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How should clinicians address intratumour heterogeneity in clear cell renal cell carcinoma?

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Purpose of review
Despite the availability of multiple targeted therapies, the 5-year survival rate of patients with metastatic clear cell renal cell carcinoma (ccRCC) rarely exceeds 10%. Recent insights into the mutational landscape and evolutionary dynamics of ccRCC have offered up a plausible explanation for these outcomes. The purpose of this review is to link the research findings to potential changes in clinical practice.

Recent findings
Intratumour heterogeneity (ITH) dominates the evolutionary landscape in ccRCC at the genetic, transcriptomic and proteomic level. Spatial and temporal separation of tumour subclones within the primary tumour as well as between primary and metastatic sites has been demonstrated at single nucleotide resolution. In the cases analysed to date, approximately two-thirds of somatic mutations are not shared between multiple biopsies from the same primary tumour. Very few of the key disease-driving events are shared across all primary tumour regions (with the exception of *VHL* and loss of chromosome 3p), whereas the majority are restricted to one or more tumour regions (*TP53, SETD2, BAP1, PTEN, mTOR, PIK3CA and KDM5C).*

Summary
ITH must be considered in the management of ccRCC with respect to diagnostic procedures, prognostic and predictive biomarkers and drug development.

Keywords
clear cell renal cell carcinoma, intratumour heterogeneity, tumour evolution

INTRODUCTION

With the reported incidence of clear cell renal cell carcinoma (ccRCC) rising at 2.5% per year [1], the number of patients undergoing surgery with curative intent is increasing, yet one-third will experience local or distal relapse [2]. Prediction normograms are useful in estimating the risk of recurrence following nephrectomy; however, the outcomes for patients with similar clinical and histological features vary and the risk is not always appropriately assigned [3]. To date, no molecular biomarkers of progression have proven sufficiently robust to enter clinical practice [4], which has clinical implications for assigning surveillance schedules and entering patients into adjuvant studies.

In spite of migration toward incidentally revealed tumours [5], 20–25% of patients still present with metastatic disease. Thus, half of all ccRCC patients will experience metastatic disease. A deficiency of prognostic biomarkers is pertinent in this setting when selecting patients for cytoreductive surgery or metastectomy. The median survival of patients with advanced disease is ~18 months, even in the context of multiple lines of therapy [6]. Inhibitors of vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR) have emerged as the main palliative therapies. Primary resistance to VEGF inhibitors (VEGFi) is seen in 20–30% of patients, and the vast majority of those who respond will relapse within 1 year because of acquired resistance [7]. Various biochemical (e.g. corrected serum calcium, alkaline phosphatase and lactate dehydrogenase levels) haematological (e.g. haemoglobin

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KEY POINTS

- Intratumour heterogeneity dominates the evolutionary landscape in ccRCC at a genetic, transcriptomic, and functional level.
- Two-thirds of the somatic mutations are not represented in single biopsies.
- ITH has a direct impact on clinical practice with solid implications in diagnostic procedures, biomarkers and drug development.

levels and thrombocytosis) and clinical (e.g. the number and site of metastases, prior nephrectomy, performance status and time from diagnosis to treatment) parameters have been used to predict the likelihood of response [8], but molecular determinants of response and mechanisms of treatment resistance remain elusive. This is in contrast to other malignancies whereby response to therapy (BRAF-mutant melanoma/BRAF inhibitors [9]), primary resistance (RAS-mutant colorectal cancer/EGFR inhibitors [10]) and acquired resistance (MET amplification/ EGFR-mutant lung cancer [11]) can be predicted on the basis of the tumour molecular profile. In the absence of such biomarkers in metastatic ccRCC, there is no consensus among the treating physicians with regards to the choice of the first-line agent, its timing (upfront or following a period of surveillance based on volume of disease and prognostic scores) nor the appropriate target switch in the second-line setting (VEGF or mTOR).

