Spatial patterns of tumour growth impact clonal diversification: computational modelling and evidence in the TRACERx Renal study

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Title:

Spatial patterns of tumour growth impact clonal diversification: computational modelling and evidence in the TRACERx Renal study

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Abstract:

Intra-tumour genetic heterogeneity (ITH) fuels cancer evolution. The role of clonal diversity and genetic complexity in the progression of clear-cell renal cell carcinomas (ccRCCs) has been characterised, but the ability to predict clinically relevant evolutionary trajectories remains limited. Here, towards enhancing this ability, we investigated spatial features of clonal diversification through a combined computational modelling and experimental analysis in the TRACERx Renal study. We observe through modelling that spatial patterns of tumour growth impact the extent and trajectory of subclonal diversification. Moreover, subpopulations with high clonal diversity, and parallel evolution events, are frequently observed near the tumour margin. In-silico time-course studies further showed that budding structures on the tumour surface could indicate future steps of subclonal evolution. Such structures were evident radiologically in 15 early-stage ccRCCs, raising the possibility that spatially resolved sampling of these regions, when combined with sequencing, may enable identification of evolutionary potential in early-stage tumours.
Introduction:

The development of cancer can be viewed as an evolutionary process (Merlo et al. 2006, Zahir et al. 2020). Acquisition of genomic alterations including mutations and somatic copy-number alterations (SCNAs) drives the emergence of genetically heterogeneous subpopulations of cancer cells or subclones (McGranahan and Swanton 2017), resulting in intra-tumour heterogeneity (ITH). A small subset of genomic alterations (drivers) endows subclones with increased fitness that manifests as growth and survival. Subclones compete for resources, including physical space, and undergo expansion or extinction according to their fitness under given selective pressures imposed by the tumour microenvironment (TME) or therapeutic intervention. With recent advances in next-generation sequencing, clonal architecture and evolutionary features have been elucidated in a variety of tumour types (Gerlinger et al 2012, Yates et al 2015, Jamal-Hanjani et al 2017, Turajlic et al 2018). However, the ability to predict clinically relevant evolutionary trajectories remains limited.

One potential to enhance this ability lies in the detection and characterisation of ongoing clonal evolution. ITH provides a substrate for the selection of competent clones under evolving TME and frequently underpins therapeutic failure (McGranahan and Swanton, 2015). Detection of ITH relies on proper selection of samples and can be enhanced by sampling many small-size regions (Lopez and Cortes, 2016). While ITH index and macrodiversity (i.e., the number of subclones in the whole tumour) reflect the overall degree of established clonal diversity and genetic complexity within a tumour, clone diversity at a narrow spatial scale, or microdiversity (i.e., the number of subclones within a single tumour sample), could represent under-detected ongoing clonal evolution and delineate future evolutionary trajectories (Figure 1a). Just like macrodiversity, microdiversity appears to have clinical implications and has been shown to underpin poor survival in paediatric kidney cancer (Mengelbier et al 2015). Microdiversity was also revealed by the presence of multiple subclones both in situ and in co-migrating cell groups during tumour invasion in breast tumours, using topographic single cell sequencing (Casasent et al. 2018). Clonal diversification could be facilitated by parallel evolution, that is selection of distinct mutations in the same gene in spatially separate subclones. Parallel evolution has been observed in the natural evolution of various tumour types (Gerlinger et al 2012, Turajlic et al 2018, Melchor et al. 2014, Murugaesu et al. 2015, Yates et al 2015) and in the context of therapeutic resistance under selective pressure of therapy (Voss et al 2014, Juric et al 2015). More recently, parallel evolution of SCNAs was characterised comprehensively through mirrored subclonal allelic imbalance (Watkins et al. 2020). As microdiversity and parallel evolution are important indicators of ongoing and clinically relevant tumour evolutionary features, their spatial resolution could offer further understanding of evolutionary potential.

To understand the emergence and spatial patterning of these features, we developed a coarse-grained cellular automata model of tumour growth and clonal dynamics (Figure 1b). Previous experimental evidence supports predominating proliferation at the surface of ccRCCs (Hoefflin et al. 2016). However, both some cases in this study and our recent work suggest that this may not be universally true (Zhao et al. 2020). As exemplified by several categories of mathematical models formulated to characterise tumour growth dynamics (Rodriguez-Brenes et al. 2013, Gerlee 2013), different growth patterns could have an impact on evolutionary features. Moreover, previous computational studies found an impact of growth patterns on the classification of neutral evolution and selection (Sun et al. 2017, Chkhaidze et al. 2019). Therefore, we further evaluated the effects of different spatial patterns of tumour growth (hereafter also referred to as tumour growth mode) on spatial features of clonal diversity in our computational model. While distinct growth patterns may predominate in different parts of a
tumour, or at different stages of the same tumour growth, we restricted our investigation in the
current study to two simple growth models: one model with uniform growth throughout the
tumour volume as a “null model” assuming that all tumour mass can proliferate (referred to as
“Volume Growth Model”) and the other model with active proliferation restricted to the tumour
surface (referred to as “Surface Growth Model”) (see Methods, Figure 1c).

In the context of the TRACERx Renal study, we reported a workflow to evaluate both the
genomic profiles and spatial coordinates of 756 regions (patient tumour regions are referred to
as “PT regions” hereafter) from 66 tumours (Zhao et al. 2020). Here, we focus on spatial
analyses of microdiversity and parallel evolution in the same dataset. We found in the model
setting that the spatial patterns of tumour growth influenced the extent and trajectory of
subclonal diversification. Furthermore, subpopulations with high microdiversity, as well as
parallel mutational events with limited clonal expansion, were frequently detected near the
tumour edge, which was corroborated in the TRACERx Renal dataset. Finally, time-course
studies on simulated tumours suggested that evolutionary steps could be predicted at an early
stage and seeded by clones in the budding structures on the tumour surface in a subset of
tumours. These budding structures were evident in 15 early-stage ccRCC tumours, raising the
intriguing possibility that spatially resolved sampling, when combined with sequencing, may
enable identification of evolutionary potential in early-stage tumours and predict the disease
course.
Results:

Generation of an agent-based model recapitulating ccRCC evolution

We developed a coarse-grained cellular automaton model to explore the evolutionary dynamics of ccRCCs, a brief outline for which is presented here (see Methods for detailed description). The model includes 12 driver genes and 14 SCNAs (Supplemental Figure 1) that were highlighted as canonical driver events in ccRCCs in the TRACERx Renal study (Turajlic et al. 2018). Each model unit, referred to as a “tumour voxel”, represents a tumour volume of 1 mm$^3$. Tumour voxels stochastically undergo growth, death, and, upon growth, processes of driver acquisition (i.e., mutations in driver genes or driver SCNAs). To keep the model minimal, several assumptions were made. In the model, time has arbitrary units, so the baseline growth probability $p^{(1)}_{growth}$ was arbitrarily defined. Three levels of growth probability, $p^{(1)}_{growth} \leq p^{(2)}_{growth} \leq p^{(3)}_{growth}$, were introduced for the 26 drivers, to broadly reflect their association with the Ki67 score in tumour regions in the experimental data (Supplemental Figure 1). As one specific implementation, growth probabilities were set at $p^{(1)}_{growth} = 0.25$, $p^{(2)}_{growth} = (1 + s)p^{(1)}_{growth}$ and $p^{(3)}_{growth} = (1 + s)^2p^{(1)}_{growth}$, where $0 \leq s \leq 1$ determines this advantage relative to the baseline growth probability. For simplicity, mutations in all driver genes were assigned with the baseline growth probability, while some SCNAs were assumed to confer greater growth probabilities. Four SCNAs with strong association with Ki67 score, 7q gain, 20q gain, 4q loss, and 8p loss, are the most “advantageous” drivers with $p^{(3)}_{growth}$. A tumour voxel grows with $p^{(1)}_{growth}$ conferred by the most advantageous driver harboured. Note that an alternative implementation for fitness advantage was also explored (Supplemental Figure 2, Supplemental Note 1, see Methods). Mutations in driver genes were assumed to be acquired with a greater probability ($p_{driver}$) than SCNAs. Based on functional evidence linking them to chromosome instability (Varela et al. 2011, Peng et al. 2015), as well as their association with a high weighted genome instability index (wGII) in the experimental data from the TRACERx Renal study (Turajlic et al. 2018), mutations in PBRM1 or BAP1, were assumed to enhance the probability of SCNA acquisition. Finally, a second mutation in the same gene was assumed to never occur in the same tumour voxel.

