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Review

Predicting cancer evolution for patient benefit: Renal cell carcinoma paradigm

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ABSTRACT

Evolutionary features of cancer have important clinical implications, but their evaluation in the clinic is currently limited. Here, we review current approaches to reconstruct tumour subclonal structure and discuss tumour sampling method and experimental design influence. We describe clear-cell renal cell carcinoma (ccRCC) as an exemplar for understanding and predicting cancer evolutionary dynamics. Finally, we discuss how understanding cancer evolution can benefit patients.

1. Introduction

Cancer has long been considered an evolutionary process [1–3], where genetic and epigenetic alterations provide a substrate for selection, facilitating adaptation. In contrast to the adaptation of species, cancer adaptation takes place over years to decades, converging onto a common set of phenotypes, reflected in the hallmarks of cancer [4]. Only a minority of genetic alterations found in any one cancer are actively selected ("driver" events) [5], with the majority having no effect on fitness ("passenger" events). The sources of selection in cancer can be endogenous, e.g. the tumour microenvironment (TME), systemic immunity; or exogenous, e.g. therapy. The definition of a driver event is context-dependent; an alteration that persisted neutrally at first may be selected under certain circumstances, e.g. if it confers resistance to therapy. Cancer evolution is further constrained by tissue and cellular contexts. For example, some cancer genes are only altered in certain cancer types (*VHL* and clear cell renal cell carcinoma (ccRCC); *APC* and colorectal cancer (CRC)), and most show an element of cell/tissue specificity (e.g. *NRAS* and melanoma; *KRAS* and lung, CRC and pancreatic cancers). Further, temporal patterns of selection are often conserved, with some cancer genes implicated as founders (clonal alterations), and others consistently selected at later stages of evolution (subclonal alterations) [6,7]. Another important feature of cancer evolution is epistasis, a non-random interaction of genetic changes. Some driver events co-occur suggesting cooperation, while others are mutually exclusive, either as a result of functional redundancy (e.g. *BRAF* and *NRAS* mutations in melanoma), or synthetic lethality (e.g. BRCA and the base excision repair PARP protein family). Epistatic interactions constrain cancer evolution and can lead to conserved, and potentially predictable, order and pairing of driver events in cancer. Cancer evolution is also constrained by germline variation, especially in cancer susceptibility genes, where cancer evolution proceeds via somatic inactivation of the wild-type allele [8]. Broader germline variation has also been shown to constrain the repertoire of somatic driver events in sporadic tumours [9]. Cancer evolutionary constraints remain poorly understood, but likely reflect both cell-intrinsic and cell-extrinsic factors [10]. Their understanding could offer substantial patient benefit in terms of predicting evolutionary routes, exploiting therapeutic vulnerabilities and designing cancer prevention strategies.

The development of primary tumours is associated with distinct modes of evolution and evolutionary tempo (Fig. 1). In linear evolution, only one population (clone) becomes fixed over time outcompeting all others via a "clonal sweep" (Fig. 1). Branched evolution results from a gradual process of evolution and fixation of multiple subpopulations (subclones), due to similar fitness or distinct adaptive traits, often involving small scale alterations. Branched evolution leads to widespread intra-tumour heterogeneity (ITH). Akin to linear evolution, punctuated evolution is defined by a clonal sweep but is distinguished

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by a rapid burst of large-scale alterations which are fixed early in tumour evolution. Critically, inferences of evolutionary mode, especially in the absence of any intermediate populations can be highly biased, and particularly in single sample studies.

The ability of primary tumour cells to disseminate and seed new tumour colonies in distant tissues is referred to as "metastatic competence". This progression can also be reckoned as an evolutionary process with two general models described. Linear and parallel models of metastatic progression differ essentially in when and where they propose metastatic competence evolved [11–14]. Linear progression model assumes metastasis competent clones to have emerged late during tumorigenesis, representing either a minor or dominant clone in the primary tumour. In the parallel progression paradigm, disseminated tumour cells lacking metastatic competence leave the primary tumour early (before it is clinically detectable) and continue to evolve towards metastatic competence. In contrast to the linear model, the degree of genetic divergence between the primary and metastasis is expected to be high [11]. Finally, the same clone may seed all metastases (monophyletic seeding), or distinct clones may do so, often in an organ-specific manner (polyphyletic seeding) [11]. All these distinctions are of critical importance for the success of systemic therapy - especially with regards to timing, e.g. adjuvant, neoadjuvant, palliative, as well as uniformity of response to therapy at different metastatic sites (mixed response, oligoprogression).

Evolutionary features have important clinical implications, yet their inference is profoundly impacted by the exact approaches to estimate population frequencies over space and time. To motivate a serious consideration of the use of evolutionary understanding for clinical decision making and therapeutic advances we first review standard tumour sampling and molecular profiling methodologies in the context of clonal reconstruction of tumours. We discuss a novel profiling technique, RepSeq, that could potentially improve clonal reconstruction in a clinical setting. We present ccRCC, the focus of our research programme, as an exemplar for understanding cancer evolution in patients, however a detailed literature review of the ccRCC evolution is beyond the scope of this review. Finally, we provide a perspective on the benefits of evaluating cancer evolutionary dynamics for patient management.