CURRENT KNOWLEDGE OF THE GENOMIC LANDSCAPE IN CLEAR CELL RENAL CELL CARCINOMA

Bi-allelic inactivation of VHL is an almost-obligatory event in the development of ccRCC and as such does not associate with clinical outcome. In the last few years, four large-scale [12,13▪▪,14,15], and several smaller next-generation sequencing studies [16,17] have highlighted other important genes and pathways. Frequent somatic mutations (single nucleotide variants or small insertion and deletions) are reported in histone-modifying enzymes and chromatin remodelling complexes: SET domain containing 2 (*SETD2*), chromatin-modulating genes polybromo 1 (PBRM1), BRCA1-associated protein-1 (*BAP1*) and lysine (K)-specific demethylase 5C (*KDM5C*); components of the phosphoinositide 3-kinase (PI3K)-AKT-mTOR pathway, Phosphatase and tensin homolog (*PTEN*), Mammalian Target of Rapamycin (*mTOR*), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*); and p53 (*TP53*). Several retrospective studies have reported associations of *PBRM1, SETD2, BAP1,* and *KDM5C* mutations with advanced stage, grade, and tumour invasiveness [18,19], whereas *SETD2* and *BAP1* mutations correlate with reduced overall survival [20–22]. Although these mutations are potentially attractive prognostic biomarkers, they require prospective evaluation.

Another important class of somatic alterations in ccRCC are copy number variants (CNVs). ccRCC tumours harbour an average of 5.8 amplifications and 6.8 deletions [18] and, the majority of these events are recurrent, indicating their role in driving the disease. Association with stage and grade has been reported for 1p, 9p, 9q, 13q and 14q loss and 12q gain [19]. Several studies have reported an association between 9p deletions and the risk of recurrence and overall survival [20,21]. More recently, *CDKN2BA*, one of the two genes which map to the recurrently deleted focus on 9p, was found to be mutated in familial ccRCC [22], highlighting once more the link between sporadic and germline ccRCC tumouregenesis.

GENETIC INTRATUMOUR HETEROGENEITY IN CLEAR CELL RENAL CELL CARCINOMA: INSIGHTS INTO SPATIAL AND TEMPORAL TUMOUR EVOLUTION

The advent of next-generation sequencing technologies has enabled insights into the specific mutations but also the hierarchy behind the adaptive landscape that regulates cancer. Significant intratumour heterogeneity (ITH) attributed to genetic diversification [23] has been revealed in leukaemia [24], ccRCC [25], breast cancer [26], lung adenocarcinoma [27▪,28▪], glioblastoma [29], prostate cancer [30] gastrointestinal [31,32] and ovarian cancer [33,34]. ITH is defined as the presence of genetically distinct subpopulations of cells within geographically distinct regions of the same primary tumour. It has been implied as an important driver of tumour adaptation and progression, with solid implications for drug discovery and biomarker validation [35].

Next-generation sequencing studies in ccRCC [12,13▪▪,14–17] have relied on genomic information from single tumour biopsies. To ascertain how representative a single biopsy is of the tumour as a whole, we performed exome sequencing on multiple, spatially separate samples obtained from 10 primary ccRCC tumours and associated metastatic sites in a subset of cases [25,36▪▪]. We showed that two-thirds of the somatic mutations were not shared between all the primary tumour regions. Sixty-three to 69% of all nonsynonymous (protein-altering) mutations

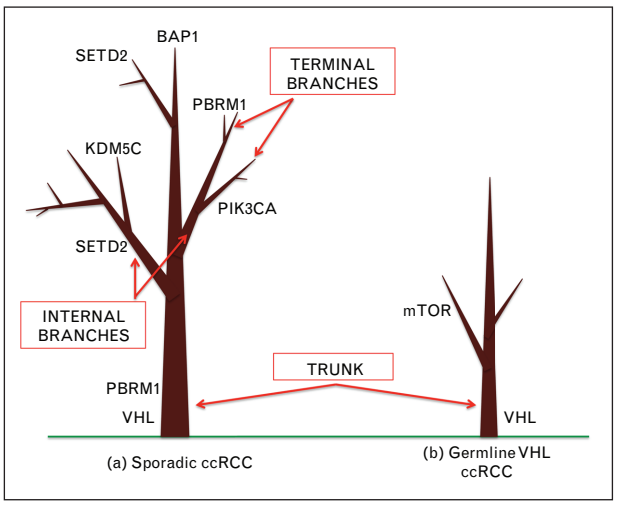

FIGURE 1. 'Trunk-branch model of tumour development' (a) Sporadic clear cell renal cell carcinoma (ccRCC). Clonal somatic events (i.e. those present in all regions of the tumour) map to the trunk. Events restricted to tumour subclones (i.e. those present in some but not all, or only a single tumour region) map to the branches. Example driver somatic events are annotated to indicate clonality. (b) Germline VHL ccRCC. Trunk-branch model of tumour development applies, but intratumour heterogeneity appears minimal in contrast to sporadic ccRCC.