Each simulation starts from a single tumour voxel carrying VHL and 3p loss and is terminated when the size of the simulated tumour exceeds 1 million tumour voxels. Simulated tumours were analysed at three spatial scales: three-dimensional (3D) whole tumour volume, two-dimensional (2D) tumour slice and tumour regions (Figure 1d). Exploratory simulations (50 per condition) were performed at 6 levels of $s$ and 11 levels of $p_{driver}$. This permits the understanding of the collective impact of fitness advantage and the rate of driver acquisition in a spatial context.

Characterisation of clonal diversity in the whole tumour

Tumours under Volume Growth commonly developed a single dominant subclone that grew to occupy a large proportion of tumour surface (panel (i) in Figure 1e). In comparison, tumours under Surface Growth developed multiple advantageous subclones in different regions of the tumour surface, and each of these subclones was characterised by a range of different driver events (panel (ii) in Figure 1e). Moreover, while the tumour surface in the Volume Growth
model was smooth overall, the tumour under Surface Growth formed bulging structures on the
surface, which reflected the outgrowth of driver subclones.

Given the disparity in the quantity and spatial distribution of driver subclones in the
representative cases under Volume Growth and Surface Growth (Figure 1e), we then asked
how the mode of tumour growth might influence the number of subclones in the whole tumour.
We first counted the number of clones (including parental clone and subclones) in the whole
tumour (Figure 2a-b). In the Volume Growth model, subclones were only observed in tumours
with greater s and greater $p_{driver}$ (panel (i) in Figure 2b). By contrast, in the Surface Growth
model, tumours with small to moderate $p_{driver}$ harboured more subclones, for a wide range of
s (panel (ii) in Figure 2b). These findings suggest that the mode of tumour growth could
impact outgrowth of subclones and their ultimate prevalence.

Noticeably, tumours also displayed distinct proportions of subclonal populations, independent
of the growth mode and evolutionary conditions (i.e., identical s and $p_{driver}$) (Figure 2c). In
the Volume Growth model, most tumours with a small $p_{driver}$ displayed a monoclonal
structure with limited evidence of clonal evolution (panel (i) in Figure 2c). With a large
$p_{driver}$, a greater extent of clonal evolution was evident in a higher proportion of tumours
(panel (ii) in Figure 2c). In a fraction of these cases, one dominant subclone was observed
with the parental clone present as a minority, suggesting early fixation of a highly fit clone and
a (near) clonal sweep (i.e., the entire tumour was taken up by the dominant subclone). In
comparison, in the Surface Growth model, extensive subclonal diversification was evident in
nearly all cases, even with a small $p_{driver}$ (panel (iii) in Figure 2d). These features observed
in individual tumours were reflected by the whole-tumour cancer cell fraction (CCF) of the
parental clone (Figure 2e) and Shannon diversity index (Figure 2f). Across a wide range of
driver acquisition probabilities, the Surface Growth model, in comparison to the Volume
Growth model, consistently displayed a relatively lower whole-tumour CCF of the parental
clonal and a greater Shannon diversity index, indicating a greater extent of subclonal
diversification. The greater extent of diversification in the Surface Growth model was also
noted for conditions with still smaller $p_{driver}$ (Supplemental Figure 3) or smaller s
(Supplemental Figure 4), where clonal evolution was generally limited. For the interest of
characterising patterns of subclonal diversification and contrasting two growth modes, we
limited our parameter analysis, for the subsequent investigation, to s = 1 and a range of $p_{driver}$
from $2 \times 10^{-4}$ to $1 \times 10^{-3}$.

In general, Surface Growth appears to enable more extensive subclonal diversification, leading
to a highly branched tumour evolution, while Volume Growth often resulted in tumours with
limited clonal evolution and occurrence of punctuated evolution. Interestingly, these were the
precise modes of evolution that underlined diverse evolutionary subtypes identified in ccRCCs,
ranging from “VHL mono-driver” tumours with limited evidence of clonal evolution, to
tumours with extensive subclonal diversification characterised by a range of drivers, and those
with early fixation of a highly fit clone resulting in rapid clonal sweep (Turajlic et al. 2018).

Having established a quantitative understanding of clonal diversity in the whole tumour and
the impact of different growth modes, we next strived for a spatial understanding of clonal
diversity.
Evaluation of genomic divergence between tumour regions

As a first step towards understanding spatial features of clonal diversity, we asked whether in-silico tumours were able to recapitulate a phenomenon of spatial clonal evolution noted in colorectal and liver cancers by others (Cross, et al. 2017; Zhai, et al. 2017) and, more recently, in ccRCCs by our group (Zhao et al. 2020), whereby subclones are patterned within a tumour such that regions farther apart exhibit more divergent genomic makeups.

To this end, we sampled uniformly spaced regions within a 2D tumour slice and measured the spatial and genomic distances between each pair of regions (Figure 3a, see Methods). Consistent with previous experimental evidence (Cross, et al. 2017; Zhai, et al. 2017; Zhao, et al. 2020), between-region spatial and genomic distances were positively correlated (hereafter referred to as “spatial-genomic correlation”) in representative tumours under Surface Growth (Figure 3b-c) or under Volume Growth (Figure 3d-e) and also in repeat simulations across multiple values of \( p_{\text{driver}} \) (Figure 3f). Moreover, different growth modes resulted in distinct trends of variation in the spatial-genomic correlation with the change of \( p_{\text{driver}} \). In the Volume Growth model, the spatial-genomic correlation was weaker in tumours with smaller \( p_{\text{driver}} \). By contrast, in the Surface Growth model, the spatial-genomic correlation was weaker in tumours with greater \( p_{\text{driver}} \), as the extensive diversification likely caused large genomic divergence even in regions separated by a short distance (Figure 3f). As a consequence of these divergent trends, the spatial-genomic correlation was stronger in tumours under Surface Growth than those under Volume Growth if \( p_{\text{driver}} \) was small, and weaker if \( p_{\text{driver}} \) was large.

Experimentally, the cohort of ccRCCs in the TRACERx Renal study displayed a spatial-genomic correlation coefficient of 0.31 (Zhao, et al. 2020).

Overall, these data demonstrate that our model is sufficient to recapitulate the spatial-genomic correlations observed across experimental data, a pattern emergent from spatial evolution of subclones in tumours. We next asked how clonal diversity itself could vary spatially within a tumour slice and whether the mode of tumour growth had an impact on the spatial patterns.

Spatial distribution of clonal diversity within tumour sections

Characterisation of clonal diversity in the whole tumour and the between-region genomic divergence provided a quantitative view of the extent of subclonal diversification. We next sought to examine whether clonal diversity was spatially uniform or variable within a tumour. To this end, a 3mm-by-3mm sliding window was applied throughout the 2D tumour slice and the number of distinct subclones (defined as “microdiversity”) was counted in that 9-mm\(^2\) area (Figure 4a). Subsequently, the spatial profile of microdiversity was analysed to identify hotspots with microdiversity equal to or greater than 5 (defined as “microdiversity hotspots”) (Figure 4a).

Consistent with the previous analysis on the whole-tumour CCF of subclones (Figure 2a), tumours under Surface Growth developed multiple subclones that occupied distinct spatially contiguous areas, reflective of a branched clonal structure (panel (i) in Figure 4b). Microdiversity hotspots were frequently observed near the tumour edge within outgrowing advantageous subclones but also at the boundaries of multiple adjacent subclones (panel (ii) in Figure 4b). By contrast, tumours under volume growth commonly developed a single dominant subclone (panel (i) in Figure 4c). In these tumours microdiversity hotspots were
observed within the area spanned by the dominant subclone with a more uniform distribution along the tumour radius (panel (ii) in Figure 4c).

To corroborate these spatial features of clonal diversity with experimental data, we examined 66 tumours in the TRACERx Renal study. Individual PT regions that contain at least two subclones were treated as a proxy for microdiversity hotspots. In total, 606 PT regions from 54 tumours were included in this analysis. Different spatial distributions of microdiversity hotspots were observed, as highlighted in representative examples: predominantly near the tumour margin (as in “G_K234”), while more uniformly distributed throughout the tumour section (as in “G_K446”) (Figure 4d).

