

## Neutral tumor evolution? [Letter]

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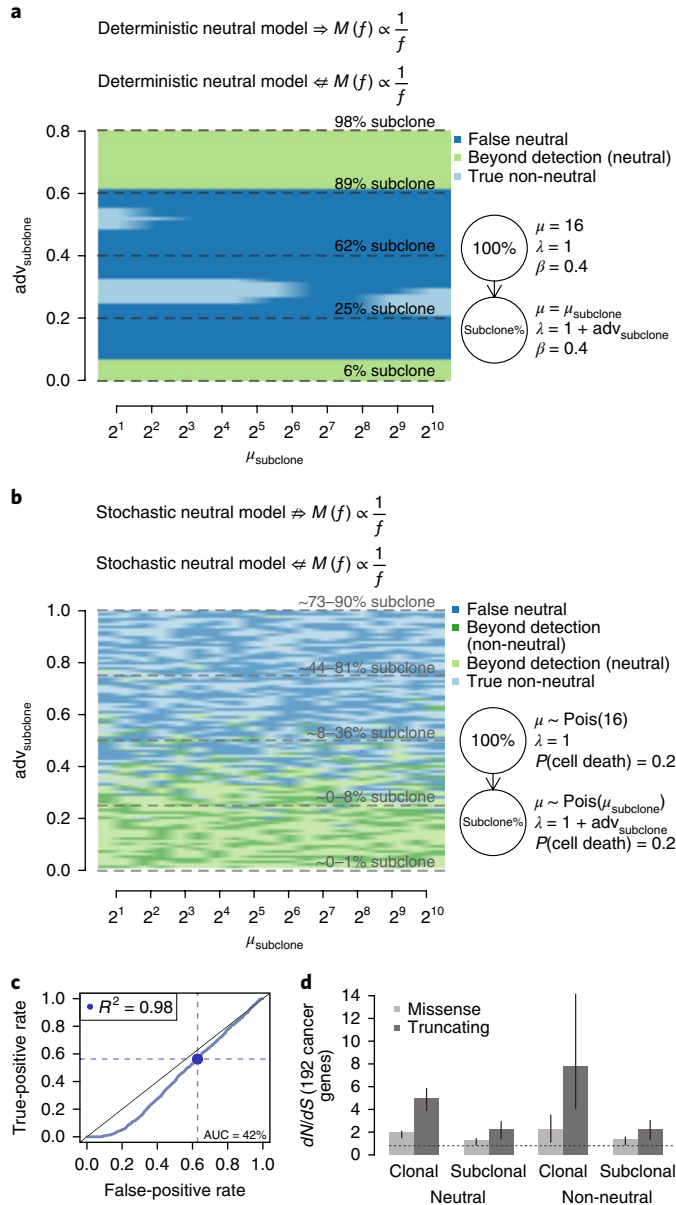
# Neutral tumor evolution?

**To the Editor** — Tumor growth is an evolutionary process that is governed by somatic mutation, clonal selection and random genetic drift, and is constrained by the coevolution of the microenvironment<sup>1,2</sup>. Tumor subclones are subpopulations of tumor cells with a common set of mutations resulting from the expansion of a single cell during tumor development, and they have been observed in a substantial fraction of cancers and across multiple cancer types<sup>3</sup>.

Peter Nowell has proposed that tumors evolve through sequential genetic events<sup>4</sup>, whereby one cell acquires a selective advantage so that its lineage becomes predominant. According to this traditional model, the selective advantage is conferred by a small set of driver mutations, but as the subclones that bear them successively expand, they also accumulate passenger mutations, which can be detected in sequencing experiments<sup>1</sup>. Genomes of

individual tumors contain hundreds to many thousands of these genetic variants at a wide range of frequencies<sup>5,6</sup>. Because genetic drift can drive novel variants to high frequencies, it is of great interest to discern the relative importance of selection and drift in shaping the frequency distribution of variants in any given tumor.

Williams et al.<sup>7</sup> have recently proposed a way to assess this relative importance. They have found that a simple model of