identified across multiple biopsies would not be detected in a single biopsy. Phylogenetic analyses provided a trunk–branch model of tumour development (Fig. 1A). Somatic events that are present in all the regions of the tumour represent the trunk, including major driver events such as inactivation of the *VHL* tumour suppressor gene and loss of chromosome 3p. Events restricted to tumour subclones that are only detected in some, but not all regions, are placed on the branches and include mutations in *TP53, SETD2, BAP1, PTEN, mTOR, PIK3CA* and *KDM5C*. Mutations in *PBRM1* were found to be either clonal or subclonal. Two other groups have reported similar heterogeneity patterns in gene panel studies [37▪,38▪▪].

Significant ITH was also evident with respect to somatic CNVs (SCNVs). 3p chromosome loss was the only driver SCNV that was clonal across all cases. Losses of chromosomes 4q, 8p and 14q and gains of chromosome 5q were detected as either clonal or subclonal, whereas the majority of other SCNVs were pervasively subclonal. Indeed, when we compared the copy-number variants obtained from multiple biopsies from eight ccRCCs primary tumours with 440 individual tumour biopsies from the Cancer Genome Atlas (TCGA), biopsies obtained from the same tumour resembled other unrelated tumours more than their tumour of origin [25,36▪▪,39].

Consistent with the mutational patterns of ITH, gene expression profiles also varied across the primary tumour. Critically, evaluation of validated gene expression signatures demonstrated that expression patterns of both good and poor prognosis [40▪▪] are detectable in different regions of the same tumour. Thus, single biopsies are not representative of the ccRCC transcriptomic landscape, and even the best current binary classification of ccRCC biomarker is subject to ITH [40▪▪].

Matched metastatic tissue was only available in a subset of the cases we examined; nevertheless, we demonstrated relative homogeneity in the metastatic lesions compared with the primary tumours. This pattern was observed in another ccRCC paired primary-metastasis analysis [41▪]. These data suggest that ccRCC metastases may be adequately portrayed by single biopsies. However, even if single metastasis is monoclonal, different metastatic sites may be populated by different subclones as has been shown in pancreatic cancer [32]. In other cases, they could prove to be polyclonal as was shown in prostate cancer [42▪] wherein spread occurred between metastases. In any of these scenarios, a single biopsy will not portray the metastatic landscape comprehensively.

We have also analyzed ITH in ccRCCs arising in the background of known VHL disease. We applied multiregional profiling to single or multiple (metachronous) ccRCC tumours arising in two cases with germline *VHL* mutations [43▪▪]. In contrast to the sporadic cases, we detected minimal ITH despite extensive sampling (Fig. 1B). Analysis of additional cases is required to confirm these findings. We confirmed the previous observation [18] of marked inter-tumour heterogeneity in the metachronous tumours highlighting the need to sample and analyse each tumour.

These studies have provided an insight into the temporal and spatial clonal diversity in ccRCC, but should they alter our approach to the management of this disease?

HOW MUCH SAMPLING IS REQUIRED?

Primary tumour

The current approach to primary tumour sampling is geared towards histological reporting, as routine genotyping is not yet standard of care. Among European uropathologists, it is common to slice the tumours at 10-mm intervals and sample one section for each centimetre of the maximum tumour diameter (Fig. 2A–C) [44]. However, compared with such conventional sampling a more comprehensive sampling approach (Fig. 2D) may

