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Overcome Tumor Heterogeneity-Imposed Therapeutic Barriers through Convergent Genomic Biomarker Discovery: A Braided Cancer River Model of Kidney Cancer

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Abstract

Tumor heterogeneity, encompassing genetic, epigenetic, and microenvironmental variables, is extremely complex and presents challenges to cancer diagnosis and therapy. Genomic efforts on genetic intratumor heterogeneity (G-ITH) confirm branched evolution, support the trunk-branch cancer model, and present a seemingly insurmountable obstacle to conquering cancers. G-ITH is conspicuous in clear cell renal cell carcinoma (ccRCC), where its presence complicates identification and validation of biomarkers and thwarts efforts in advancing precision cancer therapeutics. However, long-term clinical benefits on targeted therapy are not uncommon in metastatic ccRCC patients, implicating that there are underlying constraints during ccRCC evolution, which in turn force a nonrandom sequence of parallel gene/pathway/function/phenotype convergence within individual tumors. Accordingly, we proposed a “braided cancer river model” depicting ccRCC evolution, which deduces cancer development based on multiregion tumor genomics of exceptional mTOR inhibitor (mTORi) responders. Furthermore, we employ an outlier

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case to explore the river model and highlight the importance of “Five NGS Matters: Number, Frequency, Position, Site and Time” in assessing cancer genomics for precision medicine. This mutable cancer river model may capture clinically significant phenotype-convergent events, predict vulnerability/resistance mechanisms, and guide effective therapeutic strategies. Our model originates from studying exceptional responders in ccRCC, which warrants further refinement and future validation concerning its applicability to other cancer types. The goal of this review is employing kidney cancer as an example to illustrate critical issues concerning tumor heterogeneity.

Keywords

Intratumor heterogeneity (ITH); Intermetastases heterogeneity (IMH); Branched/divergent evolution; Parallel/convergent gene/pathway/function/phenotype evolution; Braided kidney cancer river model; Targeted therapy; Genomic predictive biomarker; Precision medicine

1. Introduction

With the advent of modern molecular technologies, especially Next Generation Sequencing (NGS), in studying cancers, it became technically and economically feasible for scientists to interrogate genomic and transcriptomic landscapes of human cancers at large scale not only in bulk but also single cell resolution with unprecedented base-pair precision [1–3]. With the international cancer genomic efforts over the past few years, including TCGA [4] and ICGC [5], we now generally regard cancer as a disease resulting from dysregulated evolution [6, 7] in acquiring inheritable genetic/epigenetic traits [8–10] and at the same time are startled by the unfathomable complexity concerning tumor heterogeneity [11–14]. In various human cancers such as lung cancer, it is now standard of care to use genetic alterations in exploring cancer biology and selecting patients for targeted therapies [15, 16]. However, clinical outcomes are highly variable among cancer patients. Among all contributing factors, tumor heterogeneity likely plays a central role [17–21]. Tumor heterogeneity is complex in many levels, including interdisease and interpatient heterogeneity, intertumor, intratumor and intermetastases heterogeneity, tumor-intrinsic genetic, epigenetic and metabolic heterogeneity, tumor-microenvironment heterogeneity, etc. A landmark paper by Gerlinger et al. demonstrated clear cell renal cell carcinoma (ccRCC) as a tumor with marked genetic intratumor and intermetastases heterogeneity (G-ITH and G-IMH) [22]. Here we utilize knowledge obtained from studying kidney cancer biology [23, 24], genomics [25–27] and therapeutics [28, 29] to illustrate how ccRCC evolves with certain inherent evolutionary principles [30] that could be exploited for therapeutic purposes through a “braided cancer river model”.