In order to quantify the observed spatial patterns, we measured the distance to the tumour centre and the distance to the closest point at the tumour contour, for every microdiversity hotspot over all repeat simulations. These two distances were then combined into a single variable termed the “normalised distance to tumour centre”, denoted as d. Interestingly, the cumulative probability distribution with respect to d depicted power law scaling (Figure 4e), suggesting that the probability of observing spots with high clonal diversity along the radius of a tumour could be estimated using a simple mathematical formula (i.e., \( P(D \leq d) \sim d^k \), where k is the power law exponent to be fitted). Furthermore, the Surface Growth model displayed a larger k compared to the Volume Growth model across multiple values of \( p_{\text{driver}} \) (Figure 4f, Supplemental Figure 5), indicating a greater likelihood of microdiversity hotspots being located near the tumour edge under Surface Growth. The power law pattern characterising the spatial distribution of microdiversity hotspots was corroborated in the experimental data (Figure 4e); ccRCC tumours displayed a k value numerically between those in the Volume Growth and Surface Growth models (Figure 4g). A greater error in matching data and fitted power law was noted at regions approaching the margin, likely attributed to under-sampling of biopsies at the tumour periphery (Supplemental Figure 5).

To summarise, regions with high clonal diversity within a tumour slice were increasingly frequent towards the tumour margin, a spatial feature particularly enhanced in the Surface Growth model and corroborated experimentally using PT regions with at least two subclones. These data suggested that subpopulations with high clonal diversity were abundant near the tumour margin.

Frequency and spatial features of parallel evolution

As subclonal diversification could involve acquisition of, and be facilitated by, distinct mutations in the same gene at spatially separate locations, we next evaluated the frequency of parallel evolution events and their spatial features.

Distinct mutational instances in the same driver gene were recorded as different driver events in the model (Figure 5a). Guided by an exploratory whole-tumour analysis, which showed parallel mutational events other than \( PBRM1 \) and \( BAP1 \) to be rare at relatively low resolution (Supplemental Note 2, Supplemental Figure 6a-b), we specifically focused on these two mutations. As might be expected, as a reflection of the extent of subclonal diversification in the whole tumour shown above (Figure 2b), parallel evolution was evident in the Volume Growth model only when s and \( p_{\text{driver}} \) were both large (panel (i) in Figure 5b), while it was pervasive in the Surface Growth model under various parameter conditions (i.e., pairs of s and \( p_{\text{driver}} \)) (panel (ii) in Figure 5b).

To further elucidate the spatial patterns of parallel mutations in \( PBRM1 \) or \( BAP1 \), we next labelled tumour regions harbouring these events (Figure 5a). Within a tumour section,
subpopulations harbouring parallel mutational events expanded to occupy a variable number of regions (Figure 5c-d). When mutational instances in \textit{PBRM1} or \textit{BAP1} occurred late, they led to limited clonal expansion (up to two tumour regions), despite the role in promoting acquisition of SCNAs (Supplemental Figure 6c-f). Those instances were commonly observed at a short distance to the tumour edge (Figure 5e). Furthermore, for a range of \( p_{\text{driver}} \), the Surface Growth model resulted in a shorter distance of the parallel evolution events to the tumour edge, compared to the Volume Growth model, likely due to the birth of these subclones occurring at the tumour surface.

In the TRACERx Renal study, parallel evolution was observed in 28 tumours, with parallel mutations in the same gene spanning distinct sets of regions (Supplemental Note 3). As highlighted previously (Turajlic, et al. 2018) and similar to the \textit{in-silico} tumours, distinct parallel mutational events could span a variable number of PT regions (Figure 5f). Consistent with the \textit{in-silico} analyses, parallel mutational events in driver genes with limited clonal expansion (spanning up to two PT regions) were predominantly located near the tumour edge (Figure 5g). This spatial distribution was also found for mutational events without evidence of parallel evolution and with limited expansion (Figure 5g), suggesting that subclones lacking significant expansion were commonly emerging near the tumour edge. The lack of expansion of these subclones could be attributed to either their narrow fitness advantage and/or late occurrence.

Together, these data suggest that parallel evolution in \textit{BAP1} or \textit{PBRM1} was more prevalent in the Surface Growth model, which underlined extensive subclonal diversification. For both \textit{in-silico} modelling and experimental analysis of ccRCC tumours, parallel mutations with limited clonal expansion were located near the tumour edge, implying that a spatially resolved sampling could target the tumour margin to identify ongoing parallel evolution.

**Predictive feature of future evolutionary trajectories**

Having so far focused on quantitative and spatial characterisation of microdiversity and parallel evolution, we next asked how features of clonal diversity evolved temporally and whether some features could enable the prediction of future evolutionary trajectories. The ability to predict likely evolutionary trajectories could aid in the clinical management of ccRCC tumours. However, the challenges of obtaining serial biopsies of the same tumour limit this opportunity in clinical practice. Therefore, we examined if our model could be used to indicate the temporal features of evolution of clonal diversity.

To this end, the number of subclones within a tumour slice was tracked over time. Tumours under Volume Growth developed a small number of subclones that were sustained over the course of tumour evolution (Figure 6a, panel (i) in Figure 6b, Supplemental Figure 7a). By comparison, the number of subclones increased over time in tumours under Surface Growth (Figure 6a, panel (ii) in Figure 6b, Supplemental Figure 7b). Nevertheless, tumours under distinct growth modes appeared indistinguishable at early stage according to this measurement. We then asked whether some spatial features could indicate early the divergent patterns of subsequent subclonal diversification in the two models.

To shed light on this question, we evaluated the Surface Growth model for the presence of early indicators of subclonal diversification. In fact, one characteristic spatial feature noticed in tumours under Surface Growth was a budding structure on the tumour surface, which indicated the beginning of outgrowth of an advantageous subclone (Figure 1e, Figure 6c).
Quantitatively, this budding structure reflected the rapid increase in the CCF of an advantageous subclone, as it spatially outcompeted surrounding subpopulations (Figure 6d). Depending on the driver acquisition probabilities employed in the model, the median tumour size for the detection of such budding structures was around 7 cm or smaller. Exploratory simulations attempting at “replaying” evolution (i.e., re-simulating clonal evolution from a historical tumour state with established clonal structure as a starting point) starting from different tumour sizes suggested that evolution was more repeatable if starting from a historical tumour state with budding structures emerging (Supplemental Figure 8, Supplemental Note 4).

With respect to the above findings, we then turned to analysing 46 tumours with a size of up to 7 cm in the TRACERx Renal study. By qualitative examination of radiological images of these tumours, 15 tumours displayed apparent budding structures at their surface. In one representative case (“G_K523”), budding structures were evident both radiologically (Figure 6e) and in the tumour contour image with clonal diversity mapped (Figure 6f). Interestingly, trailing the budding structures were regions with high clonal diversity, consistent with our in-silico tumour simulations under Surface Growth. These findings imply ongoing subclonal diversification in these regions that may delineate future evolutionary steps.

Lastly, in the TRACERx Renal study, the number of subclones depicted a non-linear relationship with tumour size (Figure 6g). This broadly reflected different modes of evolution, ranging from limited evolution (bottom-left part in Figure 6g), to punctuated evolution (bottom-right part in Figure 6g) and branched evolution (top part in Figure 6g). Furthermore, a subset of tumours (enclosed only by blue contour in Figure 6g) were better recapitulated by the Volume Growth model, while another subset by Surface Growth model (enclosed only by red contour in Figure 6g). A more intriguing question still is whether the future steps of clonal evolution in the early-stage tumours could be predicted. When 15 early-stage tumours with apparent budding structures were highlighted, a subset of these already displayed a greater extent of subclonal diversification, raising the possibility that spatially resolved sampling combined with sequencing in these regions may enable identification of evolutionary potential in early-stage tumours.
Discussion:

Intra-tumour genetic heterogeneity arises when different parts of the tumour acquire distinct genomic alterations, endowing subclones with a variety of fitness advantages. Despite our understanding of how dominant subclones sculpt evolutionary trajectories and histories, gleaned primarily from multi-region sampling and deep sequencing, under-represented subclones that reflect ongoing and clinically relevant evolution could remain undetected. Therefore, our focus on microdiversity and parallel evolution is central to the elucidation of the evolutionary potential of under-represented subclones and the emergent spatial patterns they form.

An important finding, via computational modelling, is that different spatial patterns of tumour growth impact the extent of subclonal diversification and shape divergent modes of evolution. While Surface Growth (i.e., proliferation limited to the superficial layer of a tumour) enables more extensive subclonal diversification and gives rise to branched evolution, Volume Growth (i.e., proliferation active throughout the tumour volume) commonly results in single dominant subclone coexistent with founding clone reflective of punctuated evolution. Linear evolution was not observed in our model. Experimentally, the inferences of linear evolution are generally biased by the lack of sufficient sampling to detect intermediate populations. Overall, this observation resonates with other computational studies to highlight the importance of understanding spatial patterns of tumour growth when deconstructing tumour evolution (Anderson et al 2006, Waclaw et al 2015, Sun et al 2017, Chkhaidze et al 2019, Noble et al 2019).