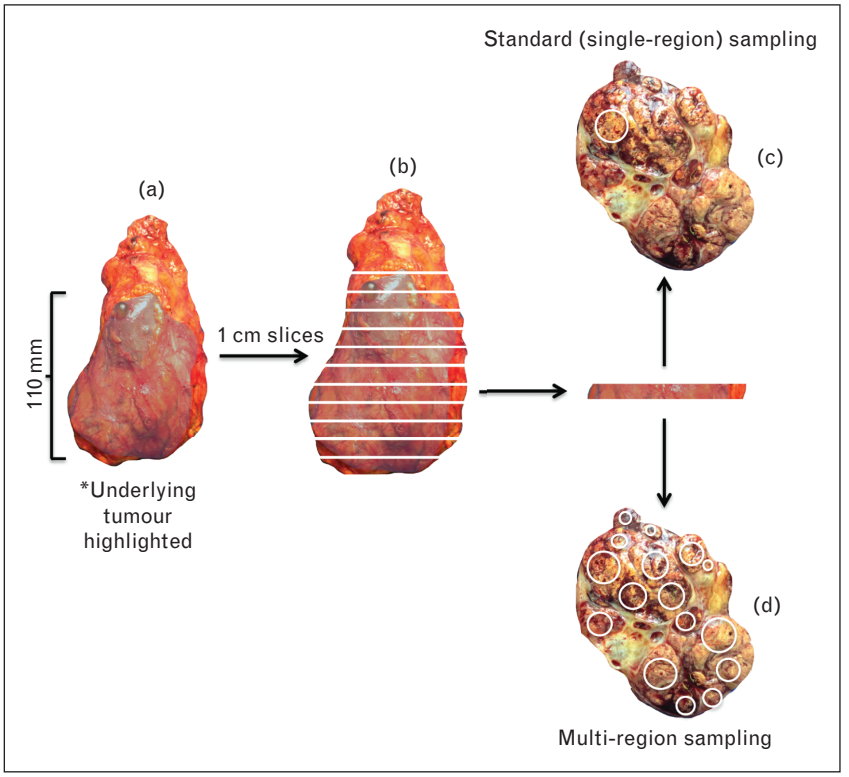

FIGURE 2. Example of tissue sampling. (a) 110-mm tumour within 170 mm right total nephrectomy specimen (underlying tumour highlighted blue). (b) 1-cm-thick slices taken from tumour specimen. (c) Standard sampling approach; a single region of the tumor slice sampled for review. (d) Multiregion sampling; multiple regions of tumour slice, representative of spatial extent and macroscopic heterogeneity, of tumour slice sampled for review.

detect a significantly higher proportion of high grade tumours [45], a finding consistent with the frequent upgrading from renal biopsies to surgical specimens [46▪]. ITH has also been observed with respect to cell type and immunohistochemical staining patterns [47]. Given that tumour grade [48] and cellular features correlate with prognosis [49] and response to therapy [50], it appears that conventional sampling may miss assign features with prognostic significance. It is not known to what extent the spatial histological heterogeneity reflects genomic variation and this area requires further investigation.

Molecular profiling is likely to be incorporated into routine care in the future to inform clinical decision-making. Recurrent mutations in the *PBRM1* [17], *SETD2* [16,51], *BAP1* [52] *and KDM5C* [16] have all been associated with advanced stage, grade and tumour invasiveness. Mutations in these genes are underestimated in single biopsies because of their frequent subclonal status [53,54] (Table 1). Our multiregion sampling studies suggest a persistent increase in the number of detected mutations with each subsequent biopsy with no evidence of saturation of genomic ITH [36▪▪] (Fig. 3A). How do we decide the level of sampling required to capture all, or most of the important genetic alterations within the primary tumour? Based on their limited driver gene profiling, Sankin *et al.* suggested that three different tumour regions need to be sampled to detect mutations in *PBRM1, SETD2, BAP1* and/or *KDM5C* with 90% certainty [38▪▪] (Fig. 3B). Our own estimates, based on more extensive sampling, suggest that this figure is likely to be higher for the detection of exome-wide driver events (Fig. 3C).

Metastases

Unless there is diagnostic uncertainty, ccRCC metastases are not sampled routinely. A proportion of synchronous metastases (e.g. lymph node, IVC thrombus or adrenal metastases) may be collected at surgery. When patients present with metachronous metastases, we frequently rely on the histological profile of the historical nephrectomy specimen. However, genotypes can be discordant between primary and metastases even with respect to *VHL* [55], and genetic alterations that drive disease progression and treatment resistance can arise *de novo*, or expand from a minor subclone, which evaded detection in the primary [56]. These observations indicate that sampling of metastases and

Table 1. Comparison of the prevalence of driver mutations per biopsy

Gene	TCGA_freq	Sato_freq	Scelo_freq	Gerlinger_freq
PBRM1	33	24	39	60
SETD2	12	11	19	30
BAP1	10	8	12	40
KDM5C	7	4	7	10
ATM	3	2	5	10
ARID1A	3	2	5	10
TP53	2	3	4	40
PTEN	4	2	3	20
MTOR	6	6	9	10
PIK3CA	3	5	0	20

Single-biopsy studies [12,13▪▪,15] are compared with all 79 individual samples for the 10 cases sequenced by multiregional profiling [36▪▪].

progressive disease sites could inform longitudinal changes in clonal dynamics especially in response to selective (treatment) pressure.