2. Methodologies for studying molecular evolution

Genomic alterations present in the 'most recent common ancestor' (MRCA), referred to as "clonal", are present in all tumour cells of a given tumour. In contrast, "subclonal" alterations are present in a subpopulation of tumour cells and hence can be used to identify and track distinct lineages in the tumour. The term "subclonal reconstruction" refers to the reconstruction of these lineages and their phylogenetic relationships from sequencing data [15]. Most methods consider single nucleotide variants (SNVs), small insertions and deletions (indels), and somatic copy number alterations (SCNAs). There are four key stages in any computational workflow for subclonal reconstruction from DNA sequencing data: i) SNV discovery, ii) SCNA reconstruction, iii) SNV clustering and iv) phylogenetic reconstruction. Variant allele frequencies (VAF) of SNVs along with tumour purity and local copy number, are used to infer cancer cell fraction (CCF), that is the fraction of

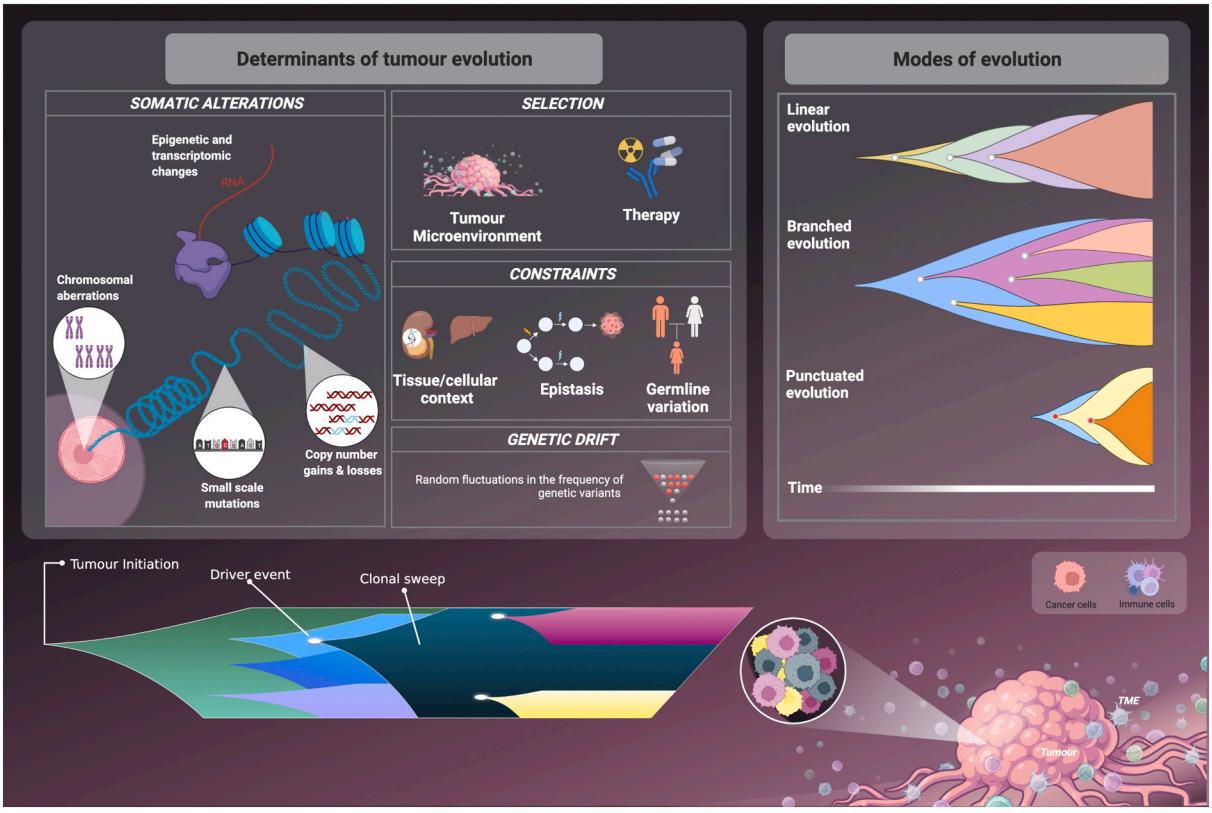

Fig. 1. Determinants and modes of tumour evolution: The fish plot represents the proportion of cells from different clones over time and depicts the evolutionary process of a tumour. Somatic alterations, selection, constraints and genetic drift are the key forces that drive tumour evolution. Somatic alterations include small scale mutations (SNVs and small insertions/deletions), copy number changes (gains, losses and loss of heterozygosity), chromosomal aberrations (aneuploidy, whole-genome doubling, translocations, chromothripsis and chromoplexy) or changes in the transcriptomic and epigenetic mechanisms. Selective pressures can be endogenous, e.g. the tumour microenvironment, or exogenous, e.g. therapy. Factors like epistatic interactions, germline variations and tissue-specific context constrain tumour evolution. Genetic drift influences smaller cell populations in particular and impacts tumour evolution. Linear, branched and punctuated evolutionary modes are current models of tumour evolution. The white dots represent driver events in the fish plots and the red dots represent a rapid burst of large-scale driver events. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sampled cancer cells carrying the SNV. Algorithms that estimate purity and ploidy and infer the allele-specific copy number from sequencing data make use of the sequencing read depth ratio between tumour and reference (normal) samples and the balance between parental alleles. Most of the existing methods infer allele-specific copy numbers on a single tumour sample, there are computational methods in development that infer allele- and clone-specific copy numbers across multiple related samples [16]. To define distinct clones, SNVs with comparable CCFs are clustered and their phylogenetic relationships are inferred. Computational methods for subclonal reconstruction including best practices and benchmarking are reviewed elsewhere [15,17], however, critical upstream factors that impact subclonal reconstruction include i) tumour sampling method ii) choice of sequencing assay (bulk or single-cell sequencing), and iii) depth and breadth of sequencing (whole genome or targeted coding region sequencing) (Table 1).

