Reply: Is the evolution of tumors Darwinian or non-Darwinian?

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Wu and colleagues' letter discusses the limitations of the use of a single sample per tumor to investigate neutral evolution in human cancers. Neutral tumor evolution describes the situation where there is no differential clonal selection amongst the population of cells within a cancer: all mutations that accrue during growth are passengers and all drivers were already present in the most recent common ancestor of the population.

In Williams et al., 2016[1] we showed, using publically available data comprising a single sample from each of ≈900 tumors of 14 different types, that the patterns of subclonal mutations within many cancer genomes (≈35%) were precisely predicted by a mathematical formula describing neutral tumor evolution. In contrast, Ling et al. 2015[2] performed multi-region whole-exome sequencing (23 samples) and high-density targeted sequencing (286 samples) of a *single* hepatocellular carcinoma case, and by examining the mutation burden across the tumor, concluded that the entire malignancy was evolving neutrally.

Wu and colleagues specifically question whether these two different approaches, namely analyzing intra-tumor heterogeneity within a sample versus dense multi-region sampling, measure the same features of tumor evolution.

Clearly, the key issue here is intra-tumor variation of the evolutionary process itself; specifically, whether some regions of a tumor are evolving neutrality and others are not. We agree with Wu and colleagues' assertion that 'local' neutrality (e.g. within a single sample) does not necessarily imply 'global' neutrality across the whole tumor.

However, there are two reasons to think that local and global neutrality are often correlated.

First, as we discussed in Williams et al., our classifications of neutrality were consistent with the detection of subclonal driver mutations in existing multi-region sequencing studies: sub-clonal driver mutations and convergent evolution (consistent with ongoing selection) were often detected in 'non neutral-like' renal carcinoma[3] and

glioblastoma[4], but less frequently in 'neutral-like' colorectal cancer[5].

Second, we note that if a single sample comprises a large portion or section across the tumor, neutrality can be assessed with our method based on the analysis of within-sample variant allele frequencies (VAF) - mutations that are subclonal within the sample. Such a large sample can provide a 'global' view of neutral evolutionary dynamics and, to a degree with which a single large sample represents the tumor as whole, mitigates sampling bias. A similar approach has been successfully applied to deconvolute the clonal architecture of a single breast cancer case[6]. We note that the TCGA data we analyzed in our study is derived from large fresh-frozen resection specimens rather than small biopsies (http://cancergenome.nih.gov/cancersselected/biospeccriteria), thus reducing the sampling bias of our approach. However, we fully acknowledge that no single sub-sampling strategy can fully capture the spatial architecture of a tumor and there is the need for extensive multi-region sequencing, which however remains at the moment impractical for large cohorts such as TCGA.

Importantly, as we noted in our study, the depth of sequencing remains a limitation as it determines the time elapsed from the common ancestor (of the sampled population) where we can investigate neutral evolution, as new mutations become progressively rarer as the population grows. We agree that for low depth of sequencing, because under neutrality subclonal VAF is proportional to time, only a short period after the common ancestor can be studied and so only 'global scale' neutrality can be characterized, while the evolutionary dynamics of small populations remain inaccessible.

Given these two points, we think it is unlikely that our analysis risks grossly misrepresenting the tendency for neutral evolution in a tumor type.

While we fully agree that multi-region profiling reduces potential sampling bias (and indeed we use multi-region sequencing ourselves for this reason[4,5]), our method has the crucial advantage of allowing us to profile existing large cohorts (such as those of the ICGC and TCGA) and so to statistically address the issue of inter-patient variation[7] within a tumor type. Clearly the optimum would be to combine the two approaches and perform multi-region sequencing on large cohorts, though this presents obvious financial and technical challenges. Indeed, our recent study shows multiple-sample analysis of VAF distributions leads to robust calling of neutrality[8]. Moreover, we note too that studying truly 'local' evolution requires the sequencing of very small and localized cancer cell populations, as we previously demonstrated[5].

