Evolution under Spatially Heterogeneous Selection in Solid Tumors

Guanghao Li,†,1,2,3,4,5 Zuyu Yang,†,2,3,6 Dafei Wu,2,3,7 Sixue Liu,2,3,5 Xuening Li,2,3,5 Tao Li,2,3,5 Yawei Li,2,3,5 Liji Liang,2,3 Weilong Zou,8,9 Chung-I Wu,2,3,10 Hung-Yi Wang,*,11,12 and Xuemei Lu*,1,2,3,4,5

1State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China
2China National Center for Bioinformation, Beijing, China
3CAS Key Laboratory of Genomic and Precision Medicine, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China
4Center for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, Kunming, China
5University of Chinese Academy of Sciences, Beijing, China
6Institute of Environmental Science and Research, Porirua, New Zealand
7Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing, China
8Surgery of Liver Transplant, The Third Medical Center of Chinese PLA General Hospital, Beijing, China
9Surgery of Hepatopancreatobiliary, Peking University Shougang Hospital, Beijing, China
10State Key Laboratory of Biocontrol, College of Ecology and Evolution, Sun Yat-Sen University, Guangzhou, China
11Graduate Institute of Clinical Medicine, National Taiwan University, Taipei, Taiwan
12Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei, Taiwan
†These authors contributed equally to this work.
*Corresponding authors: E-mails: hurngyi@g.nctu.edu.tw; xuemeilu@mail.kiz.ac.cn.
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Abstract

Spatial genetic and phenotypic diversity within solid tumors has been well documented. Nevertheless, how this heterogeneity affects temporal dynamics of tumorigenesis has not been rigorously examined because solid tumors do not evolve as the standard population genetic model due to the spatial constraint. We therefore, propose a neutral spatial (NS) model whereby the mutation accumulation increases toward the periphery; the genealogical relationship is spatially determined and the selection efficacy is blunted (due to kin competition). In this model, neutral mutations are accrued and spatially distributed in manners different from those of advantageous mutations. Importantly, the distinctions could be blurred in the conventional model. To test the NS model, we performed a three-dimensional multiple microsampling of two hepatocellular carcinomas. Whole-genome sequencing (WGS) revealed a 2-fold increase in mutations going from the center to the periphery. The operation of natural selection can then be examined by controlling the spatially determined clonal relationships and the clonal sizes. Due to limited migration, only the expansion of highly advantageous clones can sweep through a large part of the tumor to reveal the selective advantages. Hence, even multiregional sampling can only reveal a fraction of fitness differences in solid tumors. Our results suggest that the NS patterns are crucial for testing the influence of natural selection during tumorigenesis, especially for small solid tumors.

Key words: cancer evolution, tumor spatial growth model, intra-tumoral heterogeneity, natural selection, phenotypic diversity.

Introduction

Tumorigenesis starts from a single cell that grows into a population of more than 10⁹ cells through a process of somatic evolution and spatial range expansion (Sottoriva et al. 2015; Wu et al. 2016; Sun et al. 2017). As solid tumors usually form a compact structure that obstructs tumor cells from free migration, it is reasonable to expect that intratumor heterogeneity may result from independent progression of spatially distributed segments. Indeed, spatial phenotypic heterogeneity within tumors has been well documented in genomic analyses and pathological characterization of various types of cancers (Ling, et al. 2015; Sottoriva et al. 2015; Waclaw et al. 2015; Uchi et al. 2016; Yuan 2016; Carmona-Fontaine et al. 2017; Li et al. 2017; Chan-Seng-Yue et al. 2020; Gerstung et al. 2020; Losic et al. 2020; Dentro et al. 2021). For instance, Ki-67 staining suggests that proliferating cells at the tumor...
edge outnumber cells at the core (Waclaw et al. 2015; Lloyd et al. 2016; Zhao et al. 2021). In addition, cells in the central hypoxic regions are thought to be the most aggressive and therapy-resistant (Terry et al. 2017), whereas cells at the tumor margin may be replenished frequently by the continually fluctuating microenvironment, leading to more division and death (Li et al. 2020). This evidence indicates that evolutionary rates and population dynamics may vary across the range of structured tumor cell populations.

Although understanding the process of population differentiation and adaptation driven by evolutionary forces, including genetic drift, migration, and natural selection, is the central theme in evolutionary biology (Yoshida et al. 2003; Schoener 2011; Hendry 2016), most evolutionary theories attempting to explain population dynamics are based on a simplified assumption that populations are at spatial panmictia. Accordingly, many studies assume homogeneous distribution of genetic variation within tumors (Williams et al. 2016; Chowell et al. 2018; Williams et al. 2018). Since solid tumors do not evolve as the standard population genetic model due to the spatial constraint, a new “null” model for the study of intratumor genetic heterogeneity is thus necessary.

Here, we build a neutral spatial (NS) model of tumor growth. We assume tumor growth in a discrete-time birth-death process initiated from a single cell. Under neutral conditions, the tumor expanded at a constant rate as the growth rate of each subclone was similar. As a result, the shapes and relative sizes of all subclones were stable over time. Because of spatial constraint, peripheral regions exhibited higher proliferation rate than central regions which led to a higher generation numbers, the former accumulated more mutations and showed longer branch lengths than the latter. To test the NS model, we performed a three-dimensional microdissection of two tumors obtained from a single hepatocellular carcinoma (HCC) patient. The results of whole-genome sequencing (WGS) and genotyping revealed a roughly 2-fold increase in mutations going from the center to the periphery.

