Correction

EVOLUTION

The authors note that Chunyi Hao should be listed as an additional corresponding author. The corrected correspondence footnote appears below. The online version has been corrected.

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Extremely high genetic diversity in a single tumor points to prevalence of non-Darwinian cell evolution

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The prevailing view that the evolution of cells in a tumor is driven by Darwinian selection has never been rigorously tested. Because selection greatly affects the level of intratumor genetic diversity, it is important to assess whether intratumor evolution follows the Darwinian or the non-Darwinian mode of evolution. To provide the statistical power, many regions in a single tumor need to be sampled and analyzed much more extensively than has been attempted in previous intratumor studies. Here, from a hepatocellular carcinoma (HCC) tumor, we evaluated multiregional samples from the tumor, using either whole-exome sequencing (WES) (n = 23 samples) or genotyping (n = 286) under both the infinite-site and infinite-alleles models of population genetics. With the many single-nucleotide variations (SNVs) present in all samples, there were 35 “polymorphic” SNVs among samples. High genetic diversity was evident as the 23 WES samples defined 20 unique cell clones. With all 286 samples genotyped, clonal diversity agreed well with the non-Darwinian model with no evidence of positive Darwinian selection. Under the non-Darwinian model, M_all (the number of coding region mutations in the entire tumor) was estimated to be greater than 100 million in this tumor. DNA sequences reveal local diversities in small patches of cells and validate the estimation. In contrast, the genetic diversity under a Darwinian model would generally be orders of magnitude smaller. Because the level of genetic diversity will have implications on therapeutic resistance, non-Darwinian evolution should be heeded in cancer treatments even for microscopic tumors.

intronumer heterogeneity | genetic diversity | neutral evolution | cancer evolution | natural selection

The level of genetic diversity in a natural population is determined by several evolutionary forces, including mutation, genetic drift, migration, and natural selection (1–3). Tumors can be regarded as asexual populations of cells, so they are subjected to similar forces to those of natural populations (4–7). Therefore, the genetic diversity in tumors of the same patient is informative about how various forces drive their evolution. The level of diversity may also influence how tumors respond to environmental perturbations, either natural or medical (5–7). In the prevailing view, Darwinian selection for and against new mutations is the main driving force of intratumor diversity (4, 8–18). Because selection generally reduces genetic diversity within populations (19–21), studies assuming Darwinian evolution usually described M_all (the total number of coding region mutations within the whole tumor) in the range of tens to hundreds of coding mutations (22, 23).

Despite its wide acceptance, the Darwinian view has never been subjected to hypothesis testing, by which the observed diversity is compared with quantitative predictions. This study is to our knowledge the first one that uses high-density sampling in a single tumor and compares the observations with theoretical predictions. In this test, we consider a null model of non-Darwinian evolution in which M_all is a function of N (population size), u (mutation rate per generation), and growth parameters. In tumors, N is large, generally > 10^6, and u is the mutation rate of the entire functional portion of the genome (at the level of 10^-7 per cell division) (18, 24). Hence, the expected genetic diversity of tumors by non-Darwinian evolution would be large, probably on the order of millions of mutations, most of which are present at low frequencies (25).

We ask whether the observed intratumor genetic diversity can be largely explained by non-Darwinian forces and we invoke positive selection only when the null model of non-Darwinian evolution is rejected. There was a controversy in molecular evolution generally known as the neutralism–selectionism debate (1, 26, 27). In the postdebate modern view, genetic polymorphisms in natural populations are largely consistent with the non-Darwinian model (1–3, 26–28). There are further reasons to question the efficacy of selection within populations of cells that make up tumors (Discussion). For instance, although selection against nonsynonymous mutations is nearly universal in natural

Significance

A tumor comprising many cells can be compared to a natural population with many individuals. The amount of genetic diversity reflects how it has evolved and can influence its future evolution. We evaluated a single tumor by sequencing or genotyping nearly 300 regions from the tumor. When the data were analyzed by modern population genetic theory, we estimated more than 100 million coding region mutations in this unexceptional tumor. The extreme genetic diversity implies evolution under the non-Darwinian mode. In contrast, under the prevailing view of Darwinian selection, the genetic diversity would be orders of magnitude lower. Because genetic diversity accrues rapidly, a high probability of drug resistance should be heeded, even in the treatment of microscopic tumors.

The authors declare no conflict of interest.

Data deposition: The sequence data reported in this paper have been deposited in the genome sequence archive of Beijing Institute of Genomics, Chinese Academy of Sciences, prca.big.ac.cn (accession no. PRJCA000091).

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species (1, 3, 27), selection against such mutations in tumors is not apparently stronger than against synonymous ones (29).

In the recent literature, there has been increasingly more attention on assessing the non-Darwinian model of tumor evolution vs. the prevailing Darwinian view (30, 31). Tao et al. (31) studied 12 cases of multitumor hepatocellular carcinomas (HCCs) and concluded that competition often occurs between tumors large enough to be visible. In contrast, the genetic diversity contained within the same tumor does not deviate from the predictions of the non-Darwinian model. A caveat is that whereas the number of population samples used in testing Darwinian selection in natural populations is often in the hundreds, the sample number rarely exceeds 10 in intratumor studies (12, 13, 15–18, 30, 31). Therefore, the power to reject the null model in tumor studies might have been too low. Clearly, there is a need to sample a large number of regions in one single tumor. In this study we sampled close to 300 regions to examine the spatial distribution of single-nucleotide variants and to estimate the amount of genetic diversity in the tumor. We used these data to give a rigorous test of the null hypothesis of non-Darwinian evolution.

**Results**

**Sampling, Sequencing/Genotyping, and Mutation Calling.** The honeycomb-like microdissections yielded 286 tumor samples on a plane of a single HCC tumor (Materials and Methods, section 1), each sample being a cylinder of 0.5 mm in diameter and 1 mm in height (Fig. L4 and Fig. S1). A sample contained, on average, 20,000 cells (Fig. S2 and Materials and Methods, section 2) and permitted precise delineation of clones. Fig. L4 displays the spatial distribution of the 286 tumor samples, which were evenly distributed among the four quadrants of the tumor slice, labeled A–D clockwise. The 23 sequenced samples (red color in Fig. L4) were also evenly distributed, with 12 on the periphery of the tumor and 11 in the interior.