Wu and colleagues also note that non-exponential tumor growth leads to a different pattern of subclonal variant allele frequencies in a neutrally growing tumor: boundary driven growth (described by $N(t) \sim t^{\gamma}$) leads to the relationship:

$$M(f) \sim \frac{1}{f^{\frac{\gamma}{\gamma-1}}}$$

which may provide a good fit to the data in some cases, and so neutrality may be more common than we reported in Williams et al. Irrespective, in some of the 65% of non-neutral cases identified by our method, clear subclonal mutational clusters can be observed, and our computational simulations confirmed that such patterns are expected if differentially selected subclones are present (Supplementary Figure 11 in Williams et al.) – but we highlight that these clusters are not caused by boundary driven growth. The observations of 'subclonal clusters' is in line with previous

studies[6], and we note that amongst the TCGA samples we reanalysed in Williams et al., a previous analysis had detected subclonal peaks in the majority of cases[9] (though we note this analysis may have confused the 1/f tail with a low-frequency clone). Thus, irrespective of the underlying growth model, there is clear evidence of on-going selection in many tumors.

In their letter, Wu et al. describe a model of selection (Equation [1]) that predicts that the variant allele frequency (VAF) distribution of a tumour sample will be indistinguishable both in the presence and absence of selection. We urge caution against using Equation [1] reported in the letter in the context of cancer as this model appears incompatible with our current knowledge of cancer biology. First, the model assumes a constant population size, which is of course inapplicable to cancer. Second, the model also assumes that a large majority of new subclonal mutations are under selection, leading to a continuum tail of variants at higher frequency than is expected by chance (neutrality). Importantly, this model of selection does not lead to the formation of distinct sub-clonal clusters in the variant allele frequency distribution[6,10]. We argue that such a large number of driver events at high frequency in the same cancer is highly unlikely, as most evidence points to a limited number of putative drivers per tumour[5,11,12]. In Williams *et al*. we developed a model of selection consistent with the current knowledge of cancer genomics, wherein subclones under strong selection arise infrequently during tumour growth, and where the majority of mutations are neutral passenger mutations. These dynamics do give rise to subclonal clusters of mutations, as reported by multiple studies[6,10]. The VAF distribution produced by these models consistently leads to rejection of the neutral null model.

However, we agree with Wu and colleagues that weak selection is challenging to detect because it causes only slight changes in the clonal composition of the tumor that may be undetectable by current genomic profiling standards. Importantly, this is true for single sampling and multi-region profiling alike, and studies of n=1 tumours, such as the one conducted by the authors[2], result in findings that are of uncertain generality. We acknowledge that it is very important at this stage to understand the precise signature of weak and strong selection, especially because clonal selection is often hard to define and produces complex patterns (hence one of the reasons why we focused in the original manuscript on understanding *absence* of selection, which is analytically tractable). This important topic is the focus of our current and future work[13]. Nevertheless, we note that the analysis in Williams *et al.* demonstrates that in a significant proportion of cases the null-model of neutral evolution cannot be rejected.

In summary, we were very happy to see that two independent groups have now demonstrated neutral evolution in cancer, a concept that has been largely neglected by current genomic studies. While the difference between local and global neutrality should be fully addressed in future work, the salient point that we would like people to take away from Williams *et al.* is that in many cases neutral evolution is an entirely adequate description of the currently available data.

Acknowledgments

A.S. is supported by the Chris Rokos Fellowship in Evolution and Cancer. B.W. is supported by the Geoffrey W. Lewis Post-Doctoral Training fellowship. This work was supported by the Wellcome Trust (105104/Z/14/Z). C.P.B. acknowledges funding from the Wellcome Trust through a Research Career Developmental Fellowship (097319/Z/11/Z). This work was supported by a Cancer Research UK Career

Developmental Award to T.A.G. M.J.W. is supported by a UK Medical Research Council student fellowship.

Author Contributions

All authors contributed equally to this response letter.

Competing Financial Interests

The authors declare no competing financial interests.

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