2. Layering out tumor heterogeneity

2.1. Macroscopic heterogeneity

Tumor heterogeneity takes places at levels ranging from single cancer type of an individual patient through single tumor cell to single biomolecule. To ease further discussions, we would like to use this opportunity to layering out different levels of complexity in tumor

heterogeneity. At the human level factors at play include race, gender, age, genetics, and host-environment interactions, such that identical twins differentially develop despite sharing the same genetic material. There are ~7.4 billion individuals in the world and ~14.9 million new cancer cases diagnosed each year [31], presenting astronomical heterogeneity. At a macroscopic level, it is pertinent to first categorize cancer based on organ and tissue origins, such as brain, lung, liver, pancreas, stomach, colorectal, kidney, bladder, skin, bones, muscles, and blood [31], which also carry distinct genomic features [32, 33].

2.2. Microscopic heterogeneity

In 2015, there are ~1.66 million new cancer cases diagnosed in the USA, among which ~62,000 have cancer in the kidney [34]. To illustrate tumor heterogeneity at the microscopic, e.g. histological level, we will use kidney cancer as an example. Renal cell carcinoma (RCC) is a group of malignancies arising from the epithelium of renal tubules, and accounts for >90% of kidney cancer [35, 36]. RCC encompasses a heterogeneous group of tumors and is primarily classified based on histopathological features [37]. Major subtypes are clear cell RCC (ccRCC; ~75%) [38], papillary RCC (pRCC, ~15%) [39], and chromophobe RCC (chRCC, ~5%) [37, 40–43]. Unclassified RCC (uRCC) accounts for 4–5% of RCC that is not classifiable [36, 44] as one of the major (>5%) or the rare (<1%) subtypes such as medullary, collecting duct, mucinous tubular and spindle cell carcinoma, and MiTF family translocation renal cell carcinoma [36, 45]. Importantly, individual histological subtypes of RCC carry distinct clinical histories and therapeutic outcomes [37, 46], highlighting the importance of microscopic heterogeneity.

2.3. Molecular heterogeneity

Recent molecular technologies, especially NGS, have begun to provide molecular details concerning individual RCC subtypes [24, 37–39, 44, 47]. As ccRCC is most prevalent, most lethal, and best studied among RCC [27], we focus on the molecular heterogeneity of ccRCC in this section.

2.3.1. Genetics—The *Von Hippel-Lindau* (*VHL*) gene inactivation was the only known oncogenic driver in ccRCC for decades [48] until recent large-scale cancer genomic projects discovered novel, prevalent mutations including *PBRM1/BAF180* (29–41%), *SETD2* (8–12%), *BAP1* (6–10%), and *KDM5C* (4–7%) [25, 38, 49, 50]. Remarkably, these genes encode chromatin and epigenetic regulatory proteins, and most mutations are predicted to result in functional loss, favoring their roles as tumor suppressors [27]. The *VHL* tumor suppressor gene (TSG) is the most frequently mutated gene in ccRCC [48, 51], and its complete loss constitutes an early, truncal oncogenic driver event [52–54]. *VHL* is an E3 ligase that degrades Hypoxia-inducible factor (HIF) 1 α and 2 α [55]. Thus, human ccRCC is highly vascular due to uncontrolled activation of HIF α targets that regulate angiogenesis [56]. Thereby, anti-vascular endothelial growth factor (VEGF) agents are effective, first-line treatment for metastatic ccRCC (mRCC) [56, 57]. The long latency (>30 years) for *VHL* germline mutated patients to develop ccRCC [52, 58] and the inability of *Vhl* deficient mouse to develop ccRCC [59] suggest that additional genetic/epigenetic events are probably needed during the pathogenesis of ccRCC in mammals [30]. As *VHL* loss is the primary truncal event of ccRCC, its mutation status did not correlate with cancer survival [60].

Among the prevalent non- *VHL* alterations, *BAP1*, *SETD2*, and *TP53* mutations are associated with a poor prognosis [26, 61, 62]. Interestingly, patients with *BAP1*-mutant tumors had significantly shorter overall survival when compared to patients with exclusively *PBRM1*-mutant tumors [63]. Hence, somatic mutations in ccRCC are of clinical significance, lending support for the cancer genomics-based molecular classification of ccRCC, which will be discussed later in details.