2.1. Single-region sampling vs multi-region sampling

Profiling of single tumour samples especially in large patient cohorts has been a compelling approach to decipher the cancer genome and maximise statistical power for clinical inference [18]. However, the proportion of the tumour volume sampled in these settings is on average ~ 2% and even lower in the context of routine molecular profiling in clinical care (0.0005%; [19]). This tumour under-sampling, coupled with the lack of clonal mixing (subclones are typically spatially separated in solid cancers) impedes accurate subclonal reconstruction. Sampling tumours from a single spatially fixed location underestimates clonal complexity and can misinterpret subclonal populations as clonal, leading to an "illusion of clonality" [19]. One approach to circumventing this issue is spatially separated multi-region sampling [19], which has become a tool of choice for research studies evaluating spatial and temporal clonal evolution of primary and metastatic tumours.

Additional characteristics of tumour sampling can impact subclonal deconstruction. Compared to the ex-vivo sampling of surgically resected specimens, image-guided tumour biopsies can result in low tumour purity and reduce subclone discovery. Profiling of fresh frozen tumour samples outperforms formalin-fixed and paraffin-embedded (FFPE) samples which are prone to sequencing artefacts which in turn interfere with accurate estimates of clonal clusters [20].

2.2. Towards representative sampling

While multi-region sampling has proved a useful tool in the research setting it is not a feasible approach in the setting of routine clinical care. Thus, the cancer community has long been motivated to develop approaches to capture the molecular landscape of the whole tumour, such as the profiling of cell-free tumour DNA (ctDNA) [21]. While increasingly promising, this approach is still limited in sensitivity especially in certain cancer settings [22]. We recently described a novel tumour sampling methodology to address both under-sampling and the spatial sampling bias in current molecular profiling approaches [19,23]. This method, called "representative sequencing" (Rep-Seq) includes homogenization of residual fixed surgical tumour tissue into a well-mixed solution, followed by sequencing. Rep-Seq utilises tumour material that is otherwise treated as clinical waste and destroyed and so does not interfere with the existing pathology workflows. Compared to single tumour region and ctDNA profiling, Rep-Seq has higher reproducibility and sensitivity for mutation discovery. Critically, Rep-Seq leads to accurate subclonal reconstruction relative to the ground truth established from multi-region profiling [19]. An additional benefit of tumour homogenization is the preservation of cellular integrity of the tumour tissue, which enables dissociation of the homogenate into single cells. The single cells can be flow-sorted to enrich tumour cells thereby, reducing redundant sequencing and further improving subclonal reconstruction. RepSeq offers a clinically practical solution to address tumour sampling bias for a range of downstream molecular readouts. We are currently

Table 1
Methodologies for studying molecular evolution: The table summarises the factors that affect the stages of subclonal reconstruction.

		SNV discovery and SCNA reconstruction	SNV clustering and phylogenetic reconstruction
Tumour Sampling	Single-region	Accuracy of variant calling depends on the sequencing depth and purity of the tumour. Increasing sequencing breadth (targeted to whole genome) will not necessarily ensure capturing ITH, as this will depend on clone distribution of the tumour.	Sampling bias leads to underestimating the number of sub-clones and illusion of clonality. Challenging to unambiguously identify a branching phylogeny and the resulting phylogenetic tree can be less informative.
	Multi-region	Increases sensitivity to study ITH. Certain algorithms consider multi-sample to perform joint variant calling to increase the sensitivity of variant calling in low-coverage regions. Algorithms leveraging multi-sample data hold potential to increase accuracy of estimating allele-specific copy numbers.	Multi-sample approach helps resolve phylogenetic relationships among subclones. Reduces illusion of clonality. A tool of choice for studies evaluating spatial and temporal clonal evolution, however, can be limiting in terms of cost, logistics and clinical feasibility.
	ctDNA	ctDNA sampling over time enables temporal profiling of clonal events. Ultra-deep sequencing and highly sensitive algorithms are required to identify low-frequency variants.	Temporal resolution helps resolving minor subclones that evolved late (for example, evolving post-treatment resistance). However, the limited number of SNVs identified in this approach hampers SNV clustering and the subsequent phylogeny inference.
	Rep-Seq	Allows better sensitivity to capture subclonal variants and ITH. A novel sampling method addressing under- and spatial-sampling bias.	Higher sensitivity to subclonal events reduces illusion of clonality. Phylogenetic inference comparable to multi-sampling approach.
Choice of sequencing assays	Bulk sequencing	Detecting SNVs in a limited clonal expansion (low-VAF SNVs) is problematic.	Resolving phylogenetic relationships among subclones can be more challenging using bulk sequencing compared to single-cell. But multi-region sampling can address this limitation.
	Single-cell sequencing	Addresses the time bias inherent in bulk and identifies lower frequency variants but requires very high number of cells to be sequenced to achieve that. Low coverage per cell leads to limited sensitivity in detection of individual variants.	SCNAs are usually employed for clustering and phylogeny inference in single-cell studies. In comparison to bulk, single-cell sequencing can reconstruct phylogenies with higher sensitivity.