APPROACHES TO MOLECULAR PROFILING

Available tumour profiling approaches range from single-gene tests and limited gene panel sequencing, to whole exome, transcriptome and whole genome sequencing. Although the latter have the advantage of being unbiased and include all the genic or even intergenic regions, their cost is prohibitive in routine clinical practice. As driver events in ccRCC, are relatively well defined bespoke gene panels, focused on the recurrently mutated genes copy number-altered regions could offer a compromise between the need for prognostic information and

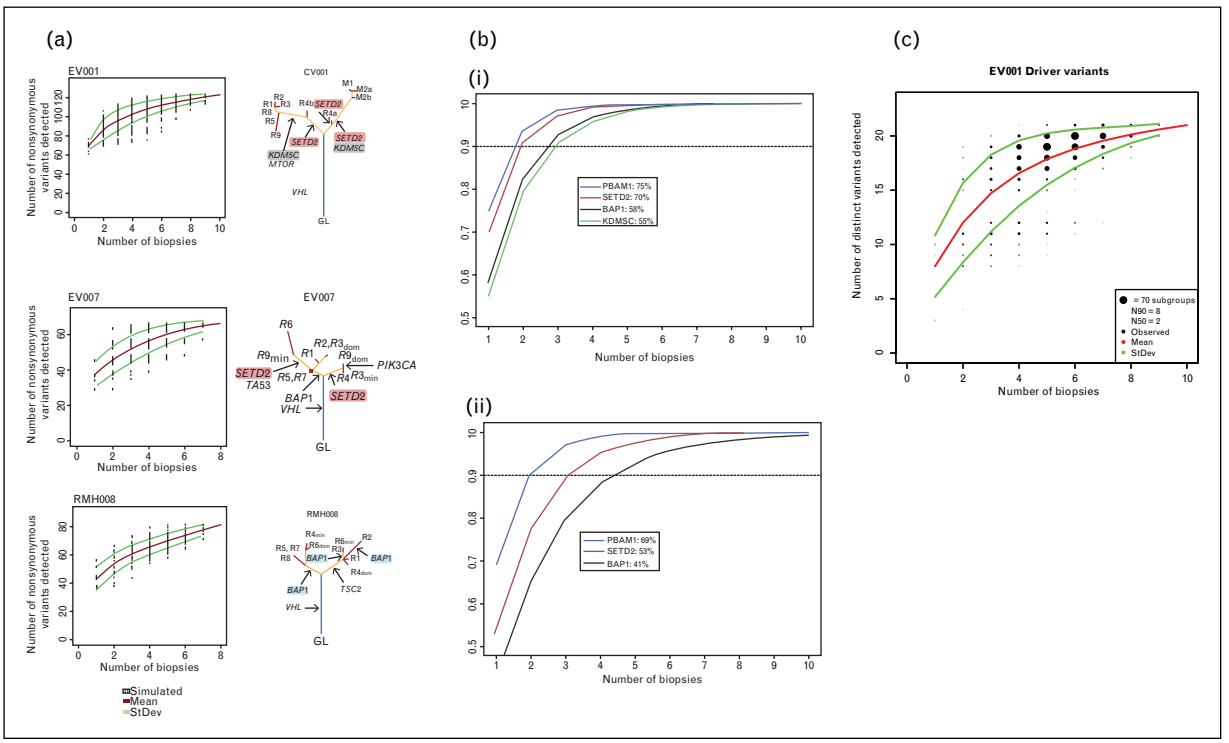

FIGURE 3. Identifying subclonal driver events. (a) Total number of nonsynonymous somatic variants detected increases with additional biopsies due to the presence of subclonal populations (represented on accompanying phylogenetic tree) in three cases of sporadic ccRCC. (b) Probability to detect a mutation in example tumour suppressor genes increases with additional biopsies (i, sample set from [36▪▪]; ii, sample set from [38▪▪]). (c) Ability to detect exome-wide driver events likely to be reliant on more extensive sampling (data not published).