2.3.2. Beyond genetics—The basic functional unit with a multi-cellular organism including human is a single cell that inherits a set of DNA which serves as template for regulated RNA synthesis to produce protein in carrying out biochemical transactions. DNA is stable and yet subject to modifications such as CpG island methylation whereas RNA, protein, and metabolite are more labile and subject to myriad modifications and regulations. DNA, RNA, protein, and metabolite generate heterogeneity by itself and in compound network, defining the epigenetic state of a live cell, which is also subject to stochastic changes. Hence, one critical component of ITH beyond genetics is epigenetics [12, 64]. Furthermore, sizable tumors normally consist of millions to billions of cells, encompassing not only cancer cells, but also endothelium, fibroblast and immune cells etc. that in aggregate constitute the tumor microenvironment. Accordingly, one key component of the tumor heterogeneity resides in the tumor microenvironment [65, 66]. The importance of the tumor microenvironment in cancer pathogenesis and therapeutics has been elucidated in many cancers [66, 67]. Exemplified in ccRCC, VEGF inhibitors are the frontline treatment and they inhibit tumor growth through disrupting tumor blood vessels instead of directly affecting tumor cells [57, 68].

2.4. Intermetastases heterogeneity (IMH): the seeding, evolving, spreading cycle

The main cause of cancer related mortality is distant metastasis that entails diverse molecular mechanisms [69–72], imposes constraints for cancer development [30, 73, 74], and presents treatment challenges. The complexity of cancer cell seeding within individual patients is best demonstrated by Gundem et al. [75]. In this study, the authors looked into not only primary-to-metastasis spread but also metastasis-to-metastasis spread in 10 lethal prostate cancer cases. They found that metastasis-to-metastasis colonization was common, metastasis-to-metastasis spread initiated through either de novo monoclonal seeding or simultaneous transfer of multiple tumor clones, and multiple colonizing events took place among metastases over time [75]. Importantly, such complex metastatic spread patterns could have conferred resistance mechanisms to targeted therapy.

3. Cancer evolution according to Darwinian principles

Charles Darwin stipulated that all species of organisms arise and develop through the “natural selection” process of small, inherited variations that increase the individual's ability to compete, survive, and reproduce. All biological materials including DNA are mutable, permitting heritable changes and thereby evolution. DNA is generally inert and multicellular organisms are equipped with DNA replication machinery that operates at high fidelity and yet allows for subtle changes. The creation, rapid proliferation, migration, and ultimate demise of individual cancer cells are at no exception. Cancer cell originates from the

identical set of DNA to its host cell and yet acquires new mutations/phenotypes at an uncontrollable pace to compete with its host for the same available resource. The drastic, uncontrollable, rapid evolution of cancer cells ends with demise of not only the host but also the renegade cancer cell that is constantly at a chaotic, heterogeneous state.

3.1. Branched evolution in cancer genomics

Central to the cancer evolution principle is the ability of cancer cells to rapidly diversify and compete as “clonal evolution of tumor cells” proposed by Nowell in 1976 [6], which helped explain the observed tumor heterogeneity by Heppner et al. in 1977 [11] and favored linear evolution in creating the fittest clone [8]. The extensiveness of genetic tumor heterogeneity in human cancer was underappreciated till Gerlinger et al. examined tumor DNA of different regions of ccRCC and reported “intratumor heterogeneity and branched evolution revealed by multiregion sequencing” [22, 76]. Since then, branched evolution has been demonstrated as a common phenomenon of many human cancers such as breast cancer [77], prostate cancer [78], and acute lymphoblastic leukemia [79, 80], whereas linear evolution has been detected in cancers including metastatic medulloblastoma [81] and acute myeloid leukemia especially at relapse [82, 83]. Altogether, the trunk-branch model concerning intratumor heterogeneity pinpoints the strategy for cancer control by targeting “trunk” lesions, which is consistent with the known successful administration of multiple kinase inhibitors in treating human cancers with obvious truncal mutations at the respective kinases [84].