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Table 1 (*continued*)

		SNV discovery and SCNA reconstruction	SNV clustering and phylogenetic reconstruction
Depth and breadth of sequencing	Whole genome sequencing	WGS identifies a higher number of SNVs (passenger mutations). Helps in understanding mutational processes better (for instance, UV-induced mutations in melanoma). WGS enables highly accurate SCNA calls and identification of subclonal copy number events.	Higher number of passenger mutations enhances the power of clustering making the clusters more apparent, and thus improving the quality of phylogeny inference. However, higher cost of WGS usually limits multi-region sequencing which helps in resolving phylogenetic relationships.
	Targeted (whole exome or panel) sequencing	Targeted sequencing, usually performed at higher depths, allows higher sensitivity to identify low frequency variants. Reconstruction of subclonal copy number events is very difficult.	Fewer SNVs identified usually limits the quality of clustering. However, in samples with many SNVs (from higher depth) and fewer SCNAs, targeted sequencing could provide better resolution of phylogenetic reconstruction than WGS.
	Depth of sequencing	Higher depth of sequencing enables discovery of low-VAF SNVs and improves precision of CCF estimates. However, tumour purity and ploidy dictate the depth needed to detect low-VAF SNVs. SNV detection methods are more affected by depth than the SCNA profiling algorithms.	Deeper sequencing generally provides better resolution of phylogenetic reconstruction. The limit of detection of minor subclones is increased by higher depth. However, choosing a sequencing depth is a compromise between breadth and choice of assay of sequencing.

evaluating Rep-Seq in a prospective clinical cohort HoLST-F (NCT03832062) of 500 patients across different cancer types.

2.3. Bulk sequencing vs single-cell sequencing

Bulk DNA sequencing has been a common tool of choice for tumour evolutionary studies due to its scalability. One of the shortcomings of bulk sequencing is the reduced sensitivity to detect low frequency (subclonal) alterations. While single-cell DNA sequencing (scDNA-seq) approaches improve the sensitivity in principle, the high levels of noise, limited genome coverage and sparsity in the data makes detection of SNVs challenging. Many single-cell studies leverage SCNAs, which are identified more reliably, but amplification techniques used in single-cell sequencing can lead to biases in subclonal reconstruction [24]. Aggregating multiple cells that have similar SCNA profiles can be used to mitigate this [25,26], but the single-cell resolution is then lost. Another limitation of scDNA-seq is that it cannot capture a tumour-wide representation of clones, as relatively small numbers of cells are usually sampled from a single tumour region. Nevertheless, in comparison to bulk sequencing, single-cell sequencing reconstructs phylogenies with greater sensitivity and certainty [15,27–29] and can be used as a complement to bulk-sequencing [27]. With the active development of scalable sequencing technologies and computational methods for subclonal reconstruction from single cells, scDNA-seq is likely to become a major tool for tumour evolutionary studies.

2.4. Depth and breadth of sequencing

Subclonal reconstruction can be performed from whole-genome sequencing (WGS), or targeted sequencing of coding regions (whole exome, WES) or selected genes (panel sequencing). The use of WGS maximises the number of clonal markers (passenger mutations) increasing the power for accurate SNV clustering and phylogenetic reconstruction. Further, WGS improves SCNA calling and enables the identification of subclonal SCNAs. The costs associated with WGS (sequencing and computational) usually limit the sequencing depth to $<100\times$ [30], which can reduce the detection of subclonal variants. On the other hand, the higher depth of sequencing achievable by WES and panel sequencing can improve subclone detection and CCF accuracy [15]. However, fewer clonal markers, especially in tumours with low tumour mutational burden limit accurate clustering and subsequent phylogenetic reconstruction. Characterisation of subclonal SCNAs is also restricted because a reduced number of genomic loci are captured in targeted approaches. The trade-off between the depth and breadth of sequencing ultimately needs to account for the study scale and *a priori* understanding of the strength of selection in the cancer type under study. For example, the focus on a small number of driver genes is justifiable in cancers where clonal expansions are usually associated with driver events (e.g. ccRCC) as opposed to those where neutral evolution is frequent (e.g. CRC) [31].