We also tested the operation of natural selection by examining the spatially determined clonal relationships and the clonal sizes. When the tumor grows under Darwinian evolution, random accumulation of driver mutations in different subclones resulted in changes in their shapes and proportions. Dominant clones with the highest fitness increased its proportions in the tumor rapidly, whereas other small clones that occurred early went extinct as the expansion of dominant clones. In addition, due to spatial constraints, only the expansion of highly advantageous clones can sweep through a large part of the tumor to reveal the selective advantages. Hence, even multiregional sampling can only reveal a fraction of fitness differences in solid tumors. This modeling yielded insights into the neutral and environmental constraints that drive the formation and progression of intratumor genetic structure, as well as the tempo and mode of tumor diversification and clonal expansion.

### Results

#### Tumor Growth under Strict Spatial Constraint

We first evaluate a neutral tumor expansion model with strict spatial constraints. Suppose that the shape of tumor is an ideal sphere consisting of layers of cells. The most recent common ancestor (TMRCA) of the tumor is at layer 0. Layer $n$ is the ring of cells with $n$ cells distant from layer 0. Cells in layer $n$ is generated from those in layer $n-1$. The number of cells in layer $n$ is $4\pi n^2$ (supplementary fig. 1A, Supplementary Material online). Assume the birth and death rates of each tumor cell are $b$ and $1-b$, respectively. The average number of divisions for cells in layer $n$ is

$$D_n = \log_{2b} \left( \frac{4\pi + 1}{1 + \left( \frac{j+1}{i} \right)^2} \right), \quad n \geq 2$$

$D_n$ is approximate linear related with $n$ (supplementary fig. 1B, Supplementary Material online).

For cells in layer $n$ and $kn$, $D_n$ approximates to $k$ as $n$ increase (supplementary fig. 1C, Supplementary Material online). Under the assumption that mutation rate is constant, mutations accumulated in cells of layer $kn$ are $k$ folds than those of cells in layer $n$. Therefore, spatial heterogeneity of solid tumors can be simply due to limited migration within the tumor mass. Under a simple spatial growth model, mutations accumulate more rapidly in the periphery than in the interior.

#### Modeling Tumor Growth with NS Structure

To investigate how spatial heterogeneity is generated under NS model in solid tumors in more detail, we propose a spatial tumor growth model (Lloyd et al. 2016). For cells in a peripheral region, the birth rate was $b_0$ and death rate was $1-b_0$, whereas cells in the central region stay quiescent when resources are limited, and the birth, quiescence, and death rates are $b_1$, $q$, and $1-b_1-q$, respectively (fig. 1A and supplementary table 1, Supplementary Material online). We demonstrated modeled tumor growth under neutral evolution, where mutations have no effect on cell growth rate. We simulated tumor growth in a discrete-time birth-death process initiated from a single cell to more than $10^6$ cells (corresponding to $10^9$ in three dimensions).

Under neutral evolution, higher birth and death rates for cells in a peripheral region than in a central region result in more generations, which further cause more mutations accumulated at the surface. To explore mutation accumulation under different birth and death rates, we performed 5,000 simulations with $q$ sampled from the $[0.1, 0.9]$ interval and $b_0$ from $[0.51, 0.6]$. The range of $b_1$ depended on the value of $q$ and $b_0$, as $b_1$ must be bigger than central cell death rate $(1-q-b_1)$ and smaller than $b_0$. Therefore, the value of $b_1$ was sampled from $[q]^2/2 + 0.02$, min $(b_0, 1-q)-0.02$.

To define the location of a cell, we first estimated the radius of the circle-like tumor, $r$. For each cell, we calculated the distance between its position with the center of the tumor, $d$. We also set up a value $m$ to determine the central and
In this study, we used $m = 0.9$. That is if $d < 0.9r$, the cells are in the central region, otherwise, it is in the periphery. It is worth of mentioning that different $m$ values should only affect the proportion of peripheral region, but not mutation accumulation and clonal structure of the tumors.

Mutations accumulate randomly according to the Poisson distribution during cell proliferation, assuming infinite site model and a constant mutation rate. The neutral mutation rate is $\mu$ and the probability that $i$ neutral mutations accumulate after each cell division is:

$$P(x = i) = \frac{\mu^i e^{-\mu}}{i!}.$$  

The neutral mutation rate $\mu = 15$ per genome (corresponding to $5 \times 10^{-9}$ per site per cell division for the human genome) (Sun et al. 2017). For each simulation, 16 samples were taken from the virtual tumor uniformly (fig. 1), sequenced in silico, and single-nucleotide variants (SNVs) were called and analyzed. The ratio of average mutation number in samples from peripheral regions to the count from central regions ($R_{p/c}$) was calculated.
The ratio of average mutation number in peripheral samples to that from the center, \( R_{PC} \), was about 1.5 on average, and the 95% confidence interval was [0.79, 2.25] (supplementary fig. 2, Supplementary Material online). \( R_{PC} \) is positively correlated with \( q \) (fig. 1C), demonstrating that if more cells are quiescent in central regions, fewer mutations are generated. In contrast, if central cells divide more frequently, the difference between the central and peripheral regions is reduced.

Thus, we observe a negative correlation between \( b_3 \) and \( R_{PC} \). The range of values for \( R_{PC} \) is relatively stable, regardless of the peripheral birth rate \( b_3 \).