3.2. Convergent evolution in genes/pathways/capabilities/phenotypes

Another key finding in the Gerlinger et al. paper is that different regions of the tumor have different mutations in the very same genes, especially at *SETD2*, which underscores the importance of changing particular tumor-cell functions as the tumor expands and evolves [17, 22]. Hence, it was suggested that tumor would do whatever is necessary to activate or inactivate “certain” genes [85] through convergent evolution [17]. This observation is consistent with the “Hallmarks of Cancer” concept proposed by Weinberg et al. [86] where certain biological capabilities are acquired during the multistep development of human tumors [73]. In other words, convergence on genes, pathways, and capabilities/functions confers the ultimate phenotypes in cancer evolution [30].

3.3. Decoding cancer genomic and therapeutic heterogeneity through a braided cancer river model

For ccRCC, the past decade heralded the development of two classes of targeted agents that have collectively improved overall survival of patients with metastatic ccRCC from approximately one year on cytokine therapy to nearly three years [30, 87]. These two classes of agents target distinct pathways involved in ccRCC tumorigenesis, namely the VEGF and mTOR pathways [56]. In metastatic ccRCC patients receiving mTORC1 inhibitors, the median PFS is <6 months; however, some patients experience markedly longer survival periods [28, 88]. For example, the outlier cohort of five patients with metastatic ccRCC that we reported had a median PFS of 28 months [28]. In three patients, multiregion analysis revealed marked intratumor heterogeneity; however, at the same time, results revealed phenotypic convergence upon mTOR pathway activation [28, 30].

One key lesson learned from studying targeted therapeutic outliers of a given cancer type is the recurrent theme about convergent evolution on select sets of oncogenic pathways [19, 28, 30]. Importantly, such pathway or phenotype convergence takes place within tumors, among tumors, and is probably shared by the same histopathological subtype of a given cancer type despite tumor heterogeneity [30]. For example, mTOR pathway activation due to either *MTOR* activation mutations [23, 89] or *TSC1* loss-of-function mutations occurred at high frequencies in ccRCC [41, 88] where *VHL* mutation is the universal tumor-initiating event [27]. This could explain why the two main categories of targeted agents approved for the treatment of metastatic ccRCC are inhibitors of VEGF or mTOR signaling pathways [56]. It also supports the notion that cancer metabolism plays key roles in ccRCC pathogenesis [24, 47, 90, 91].

As *VHL*-loss and the resulting HIF hyperactivation are nearly universal in the pathogenesis of ccRCC, it would predict VEGF inhibitors such as Sunitinib, Pazopanib, and Axitinib to be more efficacious than mTOR inhibitors such as Everolimus and Temsirolimus in ccRCC [57]. This was indeed supported by randomized clinical trials comparing these two classes of agents in metastatic RCC (mRCC) patients. For example, Record-3 a randomized trial comparing Sunitinib with Everolimus in previously untreated mRCC patients demonstrated median progression-free survival (PFS) with Sunitinib at 10.7 months and Everolimus at 7.9 months [92].

While the trunk-branch model nicely depicts ITH within a mass [22, 76, 93], it lacks the capability to describe the functional relevance of heterogeneous mutations and illustrate what may be clinically significant convergence events at the gene/pathway/function level [76]. Based on the findings of convergent pathway evolution in ccRCC therapeutic outliers [19, 28, 30], kidney cancer growth may be better visualized as a braided river with the capacity to diverge and converge (Figure 1) rather than an ever-branching tree [6]. The source of the river is analogous to the trunk mutations and contains the ubiquitous driver events, generally the initiating drivers such as *VHL* mutation and chromosome 3p loss (Figure 1). The heterogeneous mutations previously ascribed to the branches of the tree model become tributaries along the river, retaining the capability to become driver mutations and to converge with other spatially or temporally distinct mutations affecting the same gene or components along critical oncogenic or tumor suppressor pathways inherent to a given cancer type [30]. Study of one patient is highly instructive, in which two distinct and spatially separate mutations in *TSC1* and *MTOR* join along the PI3K/AKT/mTOR pathway and activate mTOR kinase through different mechanisms [28]. Our exceptional responder analysis shows that the braided river model allows for a novel visualization of subclonal events and has the potential to summarize heterogeneous genetic and epigenetic alterations into clinically relevant information [19, 30]. This model illustrates parallel and convergent events occurring throughout tumorigenesis. Starting from initiating driver mutations, this model depicts the stepwise acquisition of different driver mutations (early, intermediate, late and speedy drivers) during cancer evolution (Figure 1).