Another important finding, in both the in-silico tumours, in particular those under Surface Growth, and tumour in the TRACERx Renal study, are frequent microdiversity hotspots and parallel mutational events near the tumour edge, suggesting abundant ongoing evolution at the tumour margin. Temporally tracking the parallel mutational events in PBRM1 or BAP1 in the in-silico setting illuminated the rapid increase of their prevalence in small tumours leading to ultimately established subclones. In those under Surface Growth, this rapid transition was marked by a budding structure on the tumour surface. Interestingly, such budding structures were evident in 15 early-stage ccRCC tumours. While a prospective study in a large cohort is needed for further examination, this observation opens up the possibility that designing spatially resolved sampling strategies in early-stage tumours with assessment of clonal diversity at the tumour margin, in particular within the budding structures, could be beneficial in informing upon a tumour’s evolutionary potential.

Our computational modelling also uncovered power law scaling features that uniquely characterise clonal diversity in a spatial context. These features add to previously reported scaling phenomena describing clonal evolution (Driessens et al 2012, Williams et al 2016). In addition to the scaling feature underlying the spatial distribution of microdiversity hotspots, clonal diversity and sampling area also form a scaling relationship in both the model and experimental data (Supplemental Figure 9, Supplemental Note 5). This scaling relationship in ccRCC tumours is reminiscent of the taxa-area relationship in the macroevolution of species (Crawley and Harral 2001, Horner-Devine et al 2004, Zhou et al 2008), drawing a parallel between cancer evolution and evolution in the ecosystem.

Overall, our computational model was able to correlate with several clinical observations, despite the simplification of its primary components (probabilistic birth, death, and driver acquisition). Nevertheless, we are aware that our model has limitations. Clearly, many other
factors, such as vascularisation, necrosis, and immune predation, are all likely to (re)shape the
\textit{in-silico} patterns of clonal diversity we observed. The simple implementations of fitness
advantage endowed by ccRCC drivers could be improved in the future with wet-lab
experiments aimed at measuring and comparing growth kinetics of tumours with different
genetic backgrounds. Future time-lapse experiments could also provide evidence for growth
modes of ccRCCs and elucidate whether different modes may predominate in different regions
or at different stages of the same tumour. Moreover, while we made an assumption that only
mutations in \textit{BAP1} or \textit{PBRM1} promoted acquisition of SCNAs for simplicity, we acknowledge
that other ccRCC driver mutations (e.g., \textit{SETD2}) also link to chromosome instability. Lastly,
alternative mechanisms could explain the formation of budding structures and other protrusive
morphologies, such as mechanical properties (Fiore et al. 2020) and cell migration (Anderson
et al. 2006). In addition to these limitations from a modelling perspective, differences in size,
stage, and the number of samples among tumours in the TRACERx Renal study could
confound the patterns to which the computational outputs are compared. The current
sequencing data is panel-based with a small number of clonal markers and may have low
sensitivity to detect microdiversity. Future examination of whole-exome or whole-genome data
could lead to a more comprehensive characterisation of ongoing clonal evolution.

In conclusion, our study supports the importance of understanding spatial patterns of tumour
growth in deconstructing tumour evolution. It provides evidence, and has implications for,
focused spatial sampling of the tumour margin in order to garner richer information on ongoing
and clinically relevant evolution.
Methods:

Computational model

Tumour growth and clonal evolution in a spatio-temporal context have increasingly been studied with the aid of computational models that incorporate rule-based spatial growth and acquisition of genomic alterations (Anderson et al 2006, Waclaw et al 2015, Sun et al 2017, Chkhaidze et al 2019). Spatial patterns of tumour growth (Sun et al 2017, Chkhaidze et al 2019) have been shown to impact the ability to classify neutral evolution in contrast to selection, suggesting that spatial growth of a structured population interplays with evolutionary forces (driver acquisition, selection, and genetic drift) to shape the spatial patterning of subclones.

In the present study, a coarse-grained cellular automaton model has been constructed to simulate tumour growth and the evolution of ccRCC drivers. A basic model unit reflects a tumour volume of $1 \times 1 \times 1 \ mm^3$, referred to as a “tumour voxel”. The choice of 1 $mm$ for the size of a tumour voxel reflects the observed thickness of actively proliferative surface layer of some ccRCCs (Hoefflin et al. 2016). The full simulation lattice comprises $200 \times 200 \times 200$ lattice sites in which a tumour grows. The following sub-sections detail the model components and assumptions.

Growth and death

Tumour voxels stochastically undergo growth and death, with baseline probabilities per simulation step of $p_{\text{growth}} = 0.25$ and $p_{\text{death}} = 0.05$, respectively. Upon death, a tumour voxel is removed from the simulation lattice, rendering the site empty and available for accommodating new tumour voxels. Two different modes of spatial tumour growth are considered: Surface Growth and Volume Growth (Figure 1c). For Surface Growth, proliferation is only allowed to take place when space is available, namely, when at least one of the 26 neighbouring lattice sites of the tumour voxel selected to divide is empty. Upon duplication of a parent tumour voxel, one child tumour voxel retains the location of the parent while the other is placed at a randomly selected adjacent empty site. For Volume Growth, all tumour voxels are able to proliferate; upon duplication, one child tumour voxel retains the location of the parent while the other is placed at a selected adjacent site according to the rule described below and pushes tumour voxels in that orientation outward. The process for selecting an adjacent site includes two steps: (1) to randomly sample 10 candidate positions out of the 26 neighbouring lattice sites; (2) to select the orientation (i.e., pointing from the position of the parent tumour voxel to the candidate position) giving the smallest distance from the tumour surface, similar to the algorithm described in Waclaw et al. 2015.

Driver events

A panel of 26 ccRCC drivers that were highlighted in Turajlic et al. 2018, including mutations in 12 driver genes and 14 somatic copy number alterations (SCNAs), are considered in the present work (Supplemental Figure 1). For simplicity, the fitness advantage conferred by a driver is assumed to manifest as growth advantage. In implementation, three levels of growth probability, $p_{\text{growth}}^{(1)} \leq p_{\text{growth}}^{(2)} \leq p_{\text{growth}}^{(3)}$, were introduced for the 26 drivers, to broadly reflect their association with the Ki67 score in tumour regions in the experimental data.
In a general form, $p^{(2)}_{\text{growth}} = g(s)p^{(1)}_{\text{growth}}$ and $p^{(3)}_{\text{growth}} = h(s)p^{(1)}_{\text{growth}}$ are functions of baseline growth probability, where $h(s) \geq g(s) \geq 1$ reflect the growth advantages relative to the baseline. As one specific implementation, growth probabilities were set at $p^{(1)}_{\text{growth}} = 0.25$, $p^{(2)}_{\text{growth}} = (1 + s)p^{(1)}_{\text{growth}}$ and $p^{(3)}_{\text{growth}} = (1 + s)^2p^{(1)}_{\text{growth}}$, where $0 \leq s \leq 1$ determines this advantage relative to the baseline growth probability. The baseline growth probability $p^{(1)}_{\text{growth}}$ was arbitrarily defined, as time has arbitrary units in the model. For a given tumour voxel, its growth probability is defined by the most advantageous driver harboured (i.e., “saturated” model of fitness advantage). For simplicity, $VHL$ mutation and 3p loss are considered as truncal events in the founder tumour voxel, although in a clinical setting $VHL$ mutation are not present universally (Turajlic et al. 2018). The subpopulation of tumour voxels that only harbour these two events is referred to parental clone. For an exploratory purpose, an alternative implementation of growth advantage endowed by drivers was also evaluated (Supplemental Figure 2). In this implementation, a driver adds a certain amount of growth probability to the tumour voxel that acquires the driver (i.e., “additive” model of fitness advantage) (Supplemental Figure 2a). The growth probability of a tumour voxel depends on all the drivers it harbours, namely, $p_{\text{growth}} = p^{(1)}_{\text{growth}} + \sum_k p_{\text{growth}, k}$, where $p_{\text{growth}, k}$ reflects the amount of growth probability added by driver $k$. $p_{\text{growth}}$ is set to one if the calculated probability exceeds one. The amount $p_{\text{growth}, k}$ varies between drivers, reflecting different strengths of their association with Ki67 score (Supplemental Figure 1a). Three different scenarios were explored to reflect different amounts of growth probability endowed by drivers on average, as determined by $s_k$ of the weakest driver, namely, $\min(s_k)$, and the difference in $s_k$ between consecutive two drivers in their advantages, namely, $\Delta s_k$. (Supplemental Figure 2b).