We also explored the genetic diversity and clonal structure of the simulated tumors. In this setting, a moderate initial birth rate \( b_3 = 0.53 \) was used (Sun et al. 2017). The \( q \) and \( b_1 \) were set to 0.5 and 0.3, respectively, based on previous simulations (supplementary table 1, Supplementary Material online and see Materials and Methods). Sixteen virtual samples were taken (fig. 2A) when tumor size reached \( 5 \times 10^5, 1 \times 10^6, 1.5 \times 10^6, \) and \( 2 \times 10^6 \). SNVs were used to delineate clones and estimate phylogenetic relationships among subclones. Under neutral conditions, the tumor expanded at a constant rate as the growth rate of each subclone was similar. As a result, the shapes and relative sizes of all subclones were stable over time. Cancer cell fractions (CCFs) of all SNVs were low and always exponentially distributed (fig. 2B). Since peripheral regions exhibited higher birth and death rates than central regions, leading to higher generation numbers, peripheral cells accumulated significantly more mutations and had longer branches than central samples (fig. 2C). The \( R_{PC} \) values are 1.39, 1.23, 1.30, and 1.40 for the four panels in figure 2C.

Three-Dimensional Microsampling and Sequencing

To comprehensively trace the mutation accumulation process of solid tumors and describe their clonal distribution, we performed three-dimensional microdissection (fig. 3A and B) and WGS of two tumors, T1 and T2, obtained from a HCC patient who was a chronic hepatitis B virus (HBV) carrier and had undergone liver transplantation (see Materials and Methods). The three-dimensional microsampling of the 11 slices of T1 yielded 169 samples. We obtained 160 samples from six T2 slices. Samples were taken uniformly from each slice, with central and peripheral regions represented (fig. 4).

Sixteen samples from three slices of T1 and nine samples from two slices of T2, each containing \( \sim 3,200 \) cells, were subjected to WGS. The average depth was \( \sim 78 \times \) per sample, yielding 1,264 \( \times \) coverage of T1 and 691 \( \times \) of T2. A peripheral blood sample was collected as the normal control and sequenced to \( \sim 74 \times \) coverage with estimated purity ranging from 0.70 to 0.94 (supplementary table 2, Supplementary Material online).

We identified 254,268 SNVs in T1 and 142,032 SNVs in T2. Of these, only 6,869 (2.7%) mutations from T1 and 3,721 (2.6%) from T2 were shared by all samples from the same tumor. No SNVs were found in common between T1 and T2, indicating that they originated independently. The number of SNVs varied greatly across samples from the same tumor, from 21,345 in T1L13 to 68,149 in T1F24, and from 24,370 in T2Z11 to 70,093 in T2Z1 (supplementary tables 2 and 3, Supplementary Material online). Mutations in \( CIC, KMT2B, \) and \( DYRK1A \) were found in all T1 samples and \( SCAF4 \) and \( HNF1A \) in all T2 samples (Zhai et al. 2017; Bailey et al. 2018), whereas the \( PKHD1L1 \) mutation was detected as a subclonal event both in T1 and T2 (supplementary fig. 3 and supplementary materials, Supplementary Material online).

Diverse copy number alternations (CNA) profiles among samples within the same tumor, as well as between T1 and T2, were detected (supplementary fig. 4, Supplementary Material online). Specifically, we found significant arm-level gains of almost all chromosomes in both T1 and T2. Losses of 6q were observed in T1 and of 4p and 13q in T2. We also observed CNAs of canonical HCC driver genes at focal levels. For example, amplification of \( CCNE1 \) (Li et al. 2018) and \( TERT \) (Li et al. 2018, 2020) were found in T1 and T2, respectively, and deletion of \( RB1 \) (Wang et al. 2013) was found in T2 (supplementary fig. 4, Supplementary Material online). Genome-wide CNAs were quite different at both focal and arm levels in both T1 and T2, indicating varying evolutionary processes in tumors even in the same patient.

HBV integration into the human genome may play a crucial role in triggering oncogenic processes (Bréchot et al. 2000). We found that the HBV integrations in an intron of \( A T P 2 B 2 \) and the intergenic region between \( L O C 1 0 0 5 0 6 4 4 4 \) and \( R P L 2 1 P 4 4 4 \) were common in T1. An HBV integration in the 5′ UTR of \( T E R T \) (Sung et al. 2012; Totoki et al. 2014) was found in all T2 samples (supplementary table 4, Supplementary Material online).

We also explore mutation profiles of 96 trinucleotide changes. Five mutation signatures were identified (supplementary fig. 5A–C, Supplementary Material online) and compared with all signatures described in the Catalog of Somatic Mutations in Cancer (COSMIC) (Tate et al. 2019). The first, second, and fourth signatures were enriched for \( T: A > A: T \) mutations, which have been found in urothelial (renal pelvis) carcinomas and liver cancers with known exposure to aristolochic acid (Alexandrov et al. 2020) (COSMIC signature 22, \( \rho = 0.974, 0.972, \) and 0.731, respectively).

Mutation Accumulation and Subclone Spatial Structure

Phylogenetic trees were constructed based on SNVs from each sample using peripheral blood as the outgroup (fig. 3C and D). In the T1 phylogeny, T1Z5 branched out first (defined as clone 1), followed by five samples sharing 721 SNVs (clone 2), and ten samples sharing 16,412 SNVs (clone 4). In T2, six samples sharing 17,493 SNVs clustered together (clone 5). T2Z1 and T2Z13 shared 5,141 SNVs, constituting clone 6. Phylogenetic trees show that branch lengths vary greatly with the long-branched subclones tending to occur in peripheral regions (fig. 4C–F). The \( R_{PC} \) for T1 is 2.19 and T2 2.07, within the range of simulated results. Collectively, T1 and T2 underwent independent evolutionary paths but had similar spatial patterns, that is peripheral regions accumulated more mutations than central locations, both containing bigger subclones.