4. Case Study and the Five NGS Matters

4.1. Case presentation

A 55-year-old male presented to our genitourinary oncology clinic two years after undergoing a left radical nephrectomy. The resected tumor was found to be ccRCC, measuring 6.5 cm, with a Fuhrman grade of III/IV and no evidence of local invasion. Subsequent surveillance with cross-sectional imaging revealed multiple pulmonary nodules varying from 2 mm to 8 mm in size. He continued surveillance for subcentimeter nodules based on guidelines described in previous studies. After one year of surveillance, the patient reported a productive cough along with night sweats and fatigue. Repeat imaging showed an increase in the size of his pulmonary nodules and biopsy of a paratracheal lymph node was consistent with metastatic ccRCC. The patient was then started on Sunitinib, which was shortly discontinued because of toxicity. He was then started on Everolimus and remained on this therapy for four years without progression of disease. A timeline of his clinical course is depicted in Figure 2A.

4.2. Genomic investigation

Sequencing analysis of his primary tumor with MSK-IMPACT [94], a massively parallel ultradeep targeted exon capture NGS assay, detected chromosome 3p loss and somatic mutations in *VHL*, *AKT2*, *PBRM1*, and *BAP1* (Figure 2B and 2C). The *PBRM1* gene is the second, and the *BAP1* gene is the fourth most commonly mutated gene in ccRCC [27]. Intriguingly, the co-occurrence of *PBRM1* and *BAP1* mutations in ccRCC tumors has been shown to associate with a very poor clinical outcome [50], which contrasts the observed, exceptional clinical outcome of our patient. Accordingly, we hypothesized that the primary tumor could have multiple subclones, and *PBRM1* and *BAP1* mutations might have been diverged (Figure 3 Scenario 2) instead of converged (Figure 3 Scenario 1). Due to our patient's surprising clinical course, we elected to sequence one of his metastatic sites after his disease progressed after four years of Everolimus treatment (Figure 2). As shown in Figure 2C, genomic analysis of the metastatic bone lesion was significant for somatic mutations in *VHL*, *PBRM1*, *AKT2*, and *HIST1H1C*. Most importantly, the *BAP1* mutation found in the primary tumor was absent in the bone metastasis, which favors the intratumoral heterogeneity stipulated as Figure 3 Scenario 2 where *PBRM1* and *BAP1* mutations were present in different subclones within the primary tumor. Of note, the presence of *HIST1H1C* in his metastatic site but not his primary supports the further evolution of the metastatic cancer.

4.3. Treatment outcome

Upon the initial evaluation, the patient was diagnosed with stage IV ccRCC and began treatment with VEGF inhibitor Sunitinib as the standard first-line of care [95]. Because of toxicity, Sunitinib was discontinued after 3 months. This patient began treatment with the mTOR inhibitor Everolimus at 10mg daily, to which he demonstrated a long-lasting durable therapeutic benefit for four years without disease progression. Surveillance imaging showed progression of disease after four years on Everolimus, which demonstrated new metastatic lesions in the spine and ribs. At this time Everolimus was discontinued and the patient was started on another VEGF inhibitor, Axitinib. After a few months on Axitinib, he progressed

with an obstructive mass in the lung and received radiation therapy. Follow-up imaging showed further progression of osseous lesions with cord compression, which was managed with laminectomy. One month later, imaging showed an increase in the size of the pulmonary nodules and new pancreatic metastatic sites. This patient began checkpoint blockade immunotherapy with Nivolumab, an anti-PD1 monoclonal antibody [96], and has remained on treatment at the time of this report.