For sequencing, the average read depth was 74.4× per sample (Dataset S1), yielding a total of >1,700× for the plane of Fig. L4 (SI Materials and Methods). With the additional genotyping over 286 samples, the coverage is to our knowledge the highest ever carried out on a single tumor. The average sample purity is 85% as described in the legend of Fig. L4 (Materials and Methods, section 3). In total, we found 269 single-nucleotide variations (SNVs) in coding regions or at splice sites (Materials and Methods, Fig. 1. Sampling scheme and clonal genealogy of HCC-15. (A) Samples were taken from a 1-mm-thick slice cut through the middle of a HCC tumor, 3.5 cm in diameter. Of the 286 samples, 23 were subjected to whole-exome sequencing (red numbers) and the rest (black numbers) were used in genotyping for mutations discovered in sequencing (Materials and Methods, sections 1–5). The numbers correspond with those of Fig. 2. Across the sequenced samples, the average read depth was 74.4× (Dataset S1). On average, these samples contained 85% cancerous cells estimated by ABSOLUTE (S2). This level of purity is consistent with previous reports regarding hepatic tumor samples (12), especially when the sample volumes are small (~20,000 cells). Pathology reports, when available for the matched HCC samples, generally agreed with the purity estimates. (B) All 35 polymorphic nonsynonymous mutations in the sequenced samples are shown in the heat map, which depicts the observed frequencies (from 0 in white to 1 in yellow) with mutation names at the top of the map. Each row presents the mutations in a sequenced sample. Far Right shows six fixed mutations that are potential drivers. Left shows the genealogy of the 24 samples. Only two clones, indicated by blue bars, are represented by more than one sample. (C) The genealogy of clones arranged to reflect their spatial relationships. The ancestral clone, Ω, is in the middle and the descendant clones radiate outward. These clones are arranged on six rings with each outer ring having one more nonsynonymous mutation (indicated) than its interior neighbor. Each star symbol represents a singleton clone. (D) The expanded genealogy that includes all 286 samples. The blue stars designate the sequenced samples.
sections 4 and 5 and Dataset S2). Due to the dense sampling, SNVs found in multiple samples are unambiguous by the cross-validation among samples, using whole-exome sequencing (WES) and/or Sequenom. Singleton SNVs (i.e., occurring in only one sample) required at least 10 reads per position. Sequenom genotyping and sometimes Sanger sequencing, all singleton SNVs presented have been confirmed to be true positives (Datasets S2 and S3 and Fig. S3). Therefore, the final SNV calls for this study are considered free of false positives. Furthermore, given the large number of samples, false negatives would likely be negligible.

Copy number alterations (CNAs) are another common source of somatic genomic aberration. We used the program package CAScav to call CNAs from our data (Materials and Methods, section 3). On average, each sample contained 23.6 CNAs, distributed among 14 chromosomes (SI Materials and Methods and Dataset S4). Because the mechanisms of CNA production are very different from those for SNVs, and because the latter also are much easier to ascertain, this study focused on SNVs (Discussion).

Fixed and Polymorphic Somatic Mutations. Somatic mutations discovered in the sequenced samples were classified as either fixed or polymorphic. In this study, the terminology of population genetics is applied to facilitate theoretical analyses. Fixed mutations were those present in the entire cancerous cell population but absent in the noncancerous sample. These mutations must have already occurred at the onset of tumorigenesis. Polymorphic mutations, on the other hand, were present in some but not all cancerous samples (Materials and Methods, section 6).

Among the 269 SNVs observed in HCC-15, 209 and 35 mutations were confirmed to be fixed and polymorphic, respectively (Datasets S2 and S3 and Fig. S4). The remaining 25 mutations, divided into 22 possibly fixed and 3 possibly polymorphic SNVs, were not used in the analysis. The 35 validated polymorphic SNVs would define clone sizes and delineate clonal boundaries according to the genotypes of the 286 samples (Materials and Methods, section 7 and Dataset S3).

The 209 fixed mutations were divided into 166 protein-altering mutations (comprising 148 missense, 11 nonsense, and 7 splicing mutations) and 43 synonymous changes. In Materials and Methods, section 8, Fig. S5, and Dataset S5, a list of “driver” genes that are significantly more commonly mutated in cancer samples, especially in gastrointestinal and HCC tumors [Dataset S6; https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp; Schulze et al. (32)], was compiled from published data. In reference to this list, we identified 6 putative driver genes among the fixed mutations, which were CCAR1, CPXM2, DNAH7, TMPRSS13, TP53, and TSC1. In contrast, none of the 35 polymorphic mutations is in the driver group. The pathways represented by the fixed and polymorphic mutations are also somewhat dissimilar, as shown in Dataset S7.

Clonal Diversity and Genealogy. The 35 validated polymorphic SNVs delineated 20 cell clones among the 23 sequenced samples. A clone is defined as a cell population carrying a unique set of somatic mutations. We denoted $\Phi_i$ as the number of clones that appeared $i$ times in n samples. The vector of $[\Phi_0, i = 1$ to $n − 1]$ is the allele frequency spectrum in population genetics (2, 3). In our data, $[\Phi_0 = 18, 1, 0, 0, 0, \ldots ; i = 1−22]$ and $n = 23 = 18 \times 1 + 1 \times 2 + 1 \times 3$. In other words, $20 = (18 + 1 + 1)$ clones consisted of 18 singletons, 1 doubleton, and 1 triplont, which were, respectively, cell clones represented by one, two, or three samples. The small number of samples (3 of 23) yielding redundant information was indicative of the extensive diversity in the coding regions of the tumor. In particular, Simpson’s diversity index, $H = 1 − \sum \Phi_i n^2$, was 0.941, indicating that two random samples would have a very high probability of being genetically different.