To further delineate the size and spatial distribution of subclones and investigate the relationship between branch length and subclone size, we conducted additional
genotyping of selected 906 and 565 SNVs in 153 T1 and 151 T2 samples, using the Ampliseq method (supplementary tables 5 and 6, Supplementary Material online). The genotypes were used to construct expanded phylogenies including all the sequenced and validated samples to delineate clonal distribution in each slice. Consistent with the phylogenies based on WGS data, the three clones \(a\), \(b\), and \(c\) from T1 and the two clones \(h\) and \(d\) from T2 were identified in the expanded phylogenies (fig. 4A and B). According to the phylogenies, both \(a\) and \(d\) are of the same age as the other clones, implying that their larger sizes resulted from a growth advantage over other cells. Clonal structure of slices shows that subclones \((b)\) in T1 and \((h)\) in T2 contain long-branch samples occur in peripheral regions of the tumors (fig. 4C and E).

Most subclones defined using selected SNVs were highly consistent with those characterized by whole-genome variants. For example, 78 SNVs were chosen from the shared SNVs of subclone \(a\) from WGS, an average 95.7% of which were identified in all validation \(a\) samples. The values for subclone \(b\), \(c\), \(d\), and \(h\) were 96.3%, 97.2%, 99.1%, and 59.4%, respectively (supplementary table 7, Supplementary Material online). Consistency for subclone \(h\) is relatively low. The possible reason might be that subclone \(h\) was located at the surface of the tumor with higher genetic diversity.

Using clone distributions across slices, we inferred changes in subclone composition in three dimensions for both tumors. Subclone \(a\), accounting for 42.6% of T1, was further divided into \(a1\) and \(a2\). The subclone \(d\) (84.4% of T2) was further divided into \(d1\), \(d2\), and \(d3\) (fig. 4D and F). Clone \(a\) was the biggest in slice T1F, T1H, and T1K. \(a\), \(b\), and \(c\) were similar in size in slices T1O, T1Q, and T1S. \(b\) was the dominant subclone in slice T1V and \(y\) was similar to \(b\) in size in slices T1Y and T1AB. Different subclonal distributions across slices suggests a significant spatial

**Fig. 2.** Clonal structures and phylogenies of tumors under neutral evolution over time. (A) Clonal distributions of the same virtual tumor when cell population size is \(5 \times 10^5\), \(1 \times 10^6\), \(1.5 \times 10^6\), and \(2 \times 10^6\). Colors represent subclones. (B) Sixteen samples, each with about 200 cells, are taken uniformly from each tumor and sequenced virtually at each time point. Position of each sample is in the panel 4 of (A). Histograms are the distributions of CCFs for all SNVs detected. (C) Sample phylogenetic trees. Normal sample without any SNVs is used as the outgroup. Samples marked with red stars are from central regions. Internal branch lengths are significantly shorter than peripheral branches (\(P < 0.001\), \(= 0.043\), \(<0.001\), and \(<0.001\) for population size \(5 \times 10^5\), \(1 \times 10^6\), \(1.5 \times 10^6\), and \(2 \times 10^6\), respectively). The \(R_{\text{sub}}\) values are 1.39, 1.23, 1.30, and 1.40. Unit of branch length in (C) is evolutionary distance in maximum parsimony.
heterogeneity in T1 (fig. 4C). Clone α size decreases from the center to the surface, whereas the size of clone β and γ increases (fig. 4C). Subclones from different slices showed a similar pattern of both boundaries and sizes in T2. δ was the dominant subclone for all slices, and subclone θ was much smaller than δ (fig. 4E), indicating spatial diversity of T2 was much lower than in T1. In both T1 and T2, some samples located at the tumor peripheral regions did not belong to any subclones detected by WGS, implying higher genetic diversity on the surface of the tumor.

We noticed that peripheral regions not only accumulated more mutations, but also contained more changes in genes related to cell proliferation and cell cycle function (supplementary table 8, Supplementary Material online). For example, only three and six mutations in these genes were identified in the central region samples from T1 and T2. In contrast, S6 and 30 SNVs were found in the peripheral samples from T1 and T2, respectively. In particular, the two longest branches, T1Z1 had 19 and T2Z1 had 17 of these mutated genes. These mutations may have accelerated cell division rates in the peripheral samples, in turn coinciding with a higher number of mutations found in these regions.

**Tracing the Temporal and Spatial Tumor Growth under Neutrality and Natural Selection**

Although modeling neutrality, we observed that diversity and the subclonal structure emerged rapidly, and higher birth and death rates at the periphery compared with the center led to more mutations and longer branches, consistent with our empirical observations. It suggests that the spatial pattern of mutation accumulation is an intrinsic feature of solid tumors and can be generated in a short period of time.

