinct genomic features lead to diverse drug response across PDCs. (a–e) Clonal hierarchy of RCC.1 PDCs exhibited drug efficacies and retention of genomic alterations (GA). The gray-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, PIK3CA, 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, CNOT2A/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 at least one sample. “1” at the arrow in (a) and (f) indicating the drugs that showed substantial responses in all the PDC derived from the patient. “2” at the arrow in (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. [Color figure can be viewed at wileyonlinelibrary.com]
metastatic PDCs show shared sensitivity to temsirolimus, gemcitabine and cabazitaxel (Fig. 5j). 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 (Figs. 5l and 5m). In contrast, in the vena cava branch the PDCs were mostly sensitive to two VEGFR-inhibitors, tivozanib and regorafenib, approved for other malignancies (Fig. 5n, Supporting Information 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 our 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. Interestingly, these CNAs were also detected in the metastatic RCC.3 tissue/PDCs, but were not detectible in the primary or vena caval 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 (Figs. 4 and 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 patients.

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.4. 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.3 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 Figs. 2 and 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/PDGFRAxis 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 multikinase inhibitory action or off-side effect (Supplementary Table S5). 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. Furthermore, novel clinical trial approaches are being instituted under consideration the context of cancer evolution and subclonal oriented therapeutic approaches.8,19 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, Turañić 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 addition to the promises of patient-derived models regarding identification of the most appropriate therapeutic algorithms through integration of molecular profiling with \textit{ex vivo} drug dependencies of individual patients. In conclusion, we suggest that \textit{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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