4.4. Sequencing primary vs. metastatic tumor

This case highlights several interesting points, especially in the era when we are considering precision medicine approaches for cancer care. Our patient's exceptional response to Everolimus was particularly surprising, especially in view of his mutations of *BAP1* and *PBRM1* present in the primary tumor, which would suggest an aggressive disease course [50]. This finding led to an investigation of the clonal composition of his primary and metastatic tumors. Using this data, we postulated that there were two clinically relevant clones present in the patient's primary tumor (Figure 3). By analysing the tumor variant allele frequencies (VAF) from the sequencing results in both his primary and metastatic tumors, we identified several interesting alterations and possible clonal subtypes that may reside in the patient's tumors.

For the patient's primary tumor we found truncal mutations in both *VHL* and *AKT2*, with what were presumed to be concurrent mutations of *PBRM1* and *BAP1* as demonstrated in Scenario 1 of Figure 3. In the Scenario 2 there are two clones, both of which contained the truncal mutations in *VHL* and *AKT2*. The first subclone contained an additional *PBRM1* mutation, whereas the second subclone contained a mutation in *BAP1*. The *PBRM1* mutation subclone was the clone later identified in his metastatic tumor while the *BAP1* mutation clone was absent. This latter scenario could explain his surprising clinical course (Figure 3 Scenario 2).

The primary tumor in ccRCC could carry many subclones, but not all of these will metastasize. For this reason, sequencing of a metastatic site would be more beneficial in identifying actionable targets for tailored systemic therapy. Sequencing of the metastatic site in our patient allowed us to identify which subclone had metastasized and recognize subsequent mutations that may contribute to disease progression or drug resistance. Analysis of this patient's primary and metastatic tumors shows that the *AKT2* mutation was a truncal event in this patient's tumorigenesis. The AKT2 protein is involved in the mTOR pathway. AKT family proteins activate the mTORC1 signalling pathway. Hence, the patient's prolonged treatment response to Everolimus could be possibly explained by the *AKT2* mutation, which awaits functional confirmation.

4.5. NGS matters in cancer genomics

To improve precision cancer medicine based on NGS data, we recently proposed an NGS checklist encompassing "Five NGS Matters: Number, Frequency, Position, Site and Time" to assist physicians and researchers in evaluating cancer patients [19]. In that essay, we examined several key components for the clinical interpretation of NGS results with a step-by-step approach. The first consideration is the fact that "number matters." A higher

sequencing coverage of tumor (e.g. 500–1000×) will provide better resolution and offer confidence in identifying genomic aberrations. This is especially the case if the aberration is relatively rare or occurs at a low frequency in the study population. In our case, the patient's primary and secondary sites were examined using a panel of 341 genes and 410 genes, with coverage of 628 and 947, respectively.

Secondly, “frequency matters,” which was highlighted in this case our patient. As seen in Figure 2C, while *VHL* mutation (30%) is the truncal event of this patient, as commonly occurred in the majority of ccRCC tumors, the detected *AKT2* mutation was equally prevalent (26%), suggesting that *AKT2* mutation could also be truncal. Thirdly, “position matters”, the missense N325S mutation of this *AKT2* occurs at the kinase domain, which could have rendered enhanced *AKT2* kinase activity. Accordingly, tumor cells or clones with this *AKT2* mutation are likely highly dependent on the constant activation of the mTOR pathway, making them exquisitely sensitive to drugs like Everolimus, which is in line with the braided river model outlined in Figure 1 [30].

Important to our case is also the notion that “site matters.” We saw with our patient that sequencing from a primary and metastatic site demonstrated different mutations. This notion highlights the fact that not all subclones will metastasize and that tumor heterogeneity, especially in the primary tumor, may prevent adequate sampling and identification of all clinically relevant subclones [93]. While our patient had mutations of both *PBRM1* and *BAP1* in the primary kidney tumor, sequencing from the bone lesion from the rib suggested that the *PBRM1* subclone had metastasized.

Lastly, “time matters,” meaning that the timeline in which a patient's tumor is sequenced and whether or not they have been treated beforehand can be of significance. In our patient, the initial tumor did not show the *HIST1H1C* mutation. We can postulate that as the patient's disease progressed and his cancer evolved, further mutations occurred or adapted, possibly as a result of systemic treatment he received. Although our patient had an exceptional response to therapy, his tumor did eventually progress and the lack of predictability of treatment response based on later mutational events limits disease management.