However, the rapid clonal expansion in central regions of tumors has not appeared in our simulations. Furthermore, whereas α and δ are large, both have low intra-clonal diversity compared with other clones, implying that they might have growth advantages and experienced recent expansion.
Extended sample phylogenetic relationships. (A) Extended phylogenetic tree of T1 samples based on WGS and genotyping data. Colors are consistent with figure 1C. (B) Extended phylogenetic tree of T2 samples based on WGS and genotyping data. Colors are consistent with figure 1D. Unit of branch length in (A) and (B) is evolutionary distance in maximum parsimony method. (C) Clonal structure of 11 T1 slices (T1F, T1H, T1K, T1L, T1O, T1Q, T1S, T1V, T1Y, T1Z, and T1AB). These slices were taken from a half of the spherical tumor sequentially from the center to the periphery. T1F is the biggest and T1AB the smallest slice. (D) Proportion of each subclone within T1. (E) Clonal structure of six T2 slices (T2F, T2M, T2Q, T2R, T2Z, and T2AB). The order of slices is also from top to the bottom of the sampled half tumor. T2Q is the largest and T2AB is the smallest slice. (F) Proportion of each subclone within T2. Samples marked with red stars were used for WGS in (A) and (B). Samples in red circles are WGS samples and in gray circles are genotyped samples in (C) and (E). Colors represent different subclones. Tumor regions marked with gray do not belong to defined clones.
We tested whether the growth of the tumors was within neutral expectation by comparing the expected site frequency spectrum (SFS) of exponentially growing cancer cell populations (Durrett 2013; Ohtsuki and Innan 2017) with the observed data (see Materials and Methods). The observed SFSs in both T1 and T2 were significantly different from the expectation under the neutral model (fig. 5A and supplementary table 9, Supplementary Material online) \( (\chi^2 = 363, 125, \ p < 2.2e^{-16} \text{ for T1, } \chi^2 = 247, 540, \ p < 2.2e^{-16} \text{ for T2} ) \). We then investigated the distribution of adjusted variant allele frequencies (VAFs) (supplementary figs. 6–8 and supplementary material, Supplementary Material online) and CCFs for all SNVs (fig. 5B). If tumor growth is neutral, polymorphic snvs only occur in subclones that are at low frequencies. In contrast, Darwinian selection increases the growth rate of fitter cells, elevating frequencies of snvs they accumulate (Fay and Wu 2000). There was a peak between 0.3–0.4 for VAF and 0.5–0.8 for CCF distributions in both tumors, suggesting selection shaped their growth (Williams et al. 2018). Therefore, Darwinian selection may have driven the evolution of these tumors. We next modeled tumor growth under Darwinian evolution (supplementary table 1, Supplementary Material online). Instead of maintaining similar shapes and proportions of all subclones during tumor progression under the neutral model, random accumulation of driver mutations in different subclones changed these parameters (figs. 2A and 6A). Dominant clones with the highest fitness rapidly increased their proportions in the tumor, whereas other small clones occurring early went extinct as the dominant clone expanded. The distribution of CCFs of SNVs under natural selection had an extra peak in addition to the low-frequency peak after the appearance and expansion of the dominant clone. The peak gradually shifted to the right and finally became fixed when the selected clone accounted for all tumor cells (fig. 6B).

Tree shapes in adaptively evolving tumors also varied according to stage (fig. 6C). Before the emergence of the dominant clone, the tree structure was similar to that of neutral tumors. Once formed, the dominant clone rapidly increased in size and contained an increasing number of samples. Nevertheless, regardless of stage, the subclones that accumulated more mutations were from peripheral regions. This pattern is evident even when the selected clone arose from the central region.

We also explored the relationships between Fst and the physical distance of paired samples within each tumor. Assuming the tumor evolved neutrally, a positive correlation between Fst and physical sample distance is expected. That is because as tumor growth, cells that are farther apart shall have more mutation accumulated between them which, in turn, leads to higher Fst. Nevertheless, tumors under positive selection will increase their size shortly which leaves no time for mutation to accumulate. Thus, a positive correlation between Fst and the physical distance of samples within tumor would not be expected. As shown in supplementary figure 9, Supplementary Material online, there is no correlation between Fst and physical distance of samples within tumor. Nevertheless, a strong positive correlation between them is revealed in simulated neutral tumors (supplementary fig. 10A, Supplementary Material online). Furthermore, strong positive correlation between Fst and physical distance of samples is found before the emergence of the dominant clone (supplementary fig. 10B, Supplementary Material online). This correlation vanishes as the emergence of dominant clone and reappears after the selected clone accounted for all tumor mass.

The distribution of CCFs of SNVs and clonal structure of T1 and T2 are consistent with simulation results of tumor growth under selection. To estimate the mutation rate (\(\mu\)) and the number of driver mutations (\(N\)) needed to invade of each tumor, we developed a framework incorporating our spatial growth model into approximate Bayesian computation (ABC) (see Materials and Methods) (Beaumont et al. 2002). Mutation rates of T1 and T2 are similar, about 15 per genome per cell division (corresponding to \(5 \times 10^{-9}\) per site per cell division) (table 1). \(N\) is four in T1 and three in T2 (table 1). T2 has a smaller \(N\), consistent with the smaller T2 population, and a bigger dominant subclone.

**Discussion**

Spatial genetic and phenotypic diversity within solid tumors has been well documented (Ling, et al. 2015; Sottoriva et al. 2015; Waclaw et al. 2015; Uchi et al. 2016; Li et al. 2017; Maley et al. 2017; Losic et al. 2020). Nevertheless, how this heterogeneity affects temporal dynamics of tumorigenesis has not been rigorously examined. Here, we developed a spatial tumor growth model where molecular heterogeneity among cancer cells is governed by the rigidity of solid tumors. Relatively high turnover rates of cells at the periphery leads them to accumulate more mutations than at the center. By examining two independently originated tumors, we find that samples from the edge have twice the number of SNVs compared with samples from the core, consistent with our model prediction. As the spatial heterogeneity is generated by the constraint of solid tumors, we propose the model should be applied to at least all small tumors with limited cell migration. For large tumors, angiogenesis can generate a network of blood vessels that sustains optimal growth in the central part of a tumor mass. Therefore, the distinction between peripheral and central is blurred.