The genealogical relationship of the 20 clones is shown in Fig. 1B. The same genealogy with spatial information is given in Fig. 1C, in which clones were shown to emanate from the ancestral $\Omega$ clone in the center. For visual clarity, these clones were arranged on five rings, denoting the number of mutations away from $\Omega$. The 7 direct descendants of $\Omega$, labeled from $a$ to $i$, all carried 1–2 mutations in addition to that of the $\Omega$ clone. Their descendant clones, each having additional mutations, were denoted with primes (6′ and 6′′, for example). Some clones at the end of a branch were marked by a star symbol, which represented a singleton. On average, the number of coding mutations ($U$) accrued since the tumor began grew from a single progenitor cell was 2.65 (Fig. 1C). As shown in Table 1, $U$ is an important parameter in determining the genetic diversity of the entire tumor and, at $U = 2.65$, the mutation rate in HCC-15 is unequivocal among studies of intratumor diversity (12, 13, 16–18, 31). The genealogy of Fig. 1C was further expanded to include all 286 samples as portrayed in Fig. 1D (Materials and Methods, section 7).

Sizes of the Mutation Clones in Relation to Darwinian Selection. To delineate the size and spatial limit of each clone, the 286 samples were genotyped. Although a cell clone is typically defined by a suite of mutations (Fig. 1C), it may often be more informative to define a “mutation clone” by the collection of clones that share that mutation. For example, the MUC16 clone in Fig. 1C was composed of 6′, 6′′, 6′′′, 1′, 1′′, 1′′′, 2′, 2′′, and 2′′′, whereas the THRA clone, which included 6′′2 and 6′′′2, was a subclone of the MUC16 clone. Fig. 2 displays the sizes and spatial patterns of the mutation clones observed, with the subclones shown in increasingly darker shades. Genealogically, separate clones were observed to be segregated, revealing limited cell movement within solid tumors. The “sectoring” patterns of Fig. 2 suggested that clones grow outwardly, as the derived subclones were consistently observed on the outer flank of the parental clone.

<table>
<thead>
<tr>
<th>Table 1. Expected clonal diversity, $N_r$, according to Eq. 3</th>
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<tr>
<td>$N_r = 10^3$</td>
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<tr>
<td>Exponential growth:</td>
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<tr>
<td>$dN/dt = r N$ and $N_r = e^{rt}$</td>
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<tr>
<td>$r = \ln(2) \times 0.1$</td>
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<td>$r = \ln(2) \times 0.01$</td>
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<tr>
<td>2D growth: $dN/dt = r N^{2/3}$ and $Nt = (1 + rt)^{3/2}$</td>
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<td>$r = (36a)^{1/3} \times 0.1$</td>
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<tr>
<td>$r = (36a)^{1/3} \times 0.01$</td>
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<tr>
<td>Simulations under a well-mixed population (calculation by Eq. 3)</td>
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<td>$0.667 \pm 0.075$ ($0.643$)</td>
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<td>Simulations under spatial rigidity</td>
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$T$ and $u$ are also given. Three different growth models reaching different final cell numbers ($N_r$) are used in the calculation, $U = u x T = 2$, which corresponds to the number of coding region mutations acquired during tumor growth (main text and SI Materials and Methods). $T$ is the number of generations to reach $N_r$, and $u$ is the mutation rate per generation. When cells double every generation with no cell death, $r = \ln(2)$. Hence, $r = \ln(2) \times 0.1$ would mean 10% of the growth rate of the pure cell-doublet populations. In the 2D “simulations under a well-mixed population,” the results are checked against the theoretical values given by Eq. 3. The simulated values match the theoretical calculations well.
We now evaluate whether certain clones grew faster than others. The null hypothesis of non-Darwinian evolution was that all clones have the same (or neutral) growth rate, whereas the alternate hypothesis of Darwinian selection posits faster growth of some clones.

To test the null hypothesis, we compared the sizes of the observed mutation clones with the expected sizes, often referred to as the mutation frequency spectrum and denoted as \( [\xi_i, i = 1 \text{ to } n - 1] \). \( \xi_i \) is the number of sites where the mutant appears \( i \) times in \( n \) samples in the infinite-site model of population genetics (2, 3). In HCC-15, \( [\xi_i = 26, 7, 1, 1, 0, 0, \ldots] \) for \( i = 1-22 \) (Fig. 2 legend and Dataset S8), where \( \sum \xi_i = 35 \) was the number of mutations in the sequenced samples (Materials and Methods, section 9).

In a population with a constant effective size of \( N_r \), \( E(\xi_i) = \theta_i \), where \( \theta = 2N_r u \) (2, 3). In exponentially growing populations, the corresponding \( E(\xi_i) \) has been defined by Durrett (25) as

\[
E(\xi_i) = \frac{u}{r} \frac{n}{i(i - 1)}
\]

where \( r \) is the rate of population growth, the difference between cell birth and death rates (see below). In addition, \( u \) is the mutation rate per cell generation, and \( n \) is the sample size (Materials and Methods, section 10). Because \( \sum \xi_i = \frac{(7 + 1)}{2} = 9 = 23 \times u/r \times \sum \frac{1}{i(i - 1)} = 23 \times u/r \times 0.059 \), we obtained \( u/r = 0.041 \) by Eq. 1. For the total of 35 sites, \( E(\xi_i) = 26.0, 4.72, 1.57, 0.79, 0.47, 0.31, \ldots \), which was very close to the observed spectrum of \( [\xi_i = 26, 7, 1, 1, 0, 0, \ldots] \) (\( \chi^2 = 2.53 \) and \( P = 0.865 \) for \( \xi_{22} \)). Hence, the size distribution of the mutation clones (Fig. 2) was as expected under the neutral model, and no clones were of unusually large proportion.