Here we describe a case in which analysis of the patient's primary tumor suggested concurrent *PBRM1* and *BAP1* mutations, predicting an aggressive and unresponsive clinical course. However, the patient had an exceptional response to the mTOR inhibitor Everolimus for 4 years. Analysis of a metastatic site after the patient progressed revealed a truncal event in the mTOR pathway gene *AKT2* and a concurrent *PBRM1* mutation. No *BAP1* mutation was found in his metastatic tumor. Based on primary-metastatic tumor pair genomic sequencing, *PBRM1* and *BAP1* mutations diverged instead of converged in the primary tumor, and the *PBRM1* but not the *BAP1* mutant clone metastasized, which helps interpret the observed unexpected clinical outcome (Figure 3). Through harnessing the ultradeep NGS information gathered from the primary-metastatic tumor pair of this patient, we could offer molecular rationale underlying an unexpected clinical course.

5. The era of targeted therapeutics with genomics-precision in kidney cancer

The estimated new kidney cancer cases diagnosed in the United States and the world every year are ~63,000 and ~300,000, respectively [31, 34]. The implementation of targeted therapies, VEGF and mTOR inhibitors, in treating mRCC transformed an imminently lethal disease into a more manageable malignancy, and use of these agents improved patients' quality of life and prolonged survival. However, among patients treated with these agents, a wide range of clinical outcomes has been observed [97–100]. How to best choose patients that will have clinical benefit from individual class of targeted agents and how to sequence the use of individual targeted agents are major challenges [99, 101, 102]. Recent efforts have focused on determining whether genetic markers can predict treatment response [28, 60, 88, 103]. The largest study analyzing correlations between somatic mutations and treatment efficacy was a recently reported analysis based on the RECORD-3 trial [60]. RECORD-3 was a crossover, phase II study that compared first-line Sunitinib and second-line Everolimus versus the reverse sequence [92]. Four hundred and seventy-one patients were randomized. The primary end point, progression-free survival (PFS) non-inferiority of Everolimus as first line, was not met [92]. The median PFS was 7.9 months for first-line Everolimus, and 10.7 months for first-line Sunitinib, and combined median PFS was 22.4 months for sequential Everolimus-Sunitinib, and 32.0 months for sequential Sunitinib-Everolimus. This trial offered a unique opportunity to study correlations between somatic mutations and response to first-line VEGF-targeted therapy and mTOR inhibitors.

NGS of 260 individual mRCC patient archival tissue was performed to an average depth of ~560×. Of note, 220 of 260 have ccRCC. In general, patients had a longer PFS on Sunitinib than Everolimus (8.3 vs. 5.3 months, HR 1.85, 95% CI 1.25–2.75). Interestingly, patients with *PBRM1* mutations, however, had similar PFS to first-line Sunitinib or Everolimus (11.0 vs. 11.5 months, HR 1.07, 95% CI 0.66–1.74). In contrast to patients with *PBRM1* mutations, who did well regardless of therapy, patients with *KDM5C* mutations have a notable prolonged PFS on Sunitinib (20.6 months) versus Everolimus (9.8 months). This study provided the first evidence that somatic mutations could represent candidate predictive biomarkers [60]. A recent retrospective analysis also looked the correlation of molecular subclassification of clear cell RCC and response to targeted therapies. The investigators looked at correlations between longer responses to VEGF-targeted therapies and ccRCC mutations in a cohort of 31 patients. The authors created a heat map of the relative frequency of genomic alterations segregated by the duration of anti-VEGF treatment. Exceptional responses (defined as duration of therapy of more than 21 months) were associated with somatic genetic alterations in *KDM5C*, *PBRM1* and *VHL* [103], which was consistent with the findings from RECORD-3.