3. ccRCC as a framework to study cancer dynamics

Clear-cell renal cell carcinoma (ccRCC) is an exemplary framework to understand tumour evolution. First, sporadically occurring ccRCCs present a wide range of population sizes. Tumours that are detected incidentally during abdominal imaging measure 1–2 cms (the so-called small renal masses - SRMs), which corresponds to 10s–100s of millions of cancer cells. However, many patients are still diagnosed at an advanced stage, with primary tumours >10 cm, equivalent to >trillion cancer cells. Population size has a profound effect on mutation supply, availability of evolutionary paths, and susceptibility to genetic drift [32,33]. Second, ccRCC presents a variety of progression patterns. In the context of active surveillance, most SRMs show limited growth and almost never metastasise [34], challenging the notion that all SRMs are a precursor to more aggressive tumours. In the context of larger tumours, metastatic efficiency varies from slow-growing solitary or oligo-metastases [35], to rapidly growing widespread metastases. Lastly, following curative resection of primary ccRCC some patients develop metastases > ten years later, in a single site, typically the pancreas or the thyroid. While the observed latency is assumed to represent dormancy, the lack of broader metastatic involvement in these patients questions this notion.

Another advantage of the ccRCC framework is a well-defined landscape of recurrent driver events that characterise clonal expansions [7,36,37]. Loss of the short arm of chromosome 3 (3p loss) is a near-ubiquitous first event in ccRCC tumorigenesis. The minimum region of loss on 3p encompasses *VHL*, *PBRM1*, *SETD2* and *BAP1*, resulting in the loss of heterozygosity (LOH) in these tumour suppressor genes (TSGs). The second most common driver event, the loss of the remaining *VHL* allele by mutation or methylation, is assumed to be the second event in the sequence [38]. Bi-allelic inactivation of VHL results in a pseudo-hypoxic state, upregulation of hypoxia-inducible-factor (HIF) targets [39], and high levels of angiogenesis. Frequent alterations occur in the remaining TSGs on 3p: *PBRM1*, *BAP1*, and *SETD2*, leading to altered chromatin states and genome instability [37,40]; other chromatin modifiers, including *KDM5C* and *ARID1A*, and the components of the PI3K pathway (*TSC1&2*, *mTOR* and *PTEN*) [7]. Finally, beyond 3p loss, ccRCC is characterised by a set of recurrent copy number losses or gains involving whole chromosomes or chromosome arms [41]. Whole-

genome doubling occurs infrequently in ccRCC [42].

Chromosome 3p has limited homology in the mouse genome making it difficult for the stereotypical ccRCC genetics to be recapitulated in mouse models. In addition, the span of time over which these tumours evolve is hard to reproduce in pre-clinical models. Thus, dedicated studies of the human "model" of ccRCCs are necessary to understand how evolutionary features relate to the clinical disease course.

3.1. VHL disease as a complementary framework

Every instance of cancer evolution is unique, akin to the evolutionary biology concept of the "tape of life"; as posited by Gould in Wonderful life: "**any replay of the tape of life would lead evolution down a pathway radically different from the road actually taken**." [43]. Individuals affected by the von Hippel Lindau (VHL) disease, due to inherited *VHL* mutation [44], develop hundreds of clonally independent kidney cysts and conventional ccRCC tumours. VHL disease provides a ready-made *in vivo* model of evolutionary repeatability by recording all malignant developments starting from identical genetic backgrounds. We are currently pursuing an analysis of this readout through a collaboration with Marston Linehan's group [45]. VHL disease also provides a unique opportunity to understand how the cell of origin and tissue context constraints clonal evolution, as in addition to ccRCC, kindred develop hemangioblastomas, pancreatic cysts and pheochromocytomas, but not any other cancer types.

3.2. Evolutionary trajectories in primary ccRCCs

Given the frequent surgical resection of ccRCC primary tumours in the curative and metastatic (cytoreductive) setting, we could prospectively recruit and sample tumours at all stages of evolution in the context of the TRACERx Renal study (TRAcking renal cell Cancer Evolution through Therapy NCT03226886) [46]. We employed a multiregional, spatially aware sampling approach to maximise the recovery of distinct clonal populations. Taking advantage of the narrow repertoire of driver genes in ccRCC, we used a targeted gene panel to profile 1000s of samples, aiding robust clonal resolution. Further, because the driver SCNAs in ccRCC involve entire chromosomes/chromosomal arms and we sequenced to high depth (>600×), we successfully used off-target reads to infer SCNAs. Despite the relatively low number of clonal markers recovered by the gene panel, the phylogenies obtained were highly concordant with those based on WES and WGS multi-region data [37], consistent with the strong role of selection in ccRCC [31]. In the interim analysis of the TRACERx Renal cohort, we analysed >1200 primary tumour regions from 100 patients, observing that genetic diversity (measured as an *ITH score*) and chromosomal complexity (measured as weighted genome integrity index, *wGII*) were predictors of clinical outcome [37]; (Fig. 2). These evolutionary features provided distinct routes to progression, but also interacted such that both low ITH/high wGII and high ITH/high wGII tumours associated with worse outcomes. We found highly conserved patterns of driver event ordering,