The next question is whether the analysis would have the power to reject the non-Darwinian model if selection was indeed in operation. A key feature of the neutral model is that it has very few high-frequency mutations. In our samples, only ~0.5 site is expected to have a frequency greater than 50%. Thus, even a very small number of mutations that have been driven to a high frequency by selection would stand out, as noted before (19). For example, if only one of the 35 mutations in our samples was driven to a high frequency of 90%, or 3 of the 35 were driven to the medium frequency of 50%, the new spectra would be rejected as neutral with \( P < 0.05 \). This can be seen in the simulations based on Eq. 1 and presented in Materials and Methods, section 11 and Fig. S6. Of course, a true comparison between the non-Darwinian and Darwinian models is possible only when the mode and strength of selection are specified in the Darwinian model. It may hence be more appropriate for investigators with a defined selection scheme to carry out such a test.

The simplest form of selection does make a qualitative prediction in which larger clones, driven by selection, may have taken less time to become larger than the smaller clones. When time is measured by mutation accumulation, the larger clones may be younger, whereas in the non-Darwinian model the larger clones would be.
older (2, 3). In a previous study, Tao et al. (31) showed that, among physically separated HCC tumors, younger but larger tumors appeared to have been driven by Darwinian selection. The authors also detected many small and visible tumors, presumably neutrally growing, by molecular means. Within the same tumors, Tao et al. (31) found the expected non-Darwinian pattern in which the younger clones are smaller than the older (parental) ones. The trend is also observable in HCC-15. For example, γ→γ′→Z1, β→β′→B33, and ε→ε′→C2, where A→B means the B clone is derived from and is smaller than the A clone. Taken together, in this first study with the necessary empirical data that were analyzed by modern population genetics technology, the evolution within this single tumor appears largely non-Darwinian.

The Genetic Diversity of the Entire HCC-15 Tumor. The ability of a tumor to respond/adapt to challenges may depend on $M_{\text{ALL}}$ (the total number of coding mutations in the entire tumor). $M_{\text{ALL}}$ has not been estimated before because under a Darwinian model it would vary greatly, depending on how selection operates. Estimation is feasible under non-Darwinian evolution as shown by the four methods used to estimate $M_{\text{ALL}}$ in HCC-15. The most conservative estimate is $M_{\text{min}}$. When a tumor grows from one cell to $N_T$ cells, the minimal number of cell divisions and mutations should be $N_T - 1$ and $M_{\text{min}} = N_T \times u$, respectively. The highest estimate of diversity was obtained from exponentially growing populations ($M_{\text{exp}}$) in which the number of mutations with frequency $\geq x$ in the entire population is given by Durrett (25) as

$$M_{\text{exp}}(x) = \frac{u}{x} \left[ 1 - \frac{1}{x} \right]. \quad [2]$$

In between these two estimates are $M_{\text{eq}}$ and $M_{3D}$. The estimates of $M_{\text{ALL}}$ are given in the Fig. 3 legend, which explains the four methods, with the details given in Materials and Methods, sections 12–14.

When HCC-15 had only $10^6$ cells (~1.0 mm in diameter), less than 0.1% of its final size, all four estimates are within an order of magnitude of $10^9$ coding mutations. If $M_{\text{ALL}}$ is extrapolated to the final tumor size of $10^{10}$ cells, it would be greater than 100 million. In comparison, under the specific model of Darwinian evolution of Tao et al. (31), $M_{\text{ALL}}$ would be orders of magnitude smaller (Materials and Methods, section 15 and Dataset S9).

The estimated large diversity of HCC-15 consisted mostly of low-frequency mutations. Small local regions of the tumor are each expected to harbor some levels of diversity, which are the building blocks of the total diversity. In Fig. 4, using the rules of clonal growth and mutation accumulation for HCC-15, we simulated the total diversity. The clonal diversity of the plane through the middle of the tumor is illustrated in Fig. 4A (clones >50,000 cells were shown). Importantly, the observation in Fig. 2 and the simulation in Fig. 4A provided visual confirmation of the statistical test based on [55]. The size distribution, the growth dynamics, and the geography of the clones of HCC-15 therefore agreed well with the non-Darwinian growth model. When the simulations of Fig. 4A magnify into smaller areas, the diversity continues to increase as shown by Fig. 4B (resolution >4,000 cells) and Fig. 4C (resolution >100 cells). If we randomly sample and sequence ~50 cells from a local area at the scale of Fig. 4C, the observed genetic diversity should match the simulations. Using the 23 WES samples, we indeed verify the simulated high local diversity in the Fig. 4D legend.

Intratumor Genetic Diversity—A General Theory. This high-density study suggests that previous reports on intratumor diversity should be reevaluated in light of the non-Darwinian model (8, 11–18). Under this simpler model, the diversity estimates of Figs. 3 and 4 can be generalized because a tumor’s diversity depends only on how much time (measured by mutation accumulation) it has taken the tumor to grow to a given size (Materials and Methods, sections 16 and 17). The expected genetic diversity at generation $T$ ($H_T$, the probability that two randomly chosen cells are genetically different in the coding region) can be expressed as

$$H_T = 1 - \frac{e^{-2u}}{N_T-1} \sum_{j=2}^{N_T} \left( \frac{e^{-2uj}}{N_T-j} \prod_{k=1}^{j-1} \left(1 - \frac{1}{N_T-k} \right) \right) . \quad [3]$$

where $N_T$ is the population size at generation $i$, $T$ is the time (measured in generations) of tumor growth from a single progenitor cell, and $u$ is the mutation rate in the coding region (Materials and Methods, section 16). A generation is the time between cell divisions. An alternative formulation based on the birth-and-death process yields nearly identical results (Eq. 6 in Materials and Methods, section 16). Although $T$ and $u$ in Eq. 3 are not known, their product ($U = uT$) is observable. $U$, the number of somatic mutations accrued during tumor growth, has been well documented (12, 13, 16, 17). When a population of cells grows from a single progenitor to $N_T$ in a duration measured by $U$, $N_T$ and $U$ will largely determine the level of genetic heterogeneity (Materials and Methods, section 17). Eq. 2 shows the diversity to be the product of $N_T$ and $U$.