**Fig. 1 |  $M(f) - 1/f$  is an uninformative test for identifying (non-)neutrality in simulated and real data.**

**a**, Neutrality calls in simulations of tumor growth with subclonal expansion underlying selective sweeps. The tree topology being modeled is represented on the right together with the parameters of the neutral evolution equations for the two subpopulations of cells (Supplementary Methods). The subclone's fraction (subclone %) increases with its selective advantage ( $\text{adv}_{\text{subclone}}$ ). The  $\lambda = 1 + \text{adv}_{\text{subclone}}$  and  $\mu$  parameters of the subclone are varied along a grid. Simulations are defined as true non-neutral (light blue) or false neutral (dark blue) when the growing subclone has expanded sufficiently to be detectable and the sweep is not complete (i.e.,  $10\% \leq \text{subclone \%} \leq 90\%$ ); otherwise, the subclone is considered beyond detection (light green). Non-neutral call,  $R^2 < 0.98$ ; neutral call,  $R^2 \geq 0.98$ . **b**, As in **a**, but using the Gillespie algorithm to simulate branching processes<sup>10</sup>. Simulations leading to subclones beyond detection are called either neutral (light green) or non-neutral (dark green). Because of the stochastic nature of branching processes, different subclone % values are obtained across simulations from the same  $\text{adv}_{\text{subclone}}$  values. For five increasing  $\text{adv}_{\text{subclone}}$  values, median  $\pm$  median absolute deviation of the subclone % across the simulations are reported. **c**, Summary receiver operating characteristic curve for the neutral versus non-neutral classification based on the  $R^2$  values in 1,919 non-neutral simulations from **b** and 1,919 simulations of neutral tumors. The false-positive rate and the true-positive rate are highlighted for  $R^2 = 0.98$  used by Williams et al. AUC, area under the curve. **d**,  $dN/dS$  analysis. Maximum-likelihood estimates of the  $dN/dS$  ratios and associated 95% confidence intervals for (sub) clonal mutations in TCGA tumors categorized into neutral and non-neutral groups. Ratios for missense and truncating mutations are given.  $dN/dS > 1$  indicates positive selection

tumor growth in which all novel variants are selectively neutral, that is, whose dynamics are governed entirely by drift, predicts a linear relationship between the number of mutations  $M(f)$  present in a fraction  $f$  of cells and the reciprocal of that fraction:  $M(f) \propto \frac{1}{f}$ . They argue that deviation from this null model (i.e., if the  $R^2$  of the linear fit is below the minimum observed in neutral simulations ( $R^2 < 0.98$ )) indicates the presence of selection. Such selection can be tested by means of variant allele frequencies (VAFs) from which  $f$  can be derived. By applying this rationale to real cancer data from The Cancer Genome Atlas (TCGA), the test proposed by Williams et al. did not reject the null model (that is, neutrality was found) in approximately one-third of the cases, and the authors have concluded that those tumors were neutrally evolving. More recently, multiple myelomas with evidence of the proposed linear relationship have been associated with poorer prognosis<sup>8</sup>.

Although the analysis by Williams et al. provides an interesting approach to infer selection in human cancers, unfortunately, it is based on four major simplifying assumptions that might render the conclusions questionable.

First, inferring  $f$  of variants from their VAF requires accurate estimates of local copy number, overall tumor purity and ploidy. Williams et al. attempted to account for some of these factors by restricting their analyses to variants that had VAF between 0.12 and 0.24 and were located in copy-neutral regions of the genome. However, even in that limited VAF window, the VAF of a mutation does not reflect its true  $f$  in many cases. For example, in tumors with whole-genome duplications (37% of tumors in the analyzed dataset<sup>9</sup>) the peak of clonal mutations acquired after the whole-genome doubling event was at or below  $\text{VAF} = 0.25$  (one out of four copies in a 100%-pure tumor sample), which would have led to artificial deviation from the linear fit within that VAF window.

Second, the interpretation of the analyses is inconsistent with the use of neutrality as a null model. Failure to reject the null hypothesis is not the same as proving it true; i.e., that all neutral simulations have  $R^2 > 0.98$  does not prove that non-neutral simulations would never yield  $R^2 > 0.98$ . One would need to demonstrate that this condition is sufficient to infer neutrality, but, in addition, no equally suited models of non-neutral tumor growth should yield  $R^2 > 0.98$ .

To assess the latter, we simulated simple tumor growth in which we explicitly modeled one subclonal expansion with a selective advantage, i.e., increasing its division rate  $\lambda$  and/or the mutation rate  $\mu$

of the subclone (Supplementary Methods). On the basis of the original method described by Williams et al., neutrality was rejected only within a narrow range of  $\lambda$  and  $\mu$  values tested that would lead to detectable subclones (true rejection of neutrality in ~11% of simulations; Fig. 1a). We conclude that a linear fit with  $R^2 > 0.98$  is not sufficient to call neutrality and that improper use of this model could result in substantial overcalling of neutrality.