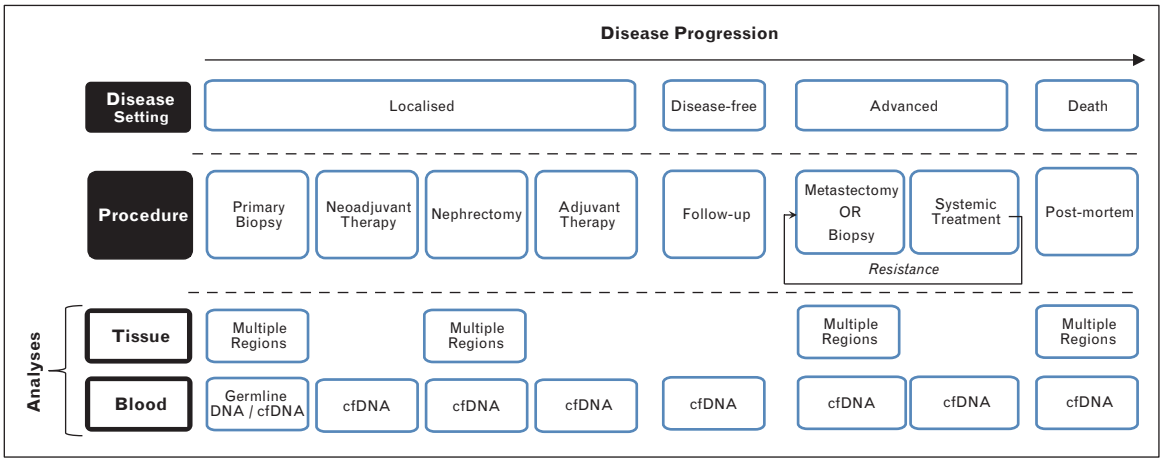

FIGURE 4. Proposed longitudinal sample collection in ccRCC. cfDNA, cell-free tumour DNA isolated from patients' plasma.

cost-effectiveness. These approaches reduce the burden on computational power and storage whilst affording a greater depth of sequencing. PCR-amplicon [57] and hybridization capture based methods are available with hybridization capture best suited for capture of larger target regions and exons from hundreds of genes [58].

It remains to be proven whether more extensive sampling and molecular profiling of ccRCC patients could determine the patient's prognosis and guide the clinical decision-making. To address this question, spatial and temporal sampling needs to be incorporated into clinical study design with histological and molecular profiling of tumours in a chronological sequence that starts with nephrectomy and concludes in postmortem sampling (Fig. 4). Integrated with robust clinical annotation of disease outcomes, such studies, already underway in lung cancer [59▪], would provide powerful biological insights but also practical guidance to disease management.

PROSPECTIVE VALIDATION OF SPATIO-TEMPORAL SAMPLING AND MOLECULAR PROFILING

Small tumour masses

For small incidentally revealed renal masses (SRMs) (defined as <4 cm), active surveillance is an alternative to surgery for patients with significant comorbidities [60]. Size is an important factor in determining the nature of renal masses. Approximately, 20% of renal masses less than 4 cm are ultimately found to be benign [61]. However, another 20% may display poor prognostic features including high grade or invasion into the perirenal fat [62]. Percutaneous biopsies are increasingly being used to support treatment decisions in this context [63,64]. Adverse genetic features such as BAP1 mutations could be used to stratify patients based on a molecular risk to either surgery or surveillance.

Locally advanced disease

For locally advanced disease (T3 and T4), wherein open radical nephrectomy is the standard of care, there is divergent practice with respect to postoperative surveillance schedule, participation in adjuvant trials and the management of patients with lymph node, adrenal or vena cava involvement [65▪]. In the context of a prospective evaluation, multiregional sampling of the nephrectomy specimen would detect the presence of adverse genetic features and their clonal status and stratify the patients accordingly. Five-year survival for Stage III disease remains ~54% almost entirely as a result of metastatic disease. Considering tumours that extend grossly into the vena cava or involve the regional lymph nodes, molecular profiling of these disease components could, in theory, anticipate the composition of future metastases and guide adjuvant therapy.