In Table 1, we computed the clonal diversity by setting low $N_T$/s, between $10^6$ and $10^7$ cells, under three different growth models (Materials and Methods, section 17). A tumor with fewer than $10^6$ cells is not detectable by current imaging technologies and $U = 2$ corresponds to two coding region mutations during tumor growth, which is also conservative (12, 13, 16, 17). Even given these parameter values, the neutral clonal diversity is still very high, in the range of 0.6–0.99. For $N_T > 10^6$ cells, two random cells should almost always be genetically different. Importantly, $H$ is not greatly affected by the assumed model (exponential, 3D, or 2D growth) of tumor growth because $T$ and $U$ would vary in opposite directions to yield similar $H$ values (Table 1). The conclusion of high diversity should therefore be generally applicable.

Discussion
Darwinian selection is undoubtedly the driving force of biological evolution but even Darwin himself was puzzled by the amount of genetic diversity within a species. As pointed out by Fisher (20), the better genotypes should have taken over the
populations, leaving little room for within-population diversity \( (19, 21) \). In the modern Darwinian view \( (26, 27) \), complex forms of selection might be able to maintain high intratumor diversity but quantitative predictions, against which observations can be compared, need to be generated first \( (4, 6, 33, 34) \).

We propose that non-Darwinian evolution be considered the null model, under which one can generate testable predictions. If the non-Darwinian predictions are rejected, it will then be necessary to incorporate some forms of selection into the model \( (1, 3) \). In this study, we test the evolution of SNV. The non-Darwinian prediction is consistent with the high \( K_a/K_s \) ratios (nonsynonymous/synonymous SNVs per site) observed in 400 cancer genomes \( (29) \) and in The Cancer Genome Atlas (TCGA) data \( (35) \). The ratio is statistically indistinguishable from 1 in most studies \( (36) \), thus indicating ineffective selection against protein sequence changes in tumors. Cases of \( K_a/K_s \sim 1 \) are rarely seen in nature; for example, \( K_a/K_s < 0.3 \) between humans and other primates \( (1) \).

The level of intratumor diversity is very different between Darwinian and non-Darwinian evolution. Under non-Darwinian evolution, HCC-15 may have 100 million coding mutations and those in the very low-frequency range account for the bulk of the diversity. Fig. 4D based on the polymorphisms within the 23 sequenced samples corroborates this estimate. Under the selection model of Tao et al. \( (31) \), the high diversity could be realized only when the selective coefficients are small, i.e., when Darwinian evolution converges with non-Darwinian evolution. In view of our estimate of the presence of hundreds of millions of SNPs in a tumor the question then arises, “Why is there little Darwinian selection?” One reason is that the bulk of the mutations are in very low frequencies. The frequency spectrum in a rapidly growing population approaches \( \theta x^2 \), where \( x \) is the mutation frequency. In fact, \( \sim 99\% \) of the mutations are found in fewer than 100 cells. Given the strong random drift on low-frequency mutations, it is not surprising that the bulk of mutations appear to be subject to no selection. However, a more important reason may be that in a solid tumor cells stay together and do not migrate, so that when an advantageous mutation indeed emerges, cells carrying it are competing mostly with themselves. These mutations may confer advantages in fighting for space or extracting nutrients but they are stifled by their own advantages. In a nonsolid tumor such as leukemia, cells are not spatially constrained and a selection sweep may indeed occur.

In a physiological sense, good mutations may emerge now and then but in solid tumors the cell populations are so structured that selection may often be blunted. The physiological effect has to be very strong to overcome those constraints. That may be what a drug treatment does—if it “loosens up” the population for effective competition to occur.

It is important to note that several types of genetic changes, including synonymous and nonsynonymous SNVs, CNAs, and epigenetic changes, are evolving in the same genomes. Although the constraints on selection discussed above may apply to all mutations, different types of changes, even synonymous and nonsynonymous SNVs in the same genes, may nevertheless experience different selective pressures and exhibit different evolutionary dynamics. The conclusion of this study applies to SNVs. Whether CNAs or other changes may evolve in the Darwinian mode cannot be tested at present because the underlying forces such as mutation rate are largely unknown.

Patient survival has been shown to be negatively correlated with the level of genetic diversity within tumors \( (5, 7–9) \). When mutations can be found in nearly all possible coding regions within a tumor, resistance to most drugs seems highly likely. Read et al. \( (37) \)
pointed out that aggressive strategies against cancerous cells are effective only in the absence of resistance at treatment and various strategies for administering drugs in the face of resistant clones have been proposed (39–42). Finally, a key feature of the non-Darwinian model is the rapidity with which mutations accrue. Even variant non-Darwinian tumor cells with fewer than 10^9 cells, which are often targets of postsurgery adjuvant therapy, would be genetically diverse (Table 1). The possibility of high intratumor diversity even in small tumors suggests a need to reevaluate treatment strategies.

Materials and Methods

The following sections present essential technical information that is referred to as Materials and Methods, sections 1–17 in the text. Additional details can be found in Supporting Information.

1) Clinical Information. The patient was a 75-y-old man with chronic Hepatitis B Virus (HBV) infection and liver cirrhosis. The tumor, ∼35 mm in diameter, was on the left lobe of the liver and well encapsulated. It was a histopathological grade III hepatocellular carcinoma (HCC) diagnosed at Peking University Cancer Hospital. The pathology report indicated that the tumor sections contain ∼90% hepatoma cells. Two sections of 35 × 35 × 10 mm from the tumor and an adjacent nontumor sample were obtained. This study was approved by the Ethics Review Committee of Peking University Cancer Hospital. Consent was obtained according to the regulations of the institutional ethics review boards.

2) Number, Volume, and Geographical Distribution of Samples. The honeycomb-like sampling is further described in Fig. S1. One 1-mm-thick slice of the tumor sample was subjected to high-density microdissection, using the Harris Micro punch with 0.5 mm inner diameter. In total, 286 microsections were obtained, equally distributed in the four quadrants (labeled A–D; Fig. S1). An adjacent nontumor sample was used as the control. Genomic DNA was extracted using the TIANamp Micro DNA Kit (Tiangen) and quantified using a Qubit 2.0 fluorometer according to the manufacturer’s instructions.