Upon proliferation of a parent tumour voxel, child tumour voxels inherit existing driver events harboured by the parent tumour voxel and stochastically acquire new drivers. Mutations in driver genes are acquired with a probability $p_{\text{driver}}$ and are assumed to be acquired in a tumour voxel more frequently than SCNAs, which have probability of $0.001p_{\text{driver}}$. Given the functional evidence that $BAP1$ (Peng et al. 2015) and $PBRM1$ genes (Varela et al. 2011) guard against chromosome instability and the experimental data in the TRACERx Renal study demonstrating the association of mutations in these genes with high wGII (Turajlic et al. 2018), we assume that the acquisition of SCNAs in a tumour voxel becomes equally likely as mutations in driver genes, if the tumour voxel harbours mutations in $BAP1$ or $PBRM1$. A range of driver acquisition probabilities have been studied to explore its impact on patterns we investigate. Lastly, only one mutation in the same driver gene is permitted in the same tumour voxel, but multiple independent, distinct mutations may be acquired in parallel within a simulated tumour in different tumour voxels.

Simulation

Each simulation starts from a single tumour voxel placed at the centre of the lattice, $(x_0, y_0, z_0)$, and runs until the tumour grows to at least 1 million tumour voxels after the last simulation step. In each simulation step, growth and death are evaluated for every tumour voxel, in random order. For those tumour voxels selected to grow, driver acquisition is evaluated for each of the 26 drivers. The computer code is written in CUDA C++.
Model analyses

Levels of analysis

Analyses have been conducted at three different levels (Figure 1D): (1) whole tumour level, which takes into account all tumour voxels in the 3D volume; (2) tumour slice level, which takes into account all tumour voxels within a 2D plane \((z = z_0)\); (3) regional biopsy level, which takes into account tumour voxels within regional biopsies. A regional biopsy is defined as all tumour voxels within a region in the 2D slice. Spatially uniform sampling is performed in this study. This process is carried out by locating the centres of candidate regional biopsies in the \(200\text{mm} \times 200\text{mm}\) 2D lattice with a spacing of \(20\text{mm}\) and collecting all voxels within a distance of \(5\text{mm}\) from each biopsy centre.

Spatial maps of driver events

In representative cases, tumour voxels that harbour selected driver events are mapped, within a 3D tumour surface and within a 2D tumour slice. In spatial maps of driver events, tumour voxels that don’t harbour drivers of interest are in grey; tumour voxels that harbour mutations in driver genes are in blue; tumour voxels that harbour gain of chromosome arm events are in green; tumour voxels that harbour loss of chromosome arm events are in red.

Spatial maps of subclones

A subclone is defined as a group of tumour voxels that harbour the same set of driver events. In spatial maps of subclones, tumour voxels that belong to the parental clone are in grey, while other subclones are visualised in randomly generated (R, G, B) colours.

Cancer cell fraction (CCF) of driver events

CCF of a driver event is calculated as the number of tumour voxels that contain the driver event divided by the total number of tumour voxels in the domain of interest, depending on the level of analyses. A driver event is considered detectable if the CCF is greater than 0.01.

Cancer cell fraction of subclones

CCF of a subclone is calculated as the number of tumour voxels that belong to a subclone divided by the total number tumour voxels in the domain of interest, depending on the level of analyses. A subclone is identified by a set of driver events, shared by a subpopulation of tumour voxels, which are accumulated within the subclone-initiating tumour voxel. A subclone-initiating tumour voxel is defined as a tumour voxel that acquires a new driver event upon birth. A subclone is considered detectable if the CCF is greater than 0.01.

Shannon diversity index

As a measure of clone diversity, Shannon diversity index is defined as \(S = \sum_i -f_i \ln f_i\), where \(f_i\) is the CCF of the subclone \(i\). All subclones are taken into account in this calculation.
Spatial and genomic distances

Spatial distance between two regional biopsies is calculated as the Euclidean distance between the positions of the centres of two biopsies. Genomic distance between two regional biopsies is calculated as:

$$d_{gen} = \sqrt{\sum_{k} (G_{i}^{k} - G_{j}^{k})^2},$$

where $G_{i}^{k}$ is the state of $k$th driver in the $i$th region and has a value of 1 if the $k$th driver is present, otherwise a value of 0.

Microdiversity

Microdiversity is defined as the number of subclones contained in a 3-mm-by-3-mm region within the tumour slice. In representative cases, microdiversity is spatially mapped within a tumour slice, by sliding a 3-mm-by-3-mm spatial window throughout the tumour slice. Microdiversity hotpots are defined as a subset of these small regions with 5 or more subclones. The distance from a microdiversity hotspot to the centre of a tumour slice is referred to as the distance to tumour centre ($d_1$). The distance from a microdiversity hotspot to the nearest point along the tumour contour is referred to as the distance to tumour margin ($d_2$). The normalised distance to tumour centre is defined as $d = d_1/(d_1 + d_2)$. Cumulative probability distribution of $d$ is generated by combining microdiversity hotspots from repeat simulations. Power law exponent is obtained by bootstrapping 100 samples of 400 hotspots per sample and fitting a power law function to cumulative probability distribution of $r$ in each sample.

Parallel evolution

Parallel evolution refers to the independent, distinct instances of mutations in the same driver gene that are acquired at different tumour locations. At the whole tumour level, the extent of parallel evolution is assessed by counting the instances of independent, distinct instances of mutations in each driver gene at a given resolution of detection. The resolution of detection is represented using a minimum CCF that has to be reached by a parallel mutational event to be detectable. At the regional biopsy level, the extent of parallel evolution is assessed by counting the number of regions that contain distinct instances of mutations for each driver gene. The distance from any region containing a parallel instance to the tumour margin is measured, to study the spatial distribution of parallel mutational instances with limited clonal expansion.

Temporal analysis

For the time-course study, 250 simulations with 5 different levels of $p_{\text{driver}}$ (50 simulations per condition) are performed for each of Surface Growth and Volume Growth models. 2D tumour slices are collected every 10 steps in Surface Growth model and 5 steps in Volume Growth model. The number of subclones is counted within each historical tumour slice. Kernel density estimation with a Gaussian kernel is performed with respect to the number of subclones and the diameter of the tumour slice, based on all simulations, to produce a continuous density estimate, using seaborn.jointplot(kind = 'kde') in Python. In a representative case, tumour voxels that harbour parallel mutational events in $BAP1$ are mapped within historical tumour slices. In all simulations, CCFs of subclones that harbouring mutations in $BAP1$ are measured within historical tumour slices over time.
Evolutionary replay

To perform evolutionary replay, historical tumour state (i.e., locations and clonal identities of tumour voxels) at a certain time point is saved and employed as a common starting state for regrowth 50 new simulated tumours. At the end of these simulations, Shannon diversity index is calculated to indicate the divergence in their evolutionary outcomes.

Experimental analyses

TRACERx Renal cohort

79 tumour sections of 66 unique primary tumours are included in this study; see the exclusion criterion in our previous publication (Zhao, et al. 2020).

CT images

Contrast-enhanced CT images were obtained using standard-of-care imaging sequences in 91 patients and curated using a local research PACS (based on the XNAT platform, (Marcus, et al., 2007)). Outlines were drawn giving volumetric tumour coverage by an oncologist (S.S.) and checked by a radiologist (D.A.), from which image strips were prepared for rapid visualisation of all tumour slices for all patients using an in-house script written in python.

Microdiversity

Spatial maps of regional clone diversity are created for two representative tumour sections. In these maps, regions are colour-coded based on the number of subclones. Regions that harbour at least one subclone are treated as a proxy for microdiversity hotspots defined in the model analysis. In total, there are 606 regions from 54 tumours that satisfy this criterion. For these regions, the normalised distance to tumour centre is measured as described above in “Model analysis - Microdiversity”.

Parallel evolution

Spatial maps of parallel mutational events in PBRM1 are created for one representative tumour section. In these maps, regions are coloured differently according to different parallel mutational events. Regions that harbour more than one event are indicated with multiple colours. To study the spatial distribution of mutational events with limited clonal expansion, the maximum distance from an event spanning up to two regions to the tumour margin is measured.