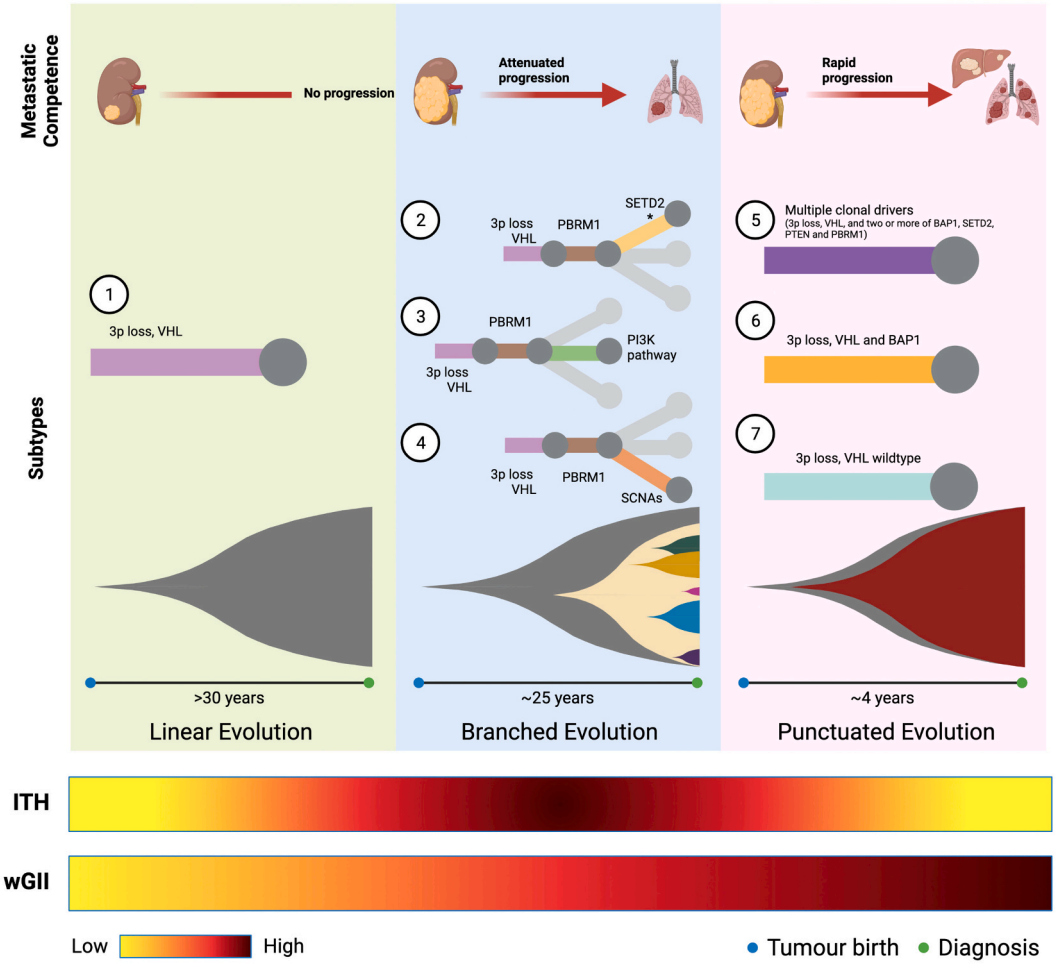

Fig. 2. Evolutionary trajectories in clear-cell renal cell carcinoma (ccRCC). Schematic illustration of seven evolutionary subtypes in ccRCC with the driver event ordering, clonal diversity (ITH) and genome instability (wGII) underpinning the phenotypic variation. The asterisk * represents parallel evolution of clones with various SETD2 mutations. ITH, intratumour heterogeneity; wGII, weighted genome integrity index; SCNA, somatic copy number alterations; PI3K, PI3K-AKT-mTOR pathway.

co-occurrence and mutual exclusivity pointing at the strong role of epistasis in shaping evolution of ccRCC. Following *VHL* inactivation, mutational order was often unidirectional, where *PBRM1* mutations preceded *SETD2* mutations, driver SCNA events, or PI3K pathway mutations [37]; (Fig. 2). The reason for this ordering was recently suggested in a pre-clinical model by Karki et al. 2021, to be the co-dependency of *PBRM1* and *SETD2* in cancer progression via the dysregulation of microtubule methylation [47]. We observed extensive parallel evolution, especially of *SETD2* mutant clones, with >10 distinct *SETD2* mutations detectable in some tumours [37]. The high frequency of parallel evolution provides further evidence of the strong role of selection in ccRCC and could also be a function of the large population size, as was suggested in non-cancer models [48].