Advanced disease

Surgery

It has been speculated that cytoreductive nephrectomy has a role in the removal of the evolutionary sink [35]. Although patients should continue to be selected on the current criteria for cytoreductive surgery [66], multiregional molecular profiling of nephrectomy specimens will show how the pruning of particular mutations affects future clonal dynamics and the long-term outcome. The most informative will be the longitudinal studies of paired

nephrectomy-metastasis(es) to determine whether ccRCC metastasis occurs as separate waves of invasion from the primary tumour. Further, analyses of larger cohorts of primary-metastasis pairs could identify the origin of the metastatic subclone and its associated variants. Although only a small number of patients will be suitable for a metastectomy [65▪], tissue sampling should be sought in the remaining patients (biopsy or postmortem sampling) to facilitate these studies. Genomic profiling of metastatic sites could conceivably prioritise those that harbour treatment resistance-driving variants for surgical resection.

Systemic therapy

Therapy constitutes a selective pressure for clonal cancer evolution [23]. Under the pressure of treatment, the tumour may become less dependent on particular drivers (Fig. 5) and repeat sampling is mandated to identify emerging clones that acquire dominance through Darwinian selection. The first challenge is to define actionable mutations that are temporally and spatially relevant. Trunk events such as *VHL* or *PBRM1* mutations represent attractive drug targets and are subject to less sampling bias. Branch events including *SETD2, PTEN* and *KDM5C* mutations may be more relevant in driving disease progression and treatment resistance. Estimating the timing of particular mutations (early versus late) is crucial in formulating the combinatorial therapy approaches that could address both trunk and branch alterations [67▪▪]. Lastly, considering the results of the early trials of PD-1/PD-L1 inhibitors [68▪], the impact of ITH on cancer immunity and response to immunotherapy needs to be incorporated into these studies and the relationship between different genotypes and immunogenicity explored further.

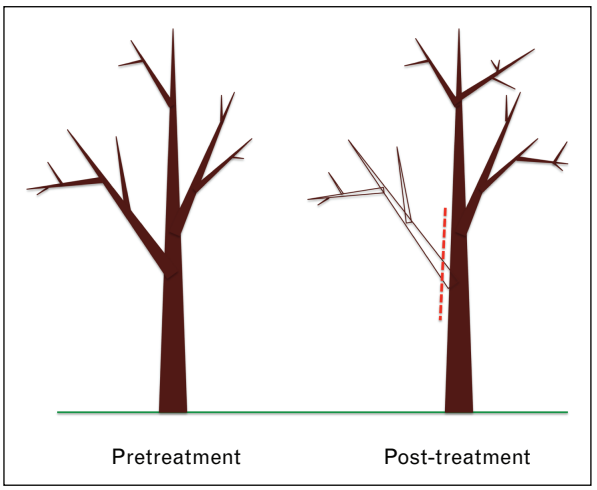

FIGURE 5. Systemic treatment as a selective pressure for clonal cancer evolution. Pretreatment and posttreatment phylogenetic trees may look very different because of the selective pressure of systemic therapy 'pruning' some branches whilst others are able to continue to 'grow'.

Minimally invasive sampling methods

Tumour cell-free DNA (cfDNA) [69] has been detected in plasma, serum and urine of ccRCC patients [70]. Levels of tumour cfDNA in plasma have been associated with the risk of relapse [71] and response to therapy [72]. In other malignancies, tumour-specific sCNAs [73] and resistance-driving mutations [74] have been detected by targeted approaches, whereas exome- and genome-wide sequencing of cfDNA has revealed the clonal structure of the primary tumour [75], de-novo genomic rearrangements [76] and variants selected by therapy [77] [78]. The utility of cfDNA to determine these outcomes in ccRCC has not been tested to date. By incorporating blood collection into prospective tissue collection and profiling studies (Fig. 4), we could potentially simplify and improve the monitoring of tumour evolution over time, detect disease progression and forecast the emergence of treatment resistance.

CONCLUSION

Recent studies have illuminated the clonal dynamics in ccRCC. By accepting the linear model of tumour evolution and investing ourselves in the concept of targeting single driver mutations, we had simplified our approach, thus limiting outcomes. ITH appears to be a consistent feature of this disease and should be considered in every aspect of its management from diagnosis, the use of prognostic and predictive tools, design of personalised treatment strategies and the stratification of patients into clinical trials. The clinical utility of molecular profiling throughout the course of disease and treatment can only be assessed in prospective trials that mandate serial tissue sampling. The success of such trials is critically dependent on all members of the multidisciplinary team, in particular oncologists, surgeons, radiologists and pathologists.

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Conflicts of interest

There are no conflicts of interest.

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