A recent study of clear cell renal cell carcinoma (ccRCC) found that cells in the tumor interior exhibited higher Ki67 staining and somatic copy number alteration burden (Zhao et al. 2021). Although the mechanism of higher cellular proliferation at the center of ccRCC requires further investigation, the results actually support the notion that elevated cellular division increases mutation burden and population dynamics actually vary across the range of structured tumor cell populations. It has to point out that, in Zhao et al. (2021), the median tumor size was nine cm and the tumor margin was one cm from the nearest boundary. As the tumors in this study were only 1.5 and 2 cm in size, the definition of central and peripheral are quite different from Zhao et al. (2021). The
results derived from the two studies should not be directly compared.

Long dendrogram branches of peripheral samples have not been previously reported in the literature. This happens due to the high proliferation rate of the cells in these samples. We can detect this pattern because we collected multiple samples that are dense enough within the tumor to capture the spatial heterogeneity. However, as we show in figure 6, natural selection can blur this phenomenon. During and right after selection, the dominant clone can grow quickly making the distinction between the interior and peripheral regions less clear.

Our observation that peripheral cells grow faster than interior in solid tumors may be important to consider in devising cancer therapy. Conventional chemotherapy aims at quickly proliferating cells (Trédan et al. 2007 ), which may effectively kill peripheral cells but leave interior cells intact. This explains why initial chemotherapy can lead to tumor shrinkage but rarely to complete elimination. That is because interior tumor cells are not dividing much, and are thus relatively insensitive to drugs that interfere with the cell cycle. After eliminating peripheral cells, interior cells get access to more resources and proliferate rapidly. Combination therapy considering diverse phenotypes and tumor microenvironments may be required (Hausser and Alon 2020).

In addition to cancer therapy, our results that tumor cells in different regions have different survival rates resemble the r and K strategies of life history in natural populations (MacArthur 1962; Pianka 1970). Individuals with high reproductive rates are favored in an r-selective environment, whereas those with high survival rates are favored in K-selective conditions. This is a trade-off between proliferation and survival (Aktipis et al. 2013; Li et al. 2020). As population density decreases from the tumor core to edge, we expect that peripheral cells may exhibit decreased survival and increased proliferation. Intriguingly, we find increased cell proliferation and cell cycle gene mutations in peripheral samples, consistent with life-history theory in natural populations.

Our model also demonstrates that detecting positive selection in tumors is a challenging task. Completely different conclusions may have been drawn if samples would have been taken at a different stage of tumor progression. For example, if we would have tested for selection at the four time points from figure 6A, the results would have been neutral, neutral, selection, and neutral, depending on the stage. The main driving forces, that is natural selection and genetic drift, may alternatively dominate during tumor progression (fig. 7). Selection may not be noticed unless samples are taken at the right time and sample size is sufficient to represent the clonal structure of the whole tumor. That is probably why
several seminal studies suggested that no dominant subclones can be found, indicating non-Darwinian evolution within tumors (Ling et al. 2015; Sottoriva et al. 2015; Uchi et al. 2016). For example, it has been proposed that tumor evolution is neutral based on a linear relationship between the expected number of mutations ($M$) and inverse allele frequency ($1/f$) from one sample per tumor (Williams et al. 2016). Using this model, one-third of the tumors tested were evolving neutrally. However, this method has been widely doubted (Wang et al. 2018; McDonald et al. 2018; Tarabichi et al. 2018). Employing this approach to our data, we find that T1 and T2 are both evolving neutrally (supplementary fig. 11, Supplementary Material online), a conclusion that conflicts with the clonal structure of the two tumors. In conclusion, extremely high spatial intratumor diversity precludes robust interference of evolution modes with only one sequenced sample, necessitating multiregion sampling.

### Materials and Methods

#### NS Model Construction

We constructed an agent-based spatial lattice model to simulate the process of tumor growth and mutation accumulation integrating a heterogeneous tumor microenvironment. Expansion of tumor cells is constrained by the spatial structure (Moore neighborhood, eight neighbors), giving rise to distinct subclones in separate tumor regions.

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**Table 1. Mutation Rate and Driver Mutation Number Estimation.**

| Parameter | Value | Posterior Probability |
|-----------|-------|-----------------------|
| $u^a$     | 6     | 0.04                   |
|          | 15    | 0.95                   |
|          | 30    | 0.01                   |
| $N^{b}$  | 2     | 0.0007                 |
|          | 3     | 0.34                   |
|          | 4     | 0.64                   |
|          | 5     | 0.02                   |

*Note—To estimate parameters using in the spatial growth model, we used ABC, generating 30,000 virtual tumors to compare with the real tumor. Details are described in Results and Materials and Methods.

$^a$Mutation rate.

$^b$Driver mutation number.
Under neutral evolution, the clonal structure is determined by both spatial constraints and random effects of cell division. Specifically, all simulations were initiated with a single cell and one generation was used as the time unit for a discrete birth-death process. At each time step, each cell either divides, stays quiescent, or dies. When tumors grow to a certain size, spatial structure limits the ranges of cell activities, leading to microenvironment differentiation for cells from distinct regions. Peripheral cells grow faster than inner cells, as there are more resources in their surroundings (Lloyd et al. 2016). The density of immune cells at tumor margins is also higher than that at the cores (Kather et al. 2018), potentially causing higher death rates. Thus, we set different growth rates of inner and outer regions of tumors in our model.