We observed all modes of evolution in ccRCC. Tumours with *VHL* as the sole driver had low wGII and ITH and appeared to evolve linearly. The time from MRCA to clinical diagnosis in these cases was >30 years suggesting long periods of tumour growth with limited evolutionary potential. Clinically they associated with indolent behaviour and specifically SRMs [37], suggesting this evolutionary entity could be suitable for surveillance. Branched evolutionary trajectories were characterised by an initial 3p loss/*VHL* mutation followed by expansion of *PBRM1* mutant clones and additional subclonal driver events, resulting in high ITH and subclonal wGII. These tumours also evolved over long periods of time [37], consistent with gradual evolution. They grew to a large size, were highly vascularised and associated with limited metastatic progression. Patients with such tumours appeared to benefit from cytoreductive nephrectomy (removal of the primary tumour in the context of metastatic disease) or metastasectomy (surgical resection of metastasis), suggesting ongoing metastatic threat from the evolving primary tumour *in situ*. Finally, we observed punctuated evolution where an early fitness peak led to a clonal sweep resulting in low ITH and high wGII. While the tumours grew to a similar size to those with branched evolutionary mode, they did so rapidly with the evidence of high proliferation and the median time of four years from MRCA to diagnosis [37]. This evolutionary short-cut was achieved via three paths: 1. Acquisition of multiple driver events within the MRCA (clonal mutations in *VHL* and ≥ 2 clonal mutations in *BAP1*, *SETD2*, *PTEN* or *PBRM1*), the order of which could not be discerned in the absence of any intermediate populations. 2. *VHL* mutation followed by *BAP1* mutation leading to a rapid clonal sweep, highlighting *BAP1* as a strong driver of tumour growth [37]. 3. Bypassing *VHL* inactivation (*VHL* wild type tumour). These patients tended to present with rapid relapse following surgery with curative intent, likely due to occult widespread metastases hence the lack of impact from the removal of the primary tumour. TRACERx Renal is an observational study, however, these findings also reconcile the results of prospective interventional studies of cytoreductive nephrectomy (SURTIME), which reported that ~20% of patients progressed four weeks after surgery [49]. We posit that these patients likely had primary tumours characterised by punctuated evolution.

Thus, in the context of ccRCC there is a clear relationship between the mode and tempo of evolution and the clinical phenotype and patient outcomes, providing strong evidence for the use of evolutionary understanding in clinical decision making.

3.3. Evolution of metastatic competence

In the TRACERx Renal study, we analysed clonally resolved primary tumours with matched metastatic tumours to understand which clones can metastasise, and to determine if metastatic potential was established at the primary site. Metastasis-competent clones had the same burden of driver mutations but were more aneuploid, with higher wGII and higher proliferation index, compared to non-metastasising clones [50]. Consistent with the linear model of metastatic progression, metastases showed little ongoing evolution [11]. Losses of chromosomes 9p and/or 14q were highly enriched in metastasising clones, and their presence drove increased overall mortality [50]. The two events frequently co-occurred suggesting cooperation. Intriguingly, genome engineered ablation of 9p21 in a mouse model of ccRCC resulted in the early emergence of chromosomal instability and selection of other aneuploidies, most prominently loss of 12q, syntenic to human 14q [51].

The timing of the emergence of these high-risk events was dependent on the evolutionary mode. In branched evolution, they emerged late, and metastatic competence was confined to a primary tumour subclone. In punctuated evolution, they were fixed early, and metastatic competence dominated the primary tumour mass [50] (Fig. 2). Punctuated evolution resulted in monoclonal metastatic seeding, while metastatic divergence (polyclonal seeding) was observed with branched evolution of the primary tumour [50]. Considering the relationship between the tempo and extent of metastases and evolutionary modes it might simply reflect the prevalence of metastatic competence in the primary tumour (widespread in punctuated evolution and limited in linear evolution), especially given the inherent inefficiency of the metastatic process [52]. However, clonal interference (multiple clones emerging in a population at the same time and interfering with each other) could potentially constrain the metastasising clone, as has been shown in pre-clinical models [53] and contribute to the patterns observed.

Metastases were invariably seeded by the most aggressive, chromosomally complex clone in the primary tumour, with one exception. Pancreatic metastases were seeded by the ancestral clone, harbouring only bi-allelic *VHL* loss without 9p or 14q loss. Pancreatic metastases tend to be indolent and emerge late, often as an exclusive metastatic site in ccRCC [54]. The limited fitness of the founder clone reconciles the lack of cross-organ seeding in these patients and raises the possibility that slow growth, rather than dormancy, underpins the late emergence of pancreatic metastases.

Metastatic cancer remain largely incurable, and our insights in ccRCC provide an evolutionary understanding of the metastatic process relevant to predicting the risk and extent of metastatic disease, with clear implications for patient surveillance, timing and nature of therapeutic interventions.

3.4. Spatial dynamics of clonal evolution

Understanding of cancer evolution is incomplete without the knowledge of how clones expand and interact in space and over time. The unique sampling framework in our cohort yields spatial maps, where each genetically defined clone is mapped back to an area in the tumour. We integrated spatial, genomic, immunohistochemistry and clinical data to evaluate the spatial characteristics of clonal growth in ccRCC [55]. Clones harbouring SCNA drivers associated with the largest clonal expansions, confirming their significant fitness advantage relative to smaller scale alterations. Clones at the tumour centre, compared to the periphery, represented an aggressive phenotype, characterised by higher Fuhrman grade, proliferation, SCNA burden, metastatic competence, and necrosis [55]. This suggests a harsh, hypoxic TME at the tumour core which selects highly fit/proliferative clones, with the subsequent high cell turn-over and death making the TME even harsher. In a corresponding mathematical model, the occurrence of necrosis at the tumour centre supports expansion of highly aneuploid clones establishing a vicious cycle of repeat selection of the most aggressive clones [55].