Statistical analysis

Two-sided Wilcox’s rank test is performed to compare several measurements between different model conditions. Statistical significance is annotated within box plots using `stat_compare_means(method = "wilcox.test", label = "p.signif")` in R.
Pearson correlation coefficients are calculated to assess the correlation between spatial and
genomic distances between tumour regions, using `cor.test(variable1, variable2, method = "pearson")` in R.

Two-sample Kolmogrov-Smirnov test is performed to compare two cumulative probability
distributions, using `scipy.stats.ks_2samp(sample1, sample2)` in Python.

Bootstrapping is performed to generate 100 random samples of 400 microdiversity hotspots
per sample with replacement, using `random.choice()` in Python. The power law
exponent is then determined by fitting a power law function to the cumulative probability
distribution from each sample, using `scipy.optimize.curve_fit()` in Python.

Quantile-Quantile (Q-Q) plot is employed to compare actual distribution of microdiversity
hotspots with a power law distribution with exponent being the median of fitted values in
bootstrapping, using `statsmodels.graphics.gofplots.qqplot()` in Python.

Kernel density estimation is performed for simulations with respect to the size of tumour
slice and the number of subclones, using `seaborn.jointplot(kind="kde")` in
Python.

R version 3.6.2 and Python version 3.7.7 are used for these analyses.

Code availability

CUDA C++ code of a representative model is available on GitHub: `https://github.com/xxxxx`
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The TRACERx Renal Consortium

Author contributions

X.F., Y.Z., E.S., K.L., S.T., and P.A.B. conceived and designed the study. X.F. and P.A.B. constructed the computational model and performed simulations. Y.Z., C.S., K.L., and S.T. provided the tumour samples and performed imaging analysis. S.D., M.O, and S.K curated and processed the radiological data in this study. S.S. performed the initial outlining of the tumours and D.A. checked and refined them. X.F. performed data analysis on simulations. X.F., Y.Z. and J.I.L. performed data analysis on tumour samples. X.F., S.T., and P.A.B. wrote the manuscript with input from all authors.

Competing interests

K.L., S.T., and C.S. have a patent on indel burden and checkpoint inhibitor response pending, and a patent on targeting of frameshift neoantigens for personalised immunotherapy pending. K. L. reports speaker fees from Roche Tissue Diagnostics. ST reports grants from Ventana, outside the submitted work. J.L. reports institutional research support from: BMS, MSD, Novartis, Pfizer, Achilles Therapeutics, Roche, Nektar Therapeutics, Covance, Immunocore, Pharmaciescics, Aveo, and consultancy support from: Achilles, AZ, Boston Biomedical, BMS, Eisai, EUSA Pharma, GSK, Ipsen, Imugene, Incyte, iOnctura, Kymab, Merck Serono, MSD, Nektar, Novartis, Pierre Fabre, Pfizer, Roche / Genentech, Secarna, Vitaccess. C.S. receives grant support from Pfizer, AstraZeneca, BMS, Roche-Ventana, Boehringer-Ingelheim, and Ono. C.S. has consulted for Pfizer, Novartis, GlaxoSmithKline, MSD, BMS, Celgene, AstraZeneca, Illumina, Genentech, Roche-Ventana, GRAIL, Medicxi, and the Sarah Cannon Research Institute. C.S. is a shareholder of Apogen Biotechnologies, Epic Bioscience, GRAIL, and has stock options in and is co-founder of Achilles Therapeutics. E.S. receives research support from AstraZeneca, Glaxo SmithKline, Merck Sharp & Dhome and is on the advisory board of Phenomic AI.

Additional information

Supplemental notes and figures are included in a separate document.
Figure Legends:

**Figure 1.** Construction of *in silico* tumours.
(a) Schematic figure illustrating future evolutionary trajectories delineated by present undetected subclones.
(b) Schematic figure of probabilistic growth, death, and driver acquisition in a coarse-grained cellular automaton model.
(c) Schematic figure of two growth modes: “Surface Growth” with proliferation predominating at the tumour surface and “Volume Growth” with proliferation throughout the tumour volume.
(d) Schematic figure of three levels of measurements: from three-dimensional (3D) tumour to two-dimensional (2D) tumour slice and 2D tumour regions within the slice.
(e) Representative *in-silico* tumours under Volume Growth (i) and Surface Growth (ii), respectively, from a 3D view. Tumour voxels harbouring select drivers, as indicated in the figure, are colour coded. Tumour voxels harbouring gain of chromosome arm events are in green; tumour voxels harbouring loss of chromosome arm events are in red. Different shades of greens or reds are employed to reflect different driver events.

**Figure 2.** Clonal diversity in the whole tumour.
(a) Schematic figure for the whole-tumour analysis of clonal diversity.
(b) Heatmap showing the average number of clones (i.e., parental clone and subclones) with respect to driver acquisition probability and proliferative advantage in the Volume Growth (i) and Surface Growth (ii) models. The average is calculated from 50 *in silico* tumours per parameter condition. Clones with a whole-tumour cancer cell fraction (CCF) of at least 0.05 are counted.
(c) Whole-tumour CCF of parental and largest subclones in *in silico* tumours under Volume Growth (i-ii) and Surface Growth (iii), respectively. Driver acquisition probabilities in these sets of simulations are $p_{\text{driver}} = 2 \times 10^{-4}$ in (i), $1 \times 10^{-3}$ in (ii), $2 \times 10^{-4}$ in (iii), respectively. “Parental (3p loss, VHL)” clone is shown along with up to five subclones with a whole-tumour CCF of 0.01 or higher. All remaining subclones are represented in the “other” group.
(d) Whole-tumour CCF of parental subclones in *in silico* tumours under Volume Growth and Surface Growth with varying driver acquisition probabilities. N = 100 for each condition.
(e) Shannon diversity index in *in silico* tumours under Volume Growth and Surface Growth with varying driver acquisition probabilities. N = 100 for each condition.
Statistical annotations in (d-e) reflect two-sided Wilcoxon tests: “****” indicates $P \leq 0.0001$.

**Figure 3.** Between-region genomic divergence.
(a) Schematic figure and procedure for the analysis in 2D tumour regions.
(b) Spatial locations of regional samples with tumour edge indicated in a representative *in silico* tumour under Surface Growth.
(c) Between-region spatial distance against genomic distance derived from the representative *in silico* tumour in panel b. Overlapping data points are counted (“n”). Pearson correlation (“r”) and p value are indicated in the figure.
(d-e) The same analysis as described in (b-c) for a representative *in silico* tumour with Volume Growth.
(f) Pearson correlation between spatial distance and genomic distance in *in silico* tumours under Volume Growth and Surface Growth with varying driver acquisition probabilities. N = 100 for each condition.
Figure 4. Spatial features of clonal diversity.
(a) Schematic figure and procedure for the analysis within a 2D tumour.
(b) Spatial maps of subclones (i) and microdiversity (ii) in a representative in-silico tumour under Surface Growth.
(c) The same analysis as described in (b) for a representative in-silico tumour under Volume Growth.
(d) Maps of regional biopsies with the number of subclones within a biopsy colour coded in two cases (G_K234 and G_K446) in the TRACERx Renal study. Hues from red to purple to blue reflect decreasing number of subclones. “Low” reflect zero subclones, while “High” reflects the maximum number of subclones found in any region (i.e., 4 subclones in G_K234, 3 subclones in G_K446).
(e) Cumulative probability distribution, $P(D \leq d)$, of the normalised distance to tumour centre in in silico tumours under Surface Growth and Volume Growth and in ccRCC tumours. Three sets of in-silico tumours with different driver acquisition probabilities are shown. N = 100 for each model condition. “S” and “V” in the figure reflect Surface Growth and Volume Growth, respectively. “p=2e-4” reflects a driver acquisition probability of $2 \times 10^{-4}$. 606 patient tumour (PT) regions from 54 ccRCC tumours are considered for the experimental analysis.
(f) Bootstrapped power law exponent $k$, as in $P(D \leq d)^{-d^k}$, fitted to cumulative probability distribution of normalised distance to tumour centre in each of bootstrap samples. For microdiversity hotspots under each model condition, 100 bootstrap samples are generated by randomly sampling 400 hotspots with replacement.
(g) Bootstrapped power law exponent $k$ in ccRCC tumours.
Driver acquisition probabilities used in representative simulations are: $p_{\text{driver}} = 2 \times 10^{-4}$ in both in-silico tumours under Surface Growth (b) and in-silico tumours under Volume Growth (c).
Statistical annotations in (f) reflect two-sided Wilcoxon tests: “****” indicates $P \leq 0.0001$.