The initial birth rate is \( b_0 \) and death rate is \( d_0 = 1 - b_0 \). Every cell has a unique spatial position in the two-dimensional coordinate system. Each cell’s destiny is decided by a random value generated from a uniform distribution \( \text{Uniform}(0, 1) \). When the value is smaller than or equal to \( b_0 \) and there is an adjacent vacancy in the Moore neighborhood, the cell proliferates. If all neighborhood positions are occupied, a cell stays quiescent. If the random value is bigger than \( b_0 \), the cell dies. We used a moderate initial birth rate and a relatively high death rate: \( b_0 = 0.53 \), \( d_0 = 0.47 \), based on the previous estimates in solid tumors (Sun et al. 2017).

When spatial structure influences tumor cell growth, cells in central and peripheral regions differ in birth and death rates. The time point where the spatial structure starts to influence cell phenotypes is at \( \sim 80 \) generations, when the tumor population size is approximately 200 cells. The quiescent rate \( q \) in central regions is 0.5. The birth rate decreases to \( b_1 = 0.3 \) and the death rate is \( 1 - b_1 - q \). The birth rate and death rate of cells at the tumor margin remain \( b_0 \) and \( 1 - b_0 \), respectively. To define the tumor regions, we first estimate the radius of the circle-like tumor, \( r \). For each cell, we calculate its distance to the tumor center, \( d \). If \( d < 0.9^* r \), the cell is assigned to the central region. Otherwise, it is considered peripheral.

Mutations accumulate randomly according to the Poisson distribution during cell proliferation, assuming infinite site model and a constant mutation rate. The neutral mutation rate is \( \mu \) and the probability that \( i \) neutral mutations accumulate after each cell division is:

\[
P(x = i) = \frac{\mu^i e^{-\mu}}{i!}
\]

The mutation accumulation parameters are set according to previous studies. The neutral mutation rate \( \mu = 15 \) per genome (corresponding to \( 5 \times 10^{-9} \) per site per cell division for the human genome) (Sun et al. 2017). Parameters used in the spatial tumor growth model are listed in supplementary table 1, Supplementary Material online.

To investigate the dynamics of clonal structure and cell lineages, we took 16 samples uniformly when tumor size reached \( 5 \times 10^5 \), \( 1 \times 10^6 \), \( 1.5 \times 10^6 \), and \( 2 \times 10^6 \) (fig. 2). To simulate the sequencing process for each bulk sample, we used a negative binomial distribution to generate read depth of each virtual point mutation and a binomial distribution to generate the mutant allele read counts. The parameters of the negative binomial distribution were estimated by fitting the depth distribution from real sequencing data, and are NB (15.8, 0.155). The binomial distribution parameters are the generated depth and the true VAF. We applied the same filtering condition to these simulated SNVs as we did to real data to make them comparable to the observed VAFs. CCFs of SNVs were two times the VAFs. CCFs and VAFs of all SNVs from whole tumors were obtained by integrating virtual sequencing results from all samples.

Clinical Information

The patient was a 57-year-old man with a chronic Hepatitis B Virus (HBV) infection and liver cirrhosis. He had undergone liver transplantation in the General Hospital of Chinese People’s Armed Police Forces. Two well-encapsulated tumors: T1, \( \sim 2 \) cm in diameter, and T2, \( \sim 1.5 \) cm in diameter, were on the right lobe and left lobe of the liver, respectively. Both tumors were diagnosed as moderately differentiated Hepatocellular carcinoma (HCC). This study was approved by the Ethics Review Committee of the General Hospital of Chinese People’s Armed Police Forces. Informed consent was signed according to the regulations of the institutional ethics review board.

Three-Dimensional Microsampling and WGS

We performed a three-dimensional microsampling of both tumors (fig. 3). Half of each tumor was embedded in the optimal cutting temperature (OCT) compound and sliced into 200-\( \mu \)m-thick pieces using the Leica CM1950 cryostat platform. We chose three slices of T1 (T1F, T1L, and T1Z) and two slices of T2 (T2F and T2Z) to conduct microdissection sampling for sequencing. To identify subclonal alterations at a high resolution, we performed a sampling of the frozen slices using syringes with blunt tip needles of 210-\( \mu \)m inner diameter. The 20 \( \mu \)l of cell lysis buffer (20 mM Tris, 2 mM EDTA, 20 mM KCl, 1 \( \mu \)g/\( \mu \)l QIAGEN protease, 1 \( \mu \)g/\( \mu \)l RNAse A) was...
piped into the syringes before sampling to maximize a sampling success rate. We obtained 16 samples from T1 and nine samples from T2. Given that tumor cell radius on the hematoxylin-eosin-stained slice is $\sim 8 \, \mu m$ and the volume of a cylinder-like microsample is $\sim 7 \times 10^6 \, \mu m^3$, there are $\sim 3,200$ cells in a sample. Cells were directly lysed to reduce DNA loss and increase the efficiency of library preparation for WGS. DNA from blood cells collected from the patient before liver transplantation was used as the normal control. Libraries were constructed using Ovation Ultralow System Kit (NuGEN, USA). Genomic DNA was fragmented into about 500 bp segments using a Covaris S220 instrument. DNA fragments were end-repaired, ligated to adapters, and amplified following the Ovation Ultralow System Kit protocol. Purified libraries were paired-end sequenced ($2 \times 150$ bp) using Illumina HiSeq X Ten sequencers at Novogene.