Special attention was paid to minimizing the sample volume (number of cells per sample) as genealogical information is better preserved in samples of smaller volume. Given that the diameter of a HCC cell is about 25 μm (20–30 μm), and the volume of a microsection is ∼0.2 mm³, the number of cells in a microsection was estimated to be ∼24,000. DNA was extracted and quantified from 10,000 tumor cells that were precisely collected by laser capture microdissection (LCM). The cell number in each of the microsections was estimated based on the reference quantity. For the 286 microsections, the median number of cells per sample was ∼20,000, which approximates the number estimated by volume (Fig. S2).

3) Detection of Copy Number Alterations and Estimation of Tumor Purity. The tumor sample was subjected to high-density microdissection, using the bioinformatics facility, including Mutect (43), SomaticSniper (44), JoinSNVmix (45), Varscan2 (46), and Samtools (47). Simulated sequencing reads produced from the MassArrayTyper 4.0 Analyzer according to the manufacturer’s specifications. To estimate the purity and ploidy for each tumor site were validated using the MassArray and iPLEX Gold assays were performed in the Beijing Institute of Genomics (BIG) computational center and the Sequenom (and occasionally by Sanger sequencing) as shown in Dataset S3 and Fig. 1 and 2 of the main text.

As described in Zhu et al. (48), two statistical tests are introduced in the program. One-sided Fisher testing calculates the statistical significance of tumor mutant allele frequency (MAF) that is higher in the tumor population than in the normal cell population. Binomial testing calculates the significance of tumor mutant allele number observed from the aligned tumor sequencing data that meet a binomial distribution. In addition, 10 filtering criteria were applied to detect somatic SNVs as described in Supporting Information. All somatic mutations are shown in Dataset S2.

5) Validation of the Observed SNVs Across the 286 Samples. SNVs discovered by WES were validated by Sequenom genotyping on the 286 samples. These discovered SNVs fall into three classes: (i) The ALL class has 178 SNVs that were discovered in all 23 WES samples (all red dots in Fig. 1A, ii) the MOST class has 53 SNVs that were present in most samples and missing in only a few (usually 1–4 where read depth was low), and (iii) the SOME class has 38 SNVs that were present in some (≤5) samples where the mutant was missing due to low control mutation calling and allele frequencies for each SNV site were determined using the MassArray/Typser 4.0 Analyzer according to the manufacturer’s specifications. To estimate the purity and ploidy for each sample were validated using the HCG19 as reference. Some SNVs found in only one sample were further validated by PCR and Sanger sequencing (Supporting Information).

4) Detection of Somatic SNV. Tagmentation-based library preparation (Fig. S7), WES, and sequence alignment are described in Supporting Information. Somatic SNV calling was performed using the in-house software, CASpoint, which has been extensively tested in the public domain (Dataset S10; also see the result in the International Cancer Genome Consortium-TCGA DREAM Somatic Mutation Challenge (SMC). (http://workshop.synapse.org/over0/5synapse.syn312572wks/70726)). We compared the false positive and negative rates, sensitivity, and accuracy of CASpoint in SNV calling with the performances of other published software installed in the Beijing Institute of Genomics (BIG) computational center and bioinformatics facility, including Mutect (43), SomaticSniper (44), JoinSNVmix (45), Varscan2 (45), and Samtools (46). Simulated sequencing reads in the SMC and a large set of whole-genome or exome sequencing reads produced from various genomics projects in solid tumors (31) and leukemia (46) in Beijing Institute of Genomics were used to evaluate the performance of CASpoint. The overall accuracy of CASpoint is comparable to the others for the SMC simulated reads. Because CASpoint showed better performance in reducing false positive rates than other programs for real sequencing data according to validation results using Sequenom and Sanger sequencing, the in-house program was used in this study to minimize the false positive rate.

As described in Zhu et al. (48), two statistical tests are introduced in the program. One-sided Fisher testing calculates the statistical significance of tumor mutant allele frequency (MAF) that is higher in the tumor population than in the normal cell population. Binomial testing calculates the significance of tumor mutant allele number observed from the aligned tumor sequencing data that meet a binomial distribution. In addition, 10 filtering criteria were applied to detect somatic SNVs as described in Supporting Information. All somatic mutations are shown in Dataset S2.

6) Identification of Fixed and Polyphasic Somatic Mutations. Based on the descriptions in Materials and Methods, section 4 and the results of Datasets S2 and S3 and Fig. S4, the 269 SNVs are classified as 209 confirmed fixed SNVs, 35 confirmed polymorphic, 5 and 25 less certain mutations. These 25 mutations, including respectively 22 possible fixed and 3 possible polymorphic mutations, were not used in the analyses. The partition of these 269 SNVs summarized in Fig. S4 is as follows:

i) The confirmed 209 fixed mutations include 178 from the ALL class and 31 from the MOST class described in Materials and Methods, section 4 above. The 178 SNVs were observed in all 23 WES samples (Dataset S2) and the limited validation among unsequenced samples indeed confirmed their ubiquitous presence. The 31 MOST class mutations were present in all but a few (1–4) WES samples, due to low depth coverage of such sites in these samples. Sequenom results validated their presence in these samples.

ii) The 35 confirmed polymorphic mutations are listed in Dataset S2 among WES samples. They were further validated across the 286 samples by Sequenom (and occasionally by Sanger sequencing) as shown in Dataset S3, which is the basis of the spatial distribution of these mutations shown in Figs. 1 and 2 of the main text.

iii) For the remaining 25 SNVs, 22 mutations are missing in 1–4 samples (Dataset S2, under “SNV in CNA regions”). These mutations occurred in regions of frequent CNAs, which would result in LOH. LOH could be inferred directly from these data when AB (mutations A and B occurred in 20 samples), A+ (mutation A but not B occurred in 2 samples), and +B
(mutation B but not A occurred in 1 sample) were all observed. In this pattern, B is lost twice and/or A is lost once. From the pattern shown in Dataset S2, it is likely that all of the 22 mutations are fixed but it is prudent to exclude them from subsequent analyses, as was done here.