Figure 5. Frequency and spatial features of parallel evolution.
(a) Schematic figure for measuring parallel evolution in the 3D tumour (i) and for analysing parallel mutational events within 2D tumour regions (ii).
(b) Heatmap showing the fraction of in silico tumours that have parallel evolution in PBRM1 with respect to driver acquisition probability and growth advantage in the Volume Growth (i) and Surface Growth (ii) models. The fraction is calculated based on 50 in silico tumours per parameter condition. In silico tumours are counted, if there are two or more parallel mutational events in PBRM1 with a whole-tumour CCF of at least 0.05.
(c-d) Spatial distribution of PBRM1 or BAP1 mutation (i) and its parallel events (ii) in a representative in silico tumour under Surface Growth (c) and Volume Growth (d), respectively. In panel (ii), different colours overlaid with “PBRM1” or “BAP1” reflect different parallel mutation events.
(e) Maximum distances to the tumour edge from parallel mutations in PBRM1 or BAP1 that span up to two regions in in silico tumours under Volume Growth and Surface Growth with varying driver acquisition probabilities. N = 100 for each condition.
(f) Maps of regions containing parallel mutations in PBRM1 in a representative case (G_K520) in the TRACERx Renal study. Distinct parallel mutations are coloured differently. For regions containing more than two parallel mutations, two colours are applied simultaneously. Double-headed arrow indicates a measurement of distance to tumour edge in this example.
(g) Maximum distances from mutational events that span up to two regions in ccRCC tumours in the TRACERx Renal study. For comparison, all mutations ("All mutations"), mutations in driver genes ("Drivers"), and parallel mutations in driver genes ("Drivers (P.E.)") are shown as separate groups.

Driver acquisition probabilities used in representative simulations are: $p_{driver} = 2 \times 10^{-4}$ in *in silico* tumours under Surface Growth and $p_{driver} = 1 \times 10^{-3}$ in *in silico* tumours under Volume Growth.

Statistical annotations in (e) reflect two-sided Wilcoxon tests: "***" indicates $P \leq 0.01$, "**" indicates $P \leq 0.05$, and "ns" indicates no statistical significance.

**Figure 6.** Early predictive indicators of evolutionary trajectories.

(a) The number of subclones as a function of the diameter of a 2D tumour slice in *in silico* tumours under Surface Growth and under Volume Growth, respectively. N = 50 simulations with $p_{driver} = 6 \times 10^{-4}$ are shown for each condition.

(b) Kernel density estimation (KDE) with respect to the number of subclones and the diameter of a 2D tumour slice in *in silico* tumours under Volume Growth (i) and under Surface Growth (ii). Each KDE plot is based on 250 simulations (50 per condition) under 5 conditions with $p_{driver} = 2 \times 10^{-4}, 4 \times 10^{-4}, 6 \times 10^{-4}, 8 \times 10^{-4}, 1 \times 10^{-3}$.

(c) The spatial patterns of parallel mutations in *BAP1* over time in a representative *in silico* tumour under Surface Growth. The red arrow indicates a budding structure at early stage of subclonal expansion.

(d) The CCFs of *BAP1* clones within the 2D tumour slice as a function of the diameter of a 2D tumour slice in each of 50 *in silico* tumours. Only *BAP1* clones with an ultimate CCF of 0.01 or higher are shown. For those with an ultimate CCF of 0.1 or higher, the diameters of tumour slices at which these clones first became detectable are recorded, with the median indicated by the dashed line. The others with an ultimate CCF of 0.1 or lower are shown in grey.

(e) Axial image in the corticomedullary contrast phase of a representative case (G_K523) showing budding structure on the tumour surface (red arrow). Outlines in red were drawn giving volumetric tumour coverage by an oncologist (S.S.) and a radiologist (D.A.).

(f) Maps of tumour regions with the number of subclones colour coded in a representative case (G_K523). Hues from red to purple to blue reflect decreasing number of subclones. “Low” reflect zero subclones, while “High” reflects the maximum number of subclones found in any region (i.e., 4 subclones in G_K523).

(g) The number of subclones as a function of ultimate tumour size in the TRACERx Renal study, overlaid with kernel density estimation based on simulated data. Tumours with a size smaller than 7 cm and with radiologically evident budding structures on the tumour surface are highlighted (orange). Contours reflect 90% probability density based on *in silico* tumours under Surface Growth (red) and under Volume Growth (blue), respective in Figure 6b.
References


Figure 1

(i) Volume Growth model

(ii) Surface Growth model

gain_20q

loss_1p

loss_4p

loss_8p

time

Surface Growth

Volume Growth

3D tumour cutting 2D tumour slice sampling 2D tumour regions

Tumour voxel New tumour voxel Dead tumour voxel Driver mutation or SCNA

Active growth No growth Tumour origin

past present future

growth death driver

parental clone "macro"-clones "micro"-clones evolutionary potential
(i) Count subclones in 3D tumour.

(ii) Calculate the whole-tumour CCF of subclones and Shannon diversity.
(i) Sample regions from tumour slice.

(ii) Collect driver information in each region.

(iii) Calculate spatial and genomic distances between each pair of regions.

(iv) Evaluate correlation between spatial and genomic distances.

Figure 3

**Surface Growth model**

- Correlation coefficient: $r = 0.6$, $p < 2.2e-16$

**Volume Growth model**

- Correlation coefficient: $r = 0.41$, $p < 2.2e-16$

Driver acquisition probability

Correlation coefficient spatial vs genomic distances

Condition
- Surface
- Volume

NS

Significance levels:
- ****: $p < 2e-04$
- *****: $p < 4e-04$
- ***: $p < 6e-04$
- **: $p < 8e-04$
- *: $p < 0.001$
- NS: $p > 0.001$
(i) Map microdiversity within a tumour slice by sliding a 3x3 spatial window within the tumour slice.
(ii) Evaluate the spatial pattern of microdiversity hotspots within the tumour slice.

Hotspots with microdiversity of at least 5

$\text{d1: distance to tumour centre}$

$\text{d2: distance to tumour margin}$

$d = \frac{d1}{d1+d2}$: Normalised distance to tumour centre

**Surface Growth model**

**Volume Growth model**

$P(D \leq d) \sim d^k$

**GrowthMode**

Surface

Volume

**Boostrapped exponent $k$**

Driver acquisition probability

**RCC**
Figure 5

2D tumour regions

Driver acquisition probability ($p_{\text{driver}}$)

Growth advantage ($\Delta$)

Frac. with P.E. of PBRM1

Surface Growth model

Volume Growth model

d: distance to tumour margin from events with limited clonal expansion

Surface Growth model

Volume Growth model

ns ** ** ** *

Max distance to tumour edge (mm)

Condition

Surface

Volume

Max distance to tumour edge (mm)

Event 1

Event 2

Event 3

Max distance to tumour edge (mm)

All mutations

Drivers

Drivers (P.E.)

G_K520

Max distance to tumour edge (mm)
**Figure 6**

(a) **Volume Growth model**

- Surface Growth
- Volume Growth

Number of subclones vs. Diameter of tumour slice (mm)

(b) **Surface Growth model**

(i) Volume Growth model

(ii) Surface Growth model

Number of subclones vs. Diameter of tumour slice (mm)

(c) Time

(i) (ii) (iii) (iv) (v)

(d) **p_{driver} = 6x10^{-4}**

- CCF of BAP1 subclone

(d) **p_{driver} = 6x10^{-4}**

- CCF of BAP1 subclone

(d) **p_{driver} = 1x10^{-3}**

- CCF of BAP1 subclone

(e) **G_K523**

(f) **G_K523**

Size of primary tumour (mm)

Number of subclones

(g) **Surface Growth Model**

- Volume Growth Model

- Early-stage ccRCCs with budding

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**Equations**

\[ p_{driver} = 6 \times 10^{-4} \]

\[ d = 73.27 \]

\[ d = 40.35 \]

\[ d = 38.43 \]
Figures

Figure 1

Construction of in silico tumours. (a) Schematic figure illustrating future evolutionary trajectories delineated by present under detected subclones. (b) Schematic figure of probabilistic growth, death, and driver acquisition in a coarse-grained cellular automaton model. (c) Schematic figure of two growth
modes: “Surface Growth” with proliferation predominating at the tumour surface and “Volume Growth” with proliferation throughout the tumour volume. (d) Schematic figure of three levels of measurements: from three-dimensional (3D) tumour to two-dimensional (2D) tumour slice and 2D tumour regions within the slice. (e) Representative in-silico tumours under Volume Growth (i) and Surface Growth (ii), respectively, from a 3D view. Tumour voxels harbouring select drivers, as indicated in the figure, are colour coded. Tumour voxels harbouring gain of chromosome arm events are in green; tumour voxels harbouring loss of chromosome arm events are in red. Different shades of greens or reds are employed to reflect different driver events.
Figure 2