Paired-end reads were mapped to the human reference genome (UCSC hg19) using the Burrows-Wheeler Aligner (BWA) (Li and Durbin 2010). All aligned reads were further processed using PICARD (http://broadinstitute.github.io/picard) and the Genome Analysis Toolkit (GATK) (DePristo et al. 2011), including de-duplication, base quality recalibration, and multiple-sequence realignment prior to mutation detection. The alignments were assessed using Qualimap (Okonechnikov et al. 2016) Sequence data summaries for each sample are listed in Supplementary table 2, Supplementary Material online. The expected SFS for each tumor is calculated using the formula (Durrett 2013):

$$E_{i} = \left\{ \begin{array}{ll}
\frac{\mu}{r} \frac{n}{k} & i = 1 \\
\frac{\mu}{r} \frac{n}{k} \frac{k}{n + k} & 2 \leq i < n
\end{array} \right.$$

Since $\sum_{i=2}^{n} \frac{1}{r(i-1)} = n$ Observed SFS, we obtained $\frac{\mu}{r}$ for each tumor and calculated all SFS values.

Spatial Tumor Growth Model under Natural Selection

We next modeled tumor growth under Darwinian evolution to further investigate the effects of different forces driving cancer progression. We assumed driver mutations occur at the rate of $10^{-6}$ per cell division (Bozic et al. 2010). The probability of $j$ driver mutations after each cell division is:

$$P(y = j) = \frac{u b e^{-u b}}{j!}.$$  

Driver mutations elevate the birth rate in models with selection. When a cell gains a beneficial mutation, the birth rate $b_j$ increases by the selection coefficient ($s$) and the death rate decreases. We calculated birth and death rates for each cell at the tumor core as

$$b = b_1 (1 + s)^n, \quad d = 1 - b - q.$$  

In this equation, $b_1$ is the initial central cell birth rate, $q$ is the quiescence rate, and $n_o$ is the number of accumulated driver mutations. We modeled tumor growth with a strong selective advantage ($s = 0.1$) (Uchi et al. 2016). The birth and death rates for every cell at the tumor margin were $b_0$ and $1 - b_0$, respectively (fig. 6A), with $b_0$ higher than $b_1$. Given that the number of drivers accumulated is limited in tumor progression (Tomasetti et al. 2015; The ICGC/TCGA PanCancer Analysis of Whole Genomes Consortium 2020), we assumed that cells gained ability to expand quickly when they have $N$ driver mutations. In our simulations, $N$ is chosen to be four, according to previous estimates from solid tumors (Martincorena et al. 2017).

Estimation of Tumor Growth Model Parameters

We combined our spatial tumor growth model with the approximate Bayesian computation (ABC) (Beaumont et al. 2002) framework to estimate mutation rate ($\mu$) and the number of driver mutations ($N$) needed for tumors to expand quickly. Mutation rate ($\mu$) was randomly sampled from a discrete uniform distribution with five values from one to 30 (1, 3, 6, 15, 30, per genome per cell division). The prior distribution of $N$ was also a discrete uniform distribution with six values from one to six. We simulated 1,000 tumors for each combination of the two parameters, generating 30,000 virtual tumors. We estimated parameters of T1 and T2 independently considering their size and evolutionary process. Cell numbers for a two-dimensional slice of T1 and T2 were estimated at about $1.5 \times 10^6$ and $10^6$, respectively.
When virtual tumors grew to about \(10^6\), we took nine samples uniformly and simulated the sequencing process described above to compare the simulated to T2 sequence data. We performed the same procedure for \(1.5 \times 10^6\) T1 cells. The ABC procedure is as follows:

1. Sample parameter \(\theta = [\mu, N]\) from the prior distribution \(f(\theta)\).
2. Simulate tumor growth with the sampled parameters \(\theta\) and calculate summary statistics \(S\).
3. Compare \(S\) with the observed \(S\) using a distance function \(d\) and tolerance rate \(\varepsilon\). If \(d(S, S) < \varepsilon\), accept \(\theta\).
4. Go to step (1).

We accepted parameters which generated sequencing results similar to those observed in T1 and T2 using a distance function \(d\) and calculated the summary statistics. abc (Csilléry et al. 2012) package was used to calculate distances and generate posterior distributions of the two parameters.

### Supplementary Material

Supplementary data are available at Molecular Biology and Evolution online.

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### Author Contributions

Xuemei Lu and Hurng-Yi Wang designed the project. Guanghao Li, Zuyu Yang, Dafei Wu, Xuening Li, and Liji Liang performed microsampling, DNA extraction and library construction experiments. Guanghao Li, Zuyu Yang, and Sixue Liu performed sequencing data analysis. Guanghao Li constructed tumor spatial growth model with the help of Chunjia Wu, Hurng-Yi Wang, Tao Li, and Yawei Li. Welong Zou provided clinical samples. Xuemei Lu and Hurng-Yi Wang supervised the project. Xuemei Lu, Hurng-Yi Wang, Guanghao Li, and Zuyu Yang wrote the manuscript.

### Data Availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Chen et al. 2021) in National Genomics Data Center (Members and Partners 2021), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA000188) that are publicly accessible at https://ngdc.cnbc.ac.cn/gsa-human.

### Code Availability

Tumor spatial growth model code is available at https://github.com/guanghao/Tumor-spatial-growth-model. The code of ABC model is available at https://github.com/guanghao/Tumor_growth_ABC.

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