We further applied mathematical modelling to understand the different modes of evolution we observed in patients' tumours (see 3.2) [56]. We observe that surface growth (active growth at the tumour surface) enables more extensive subclonal diversification and branching; while volume growth (uniform growth throughout the tumour) associates with punctuated evolution. In both modelling and experimental data, parallel evolution and hotspots of microdiversity (diversity within a single tumour region) emerged towards the tumour edge [56]. Temporal features of tumour evolution are challenging to discern without serial tumour biopsies which are impractical in most cases. We thus used our model to “re-wind” the simulation and find the early

indicators of subclonal diversification. Intriguingly we observed budding structures on the tumour surface indicating recent clonal expansions, in the early stages of tumour growth [56]. When we evaluated radiological images from patients with early stage ccRCC we also observe budding structures sometimes corresponding to the areas of confirmed microdiversity, indicating a recent clonal expansion [56]. Surprisingly, radiological imaging appears to bear a footprint of clonal dynamics.

Thus, combining mathematical modelling and large-scale real-tumour analyses is very powerful and clinically relevant. The model can be accurately parameterized from the primary data, while features which remain hidden in the primary data can be explored through in silico tumour evolution. Our findings indicate that spatially aware tumour sampling could detect clinically relevant clones: metastasising clones in the tumour-centre and emergent parallel evolution and microdiversity in the periphery. Finally, radiological features may predict future evolutionary steps in early-stage tumours which could aid risk stratification and clinical management.

3.5. Future directions

In the follow up analyses of the TRACERx Renal cohort involving 200 patients, we will refine the evolutionary classification to detect less common evolutionary trajectories. In the interim analysis we recovered successful clonal expansions through bulk sequencing, with the sensitivity to detect variants present in ~5 million cells. In the future, we will incorporate the features of microdiversity through microclone (sensitivity to detect variants present in 100–1000s of cells) and single cell sequencing, to systematically map clone fitness and evolutionary potential/ evolvability of individual tumours. To explore evolutionary constraints, we will dissect the individual contribution of the driver events in pre-clinical models and test the fitness costs of reversing their order and combination. We are investigating the TME through spatial biology approaches to define clone-specific and evolutionary mode specific niche and derive stable eco-evo predictions. Evolutionary predictions in routine practice will require scalable methods that consider vast intratumor heterogeneity (e.g. Rep-Seq). Increasingly, the application of artificial intelligence could capture evolutionary features from routinely available histological and radiological data which offer resolution of spatially distributed features.

4. Conclusion and perspective

The focus of our group is fundamentally understanding cancer evolution. However, the ultimate objective is to improve patient outcomes by leveraging our understanding of cancer dynamics. Targeted therapies and immune checkpoint inhibition have drastically improved cancer outcomes in the last decade. However, many patients relapse following surgery with curative intent and/or adjuvant therapy, while metastatic tumours remain largely incurable due to the emergence of resistance to systemic therapies. Understanding clonal evolution that had already taken place, especially its mode and tempo, offers the opportunity to predict the next evolutionary step, and possibly re-direct the tumour to a more favourable outcome. The knowledge of evolutionary constraints can shed light on tumour vulnerabilities, such as synthetic lethality, that can be exploited for novel therapies.

Evolutionary dynamics can inform flexible strategies of therapeutics to limit tumour adaptation and/or prevent resistance, such as ‘adaptive therapy’ [57] or ‘sucker's gambit’ [58]. Such strategies require dynamic monitoring, and approaches such as Rep-Seq could be implemented in a clinical setting to inform the optimal ctDNA panels to facilitate such monitoring.

It has become clear that the clonality of drug targets (for example HER2 in early-stage breast cancers [59] and FGFR in gastric cancers [60]) can impact treatment responses, while clonal neoantigens are superior to subclonal neoantigens in generating anti-immune responses following immune checkpoint inhibitor therapy [61]. Thus evaluating evolutionary features could predict the likelihood of emergence of resistance prior to therapy initiation and inform upfront combinatorial approaches.

The heterogeneity in transcriptional cell states, epigenetic profiles and interactions of tumour clones with the TME provide critical cues in our understanding of cancer evolution. Ultimately, a holistic understanding can only be achieved by an integrated approach involving multi-omics, spatial biology approaches, histopathology and radiology.

In conclusion, understanding the dynamics of cancer evolution will benefit patient management and clinical decision-making by using an ‘evolution-aware’ framework. While our current knowledge of tumour evolution is growing, a comprehensive tumour evolution ‘rule book’ is far from complete. Transformational, large-scale clinical studies coupled with pre-clinical models and cutting-edge methods are required to realise this aspiration. In the meanwhile, methods that can accurately capture tumour evolution must be considered for clinical adoption through prospective studies.

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Declaration of Competing Interest

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Data availability

No data was used for the research described in the article.

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Figs. 1 and 2 were created with BioRender.com. Fig. 1 makes use of elements from the BioRender.com template ‘New Strategies for Treating Cancer’ and ‘Cancer Evolution’ (2022). The figures are unpublished

original works, created for the express purpose of publication in this review. H. Pallikonda holds an institutional BioRender license through the Francis Crick Institute.

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