The 3 possibly polymorphic mutations were detected in some WES samples but could not be reliably genotyped across the 286 samples by Sequenom. They are almost certainly polymorphic mutations but could not be used in this study to delineate the spatial boundaries of clones or their sizes.

7) Clone Map Delineation and Phylogenetic Analysis. The 35 polymorphic SNVs unaffected by CNAs were validated in the 286 tumor samples, using Sequenom and/or Sanger sequencing (Dataset S3). The neighbor-joining method of Saitou and Nei (50) was used to construct the phylogenetic tree (Fig. 1B and D). A constrained matrix was created, containing the mutations of all samples with “1” and “0” representing the presence and absence of a mutation based on genotyping results of the 35 SNVs. We used the “APE” R package (51) and ITOL (itol.embli.de) for constructing and plotting the phylogenetic trees (Fig. 1B and D).

The positions of eight samples (A3, B17, B19, B20, C78, D6, D9, and Z1) that carried mutations of two neighboring clones were marked with blue stars in the phylogenic tree in Fig. 1D. The boundaries and space of the subclones in HCC-15 were delineated in the two-dimensional clonal map based on both the presence of the polymorphic SNVs and the phylogenetic relationship (Fig. 2).

8) Identification of Putative “Driver” Genes. We attempted to identify driver genes from among the 269 mutated genes in our study. As in common practice, driver genes are defined as those that are significantly over-represented in the cancer databases. The data we used here comprise 460,967 somatic mutations (402,716 SNVs, 42,886 small deletions, and 12,249 small insertions) detected in whole-exome sequencing data of 1,363 patients with gastrointestinal cancer (Dataset S6), including 202 hepatocellular carcinoma (HCC), 183 esophageal carcinoma (ESCA) (54,042 mutations), 288 stomach adenocarcinoma (STAD) (115,357 mutations), 220 colon adenocarcinoma (COAD) (114,594 mutations), 81 rectum cancers (READ) (25,003 mutations), and 147 pancreas cancers (PAAD) (56,815 mutations) from TCGA datasets and 242 hepatocellular carcinomas (22,294 mutations) in Schulte et al. (32). We applied the program MutSigCV 1.4 (52), which corrects for variation by incorporating a patient-specific mutational spectrum and gene-specific background mutational burden, and by measuring gene expression and replication time as well, to detect significantly mutated genes.

In total, we identified 372 driver genes from the somatic mutations dataset of 1,363 gastrointestinal cancer cases (Dataset S3). Comparing the 166 fixed protein-altering mutations in our study to the driver genes identified from the databases, we identify 6 putative driver genes (p-value < 0.2): CCAR1, CPXM2, DNAH7, TMPRSS13, TSC1, and TPS3; the last of the 6 genes has a high frequency in all gastrointestinal cancers (Fig. S5). We note that none of the genes carrying any of the 35 polymorphic mutations in this study belongs in the driver group. Ingenuity pathway analysis (IPA) (www.ingenuity.com) and Fisher’s exact test were carried out to identify significantly enriched pathways for the genes with polymorphic and fixed protein-sequence altering SNVs (Dataset S5).

9) Observed Mutation Frequency Spectrum. The mutation frequency spectrum is denoted as [zi; i = 1 to n – 1] in the main text, i is the number of sites where the mutant appears i times in n samples in the infinite-site model of population genetics. In Fig. 1B, the heat map is equivalent to a spectrum of [zi; i = 24, 2, 3, 2, 1, 3, 0, 0, 0,...] for i = 1–22, where Σzi = 35 is the number of mutations in the 23 WES samples. In this spectrum, the mutation in each sample is scored as either present or absent.

Because the frequency of each mutation was more accurately determined by genotyping, zi is represented by the number of sites where the frequency of the mutation was between (i − 1/2)2 and i(2)2 from the 286 samples. We kept the number of frequency bins at 23 because the mutations discovered were still based on the initial 23 samples. The spectrum, as given in Dataset S8, is [zi; i = 26, 7, 1, 1, 0, 0, 0, 0,...] for i = 1–22. There are two methods to compute the frequency spectrum using the data from the 286 samples. One is to score the presence/absence of each mutation in each sample. This will tend to underestimate the true diversity. This is because the imposition of the equilibrium conditions on the data would result in adequate estimation of diversity only in the observable portion of the spectrum. Low-frequency mutations were expected to be underestimated. In Materials and Methods, section 13, we provide the details of obtaining E(z) as well as the simulation data that corroborated the conjecture of E(z) < Ed. As most tumors are growing, albeit not necessarily in any specific mode, the mode of population growth is specified. If N = 1 at generation t, the population would be growing exponentially. The net growth rate (i.e., the difference between the birth and death rates) could be positive, negative, or net zero. Under this exponential growth rate. The validation rates suggest that the calls in bins > 5 reads are of high confidence. We disregard calls with ≤5 reads in Fig. 4D, which gives the mean and SD of mutation number in each of the larger size bins.