Clonal diversity in the whole tumour. (a) Schematic figure for the whole-tumour analysis of clonal diversity. (b) Heatmap showing the average number of clones (i.e., parental clone and subclones) with respect to driver acquisition probability and proliferative advantage in the Volume Growth (i) and Surface Growth (ii) models. The average is calculated from 50 in silico tumours per parameter condition. Clones with a whole-tumour cancer cell fraction (CCF) of at least 0.05 are counted. (c) Whole-tumour CCF...
of parental and largest subclones in in silico tumours under Volume Growth (i-ii) and Surface Growth (iii), respectively. Driver acquisition probabilities in these sets of simulations are $= 2 \times 10^{-1}$ in (i), $1 \times 10^{-1}$ in (ii), $2 \times 10^{-1}$ in (iii), respectively. “Parental (3p loss, VHL)” clone is shown along with up to five subclones with a whole-tumour CCF of 0.01 or higher. All remaining subclones are represented in the “other” group. (d) Whole-tumour CCF of parental subclones in in silico tumours under Volume Growth and Surface Growth with varying driver acquisition probabilities. N = 100 for each condition. (e) Shannon diversity index in in silico tumours under Volume Growth and Surface Growth with varying driver acquisition probabilities. N = 100 for each condition. Statistical annotations in (d-e) reflect two-sided Wilcoxon tests: “****” indicates $1 \leq 0.0001$. 

Figure 3
Between-region genomic divergence. (a) Schematic figure and procedure for the analysis in 2D tumour regions. (b) Spatial locations of regional samples with tumour edge indicated in a representative in silico tumour under Surface Growth. (c) Between-region spatial distance against genomic distance derived from the representative in silico tumour in panel b. Overlapping data points are counted (“n”). Pearson correlation (“r”) and p value are indicated in the figure. (d-e) The same analysis as described in (b-c) for a representative in silico tumour with Volume Growth. (f) Pearson correlation between spatial distance and genomic distance in in silico tumours under Volume Growth and Surface Growth with varying driver acquisition probabilities. N = 100 for each condition. Statistical annotations in (f) reflect two-sided Wilcoxon tests: “****” indicates 1 ≤ 0.0001 and “ns” indicates no statistical significance.
Figure 4

Spatial features of clonal diversity. (a) Schematic figure and procedure for the analysis within a 2D tumour. (b) Spatial maps of subclones (i) and microdiversity (ii) in a representative in-silico tumour under Surface Growth. (c) The same analysis as described in (b) for a representative in-silico tumour under Volume Growth. (d) Maps of regional biopsies with the number of subclones within a biopsy colour coded in two cases (G_K234 and G_K446) in the TRACERx Renal study. Hues from red to purple to blue
reflect decreasing number of subclones. “Low” reflect zero subclones, while “High” reflects the maximum number of subclones found in any region (i.e., 4 subclones in G_K234, 3 subclones in G_K446). (e) Cumulative probability distribution, $1(2 \leq 0)$, of the normalised distance to tumour centre in in silico tumours under Surface Growth and Volume Growth and in ccRCC tumours. Three sets of in-silico tumours with different driver acquisition probabilities are shown. N = 100 for each model condition. “S” and “V” in the figure reflect Surface Growth and Volume Growth, respectively. “p=2e-4” reflects a driver acquisition probability of 2e-4. 606 patient tumour (PT) regions from 54 ccRCC tumours are considered for the experimental analysis. (f) Bootstrapped power law exponent 4, as in $1(2 \leq 0)\sim 0.2$, fitted to cumulative probability distribution of normalised distance to tumour centre in each of bootstrap samples. For microdiversity hotspots under each model condition, 100 bootstrap samples are generated by randomly sampling 400 hotspots with replacement. (g) Bootstrapped power law exponent 4 in ccRCC tumours. Driver acquisition probabilities used in representative simulations are: $\$.#.-/\# = 2 \times 1001$ in both in-silico tumours under Surface Growth (b) and in-silico tumours under Volume Growth (c). Statistical annotations in (f) reflect two-sided Wilcoxon tests: “****” indicates $1 \leq 0.0001$. 
Figure 5

Frequency and spatial features of parallel evolution. (a) Schematic figure for measuring parallel evolution in the 3D tumour (i) and for analysing parallel mutational events within 2D tumour regions (ii). (b) Heatmap showing the fraction of in silico tumours that have parallel evolution in PBRM1 with respect to driver acquisition probability and growth advantage in the Volume Growth (i) and Surface Growth (ii) models. The fraction is calculated based on 50 in silico tumours per parameter condition. In silico tumours are counted, if there are two or more parallel mutational events in PBRM1 with a whole-tumour CCF of at least 0.05. (c-d) Spatial distribution of PBRM1 or BAP1 mutation (i) and its parallel events (ii) in
a representative in silico tumour under Surface Growth (c) and Volume Growth (d), respectively. In panel (ii), different colours overlaid with “PBRM1” or “BAP1” reflect different parallel mutation events. (e) Maximum distances to the tumour edge from parallel mutations in PBRM1 or BAP1 that span up to two regions in in silico tumours under Volume Growth and Surface Growth with varying driver acquisition probabilities. N = 100 for each condition. (f) Maps of regions containing parallel mutations in PBRM1 in a representative case (G_K520) in the TRACERx Renal study. Distinct parallel mutations are coloured differently. For regions containing more than two parallel mutations, two colours are applied simultaneously. Doubleheaded arrow indicates a measurement of distance to tumour edge in this example. (g) Maximum distances from mutational events that span up to two regions in ccRCC tumours in the TRACERx Renal study. For comparison, all mutations (“All mutations”), mutations in driver genes (“Drivers”), and parallel mutations in driver genes (“Drivers (P.E.)”) are shown as separate groups. Driver acquisition probabilities used in representative simulations are: $,\#-./# = 2 \times 1001$ in in silico tumours under Surface Growth and $,\#-./# = 1 \times 100!$ in in silico tumours under Volume Growth. Statistical annotations in (e) reflect two-sided Wilcoxon tests: “**” indicates $1 \leq 0.01$, “*” indicates $1 \leq 0.05$, and “ns” indicates no statistical significance.
Figure 6

Early predictive indicators of evolutionary trajectories. (a) The number of subclones as a function of the diameter of a 2D tumour slice in in silico tumours under Surface Growth and under Volume Growth, respectively. N = 50 simulations with $5.6 \times 10^4$ are shown for each condition. (b) Kernel density estimation (KDE) with respect to the number of subclones and the diameter of a 2D tumour slice in in silico tumours under Volume Growth (i) and under Surface Growth (ii). Each KDE plot is based on 250
simulations (50 per condition) under 5 conditions with $\times 1001, 4 \times 1001, 6 \times 1001, 8 \times 1001, 1 \times 1001$. (c) The spatial patterns of parallel mutations in BAP1 over time in a representative in silico tumour under Surface Growth. The red arrow indicates a budding structure at early stage of subclonal expansion. (d) The CCFs of BAP1 clones within the 2D tumour slice as a function of the diameter of a 2D tumour slice in each of 50 in silico tumours. Only BAP1 clones with an ultimate CCF of 0.01 or higher are shown. For those with an ultimate CCF of 0.1 or higher, the diameters of tumour slices at which these clones first became detectable are recorded, with the median indicated by the dashed line. The others with an ultimate CCF of 0.1 or lower are shown in grey. (e) Axial image in the corticomedullary contrast phase of a representative case (G_K523) showing budding structure on the tumour surface (red arrow). Outlines in red were drawn giving volumetric tumour coverage by an oncologist (S.S.) and a radiologist (D.A.) (f) Maps of tumour regions with the number of subclones colour coded in a representative case (G_K523). Hues from red to purple to blue reflect decreasing number of subclones. “Low” reflect zero subclones, while “High” reflects the maximum number of subclones found in any region (i.e., 4 subclones in G_K523). (g) The number of subclones as a function of ultimate tumour size in the TRACERx Renal study, overlaid with kernel density estimation based on simulated data. Tumours with a size smaller than 7 cm and with radiologically evident budding structures on the tumour surface are highlighted (orange). Contours reflect 90% probability density based on in silico tumours under Surface Growth (red) and under Volume Growth (blue), respective in Figure 6b.

**Supplementary Files**

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