Third, the deterministic model of tumor growth described by Williams et al. relies on strong biological assumptions, including synchronous cell divisions, constant cell death, and constant mutation and division rates. Stochastic models of tumor growth are biologically more realistic because they allow for asynchronous divisions and probabilistic mutation acquisition, cell death and division rates. Using simple branching processes to simulate neutral and non-neutral growth<sup>10</sup> (Supplementary Methods), we show that  $R^2 > 0.98$  for  $M(f) \propto \frac{1}{f}$  is neither a necessary nor a sufficient property of neutrally evolving tumors (Fig. 1b). Although the expected cumulative number of mutations at a given mutation frequency (i.e., the average over many independent samples) is inversely proportional to the frequency of the mutations  $\overline{M}(f) \propto \frac{1}{f}$  (ref. <sup>10</sup>), because of the biological noise modeled in branching processes, a typical realization of the neutral process in a single sample can substantially deviate from the expected linear fit, thus rendering an  $R^2$  threshold inaccurate to infer neutrality. As a result, discrimination of neutral and non-neutral simulated tumors by using a linear fit is almost arbitrary, with 53.5% false-positive neutral calls in non-neutral tumors (Fig. 1b) and an area under the receiver operating characteristic curve of 0.42 for the classification of 1,919 neutral and 1,919 non-neutral tumors (Fig. 1c).

Fourth, we reason that in tumors called neutral, no subclonal selection should be detected. To evaluate this possibility, we identified selection by using an orthogonal method based on the observed variants themselves rather than on their allele frequencies.  $dN/dS$  analysis derives the fraction of mutated nonsynonymous positions relative to the fraction of mutated synonymous positions in the coding regions. This method has been widely used to detect the presence of negative or positive selection of nonsynonymous variants in coding regions<sup>11,12</sup>. We applied a  $dN/dS$  model optimized for the detection of selection in somatic cancer variants<sup>13</sup> to TCGA exome data, by using a published list of 192 cancer-driver genes<sup>14</sup> (Supplementary Methods). The analysis was performed separately by

using variants called as clonal or subclonal (Supplementary Methods), in tumors called neutral and non-neutral, according to the rationale outlined by Williams et al.<sup>7</sup>. The  $dN/dS$  ratio analysis showed significant positive selection in subclonal mutations of tumors classified as neutral (Fig. 1d), thus further suggesting that the approach described by Williams et al. is under-equipped to detect the presence or absence of selection.

In summary, Williams et al. proposed that approximately one-third of tumors are neutrally evolving. However, we highlighted four simplifying assumptions that, to our knowledge, have not previously been highlighted, and we found that the proposed approach often identifies individual tumors as being neutral when they are non-neutral and as being non-neutral when they are neutral. A new paper by the same group<sup>15</sup> has introduced a Bayesian test for detecting selection from VAFs. The test estimates selection coefficients and as such is an important advance over Williams et al.'s frequentist test, which does not. The authors acknowledge that the test can detect only large fitness differences, but they nevertheless call tumors that fail the test 'neutral' when they are merely those in which a weak test has failed to detect selection. We note that neutral theory has been developed in population genetics, ecology and cultural evolution, and that in all of these fields, similar tests have been proposed and have eventually been found lacking for the same reason: variant abundance distributions do not contain sufficient information to exclude selection<sup>16–18</sup>. Identifying and better understanding the drivers of the potentially more aggressive (sub)clones expanding under selective biological or therapeutic pressure is critically important, because these are good candidates for predicting resistance and exploring combination therapy. Williams et al. should be commended for introducing explicit neutral tumor growth models into tumor genomics. However, quantifying the relative importance of drift and selection in shaping the allele frequencies of single tumors clearly remains an open challenge. Studies relying on their proposed test (e.g., ref. <sup>8</sup>) might then need reevaluation.

## Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

This study used data generated by the The Cancer Genome Atlas (TCGA, a collection

of publicly available data from human tissues; <http://cancergenome.nih.gov/>). We ran  $dN/dS$  analyses on the data from TCGA, using published CaVeMan<sup>19,20</sup> single-nucleotide variant calls, and ASCAT<sup>21</sup> copy number calls, as described in Martincorena et al.<sup>13</sup>. Simulation data can be reproduced by using the R and Java scripts provided. □

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M.T., I.M., M.G., A.M.L., F.M., P.T.S., Q.D.M., O.C.L., D.C.W. and P.V.L. participated in argumentation. M.T., O.C.L., D.C.W. and P.V.L. derived the deterministic equations. M.T. wrote the code and generated the figures, with input from I.M., M.G., O.C.L., D.C.W. and P.V.L.; M.T., O.C.L., D.C.W. and P.V.L. drafted the manuscript, which was revised by I.M., M.G., A.M.L., F.M., P.T.S. and Q.D.M. All authors read and approved the manuscript.