The mechanistic target of rapamycin (mTOR) pathway regulates cellular metabolism and growth and is important for the development of RCC [23]. MTOR is a serine/threonine kinase contained within the mTOR complex 1 (mTORC1), and is regulated in part through the TSC1/TSC2 protein complex [104, 105]. Temsirolimus and Everolimus are allosteric inhibitors of the kinase activity of mTOR and are approved for the treatment of advanced

renal cell carcinoma [95]. Although the pivotal trials of Temsirolimus and Everolimus showed a benefit over Interferon-alpha and placebo, respectively, only a minority of patients has significant responses [106, 107]. Nevertheless, some of these responses can be long lasting, even after treatment with VEGF-targeted therapies, so there is interest in identifying *a priori* the patients who will benefit from mTOR inhibitors. One method to identify markers predictive of response is to sequence tissue of extreme responders. In RCC, mutations in *TSC1* and *MTOR* are associated with response to mTOR inhibitors [28]. A large retrospective study of 79 patients with RCC treated with mTOR inhibitors confirmed that mutations in *MTOR*, *TSC1*, or *TSC2* were more common in responders [88]. Interestingly, in some responders with mutations, the allele frequency was as low as 7%, suggesting that subclonal mutations could also be clinically important. Notably, although the presence of these mutations can suggest patients more likely to respond to mTOR inhibitors, the inverse is not true. In that study, the majority of responders had no mutations in *MTOR*, *TSC1*, *TSC2* or other genes in the pathway (including *PIK3CA* and *PTEN*) [88]. Thus, the absence of actionable mutations should not limit the use of these medications in appropriate patients, which could be due to ITH and the issues concerning the five NGS matters.

In conclusion, we have begun to elucidate how the somatic mutation landscape and genetic heterogeneity of ccRCC tumors could impact clinical and therapeutic outcomes. Based on available data, distinct molecular subtypes of ccRCC according to the mutation status of *PBRM1*, *BAP1* and *KDM5C* could have potential biomarker values for patients with metastatic ccRCC treated with targeted agents. The treatment option for mRCC has continued to expand now to 12 drugs with 3 newly approved ones, i.e. immune checkpoint-blockade PD-1 antibody Nivolumab [96], VEGF/cMET inhibitor Cabozantinib [108], and VEGF/FGF inhibitor Lenvatinib [109]. Hence, the tumor heterogeneity of ccRCC not only provides challenges but also offers opportunities for precision cancer therapeutics aiming at increasing effectiveness, reducing cost, and most importantly prolonging and ultimately curing metastatic cancers.

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Hypothetical Gene/Pathway/Phenotype Convergence Model (Braided Cancer River)

Fig. 1. The cancer river model illustrates parallel and convergent events occurring throughout ccRCC tumorigenesis

The model depicts the step-wise, ordered accumulation of different driver mutations, the distinct time point in cancer development when CANCER (Continuous replication, Angiogenesis, iNvasion, Checkpoint bypass, Apoptotic evasion, Reprogramming in metabolism and cell fate) hallmarks have been acquired, and the chaotic point beyond which targeted therapies have limited effectiveness due to presence of aggressive subclonal “speedy” drivers that circumvent and resist therapy.

Fig. 2. Clinical and genomic overview of the two tumor samples taken from our patient
 (A) Timeline of clinical events including treatment history. (B) Copy number aberrations: copy number segments with mean log2 value greater than 0.2 were plotted (red indicates copy number gain and blue indicates copy number loss; the darker the color the more change). (C) Somatic non-synonymous mutations: the values are variation allele frequencies (VAFs).

Fig. 3. Two braided river models concerning this patient based on sequencing of the primary tumor

Both scenarios depicted mutations of *VHL* (30%), *AKT2* (26%), *PBRM1* (9%) and *BAP1* (9%). This tumor contains higher comparable mutation frequencies (26–30%) on *VHL* and *AKT2* and lower comparable mutations (9%) on *PBRM1* and *BAP1*. Scenario 1 assumes that mutations of *PBRM1* and *BAP1* occurred concurrently. Scenario 2 assumes that mutations of *PBRM1* and *BAP1* occurred independently in different subclones.