10) Expected Mutation (Site) Frequency Spectrum in Exponentially Growing Populations.

\[ E(z) = \left\{ \begin{array}{ll} \rho \sum_{n=1}^{\Sigma} \frac{1}{n + k + n - 1} & \text{for } 1 \leq j \leq n \\ \frac{\rho}{r} & \text{for } 2 \leq n < r \end{array} \right. \]

where \( r \) is the rate of exponential growth, \( u \) is the mutation rate per cell generation, \( n \) is the sample size, and \( N_r \) is the cell population size at time \( T \) (25). For HCC-15, \( [22] = [26, 7, 1, 0, 0, 0, ...] \) for i = 1–22. Because \( \Sigma_{i=1}^{26} = (7 + 1) = 9 \) = 23 u/r, we obtain \( u/r = 0.41 \). The expected site frequency spectrum for 35 mutations is hence \( E(z) = [26.0, 4.72, 1.57, 0.79, 0.47, 0.31, ...] \).
model developed by Durrett (23), the number of mutations with frequency >x in the entire population is given by Eq. 2.

\[
N_r = \frac{rN}{\theta} \frac{e^{\theta r}}{\theta r}
\]

The elegant simplicity of Eq. 2 is not unexpected because the genetic diversity in a tumor is largely determined by two parameters: the number of mutations (U) each cell accumulates during tumor growth and the population size (N_f = \theta^r x). The expression, \( U = u = (\theta r) \ln(N_f) \), thus anticipates the simplicity. The total number of mutations in the tumor, \( M_{\text{mut}}(x = 1/N_f) \), is projected to be \( (\theta r) \times 1/(\theta r) \). From the observed mutation frequency spectrum [\( r_{\text{obs}} \) and Eq. 1], we have obtained \( u/\theta = 0.41 \).

Given \( N_f \approx 10^6 \) cells, HCC-15 would have \( M_{\text{mut}} \approx 4.1 \times 10^6 \) coding mutations (Fig. 3), which was more than 10-fold larger than \( M_{\text{mut}} \). The mutation frequency spectrum is also given in Fig. 3. Even for such a small tumor, there would still be 5,000 mutations, each of which can be found in more than 100 cells. In a different approach to estimating \( u/\theta \), we used an approximate Bayesian computation method (53) by simulating a branching process with cell birth, death, and mutation often used for modeling tumor growth (54). We obtained the posterior mean \( u \) and \( \theta \) that showed \( u/\theta = 0.412 \) (Fig. S8), which was nearly equal to 0.41 obtained from Eq. 1.

In the fourth estimate, \( M_{\text{grow}} \), the growth mode is also specified and the increase in cell number is assumed to occur only on the periphery of a tumor in 3D (Materials and Methods, section 14). In the interior, each cell division results in the birth of one cell, which would replace a neighboring cell. Because the birth and death rates cancel out in the interior, the growth rate of the tumor (\( dN/dt \)) is proportional to \( \theta^2/N_f \), instead of \( \theta^2/N_f \) as in the exponential growth. Simulation results of Fig. 3 showed that the 3D growth mode yielded similar mutation numbers \( M_{\text{mut}} \), except in the lowest-frequency bin of fewer than 10 cells.

13) Computer Simulations of \( M_{\text{mut}} \): A lower bound of \( M_{\text{ALL}} \). \( M_{\text{mut}} \) is the number of mutations in the population by artificially imposing the mutation-drift equilibrium on the tumor. Thus, \( M_{\text{mut}} = 2M(\theta) \), where \( \theta = 2M(\theta) \) is the scaled mutation parameter in tumor growth and \( N_f \) is the effective cell population size. We implemented computer simulations to prove that \( M_{\text{mut}} \) is a proper lower bound of mutation number \( M \) in a growing population. \( M_{\text{mut}} \) is expected to always be smaller than \( M \) under any mode of tumor growth. Three typical growth models were simulated, including exponential growth, 2D growth, and 3D growth. It should be noted that the cell populations with models of 2D growth (\( dN/dt = rN \)) and 3D growth (\( dN/dt = rN \)) are extremely well mixed and belong to the power law family of tumor growth models.

For exponential growth, we simulated a discrete-time birth-death process, in which an individual divides and gives birth to two daughter cells with probability \( b \) and dies with probability \( d \) under any mode of tumor growth. The Wright-Fisher model of tumor growth assumes Poisson distribution for the number of offspring cells that a cell generates in a division, which may not be rigorous in modeling cell dynamics. To investigate the generality of Eq. 3, we also derived the exact formula of clonal diversity (H) under a discrete-time birth-death process of tumor growth. In particular, a cell gives birth to two daughter cells with probability \( b \) and dies with probability \( d \). Suppose that two cells are randomly selected from generation \( t \); the probability that coalescence occurs in the previous generation between the two cells is \( Pr(\text{coalescence}) = 1/N_f - 1 \). Solving previous recursion in the same way gives rise to

\[
H_T = 1 - J_t = 1 - \frac{e^{-2u}}{N_f - 1} \sum_{j=0}^{\infty} \frac{e^{-2u} j^2}{N_f - 1} \prod_{j=1}^{j-1} \left( 1 - \frac{1}{N_f - 1} \right).
\]

which is Eq. 3.

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\]

which is Eq. 3.

17) Estimating Clonal Diversity, \( H \), Under Different Growth Models. Eq. 3 can be applied to arbitrary time variable-size cell populations. However, we examined three models in this study to estimate the clonal diversity \( H \) in a growing cell population (Dataset S8), including an exponential growth model and two power-law growth models (resembling 2D and 3D growth, respectively).

In the exponential model, the population dynamics follow \( dN/dt = rN \) in continuous time. It can be solved as \( N_t = e^{rt} \). In discrete time, \( r = \ln(2) \) corresponds to the situation that all cells duplicate with no cell death in each generation. We showed the results using two growth rate values as \( r = \ln(2) \times 0.1 \) and \( r = \ln(2) \times 0.01 \). The other two models, 2D growth (\( dN/dt = rN \)) and 3D growth (\( dN/dt = rN \)), belong to the power-law family of tumor growth models. These two models, \( r = 2 \times 1/3 \) and \( r = 3 \times 1/3 \), correspond to the condition that the population generates exactly one layer of cells in the periphery in each discrete generation for 2D growth and 3D growth, respectively. In the 3D growth model, two growth rate values were tested, where \( r = 3 \times 1/3 \times 0.1 \) and \( r = 3 \times 1/3 \times 0.01 \).

We set \( N_f \) at three levels between \( 10^3 \) and \( 10^6 \) cells and \( N_f \) for \( t \) between 0 and \( T \), depending on which of the three growth models were used. Once the growth model and the \( r \) value were defined, the number of cell divisions required to reach \( N_f \) could be calculated. We calculated the expected clonal diversity \( H_t \) from Eq. 3 (Table 1).

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