Positive Selection as the Unifying Force for Clonal Evolution in Multiple Myeloma

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To the Editor:

Multiple myeloma (MM), the second most common hematologic malignancy in the U.S in 2020, is known for a lengthy progression from premalignant clonal dyscrasia to frank plasma cell malignancy, spanning 30-40 years until clinical detection [1, 2]. Traditionally, Darwinian forces have been thought to positively select sequential advantageous mutational events to shape tumor evolution [3]. Analyses of serial tumor samples chronicling evolution from smoldering to multiple myeloma have been explained by punctuated and/or static evolutionary models [4, 5]. Recently, an alternative neutral evolutionary model has been posited broadly across malignancies [6-8]. Neutrality predicates that a tumor will ultimately reach a threshold at which all of the drivers necessary to confer malignant potential will be acquired. Following this clonal, malignant transformation (i.e. the "Big Bang"), the tumor expands neutrally such that further (subclonal) mutations no longer provide an appreciable fitness advantage and thus are not selected for, as the tumor architecture remains in relative equilibrium [6]. Following the "Big Bang," new mutations adhere to the rules of a simple power-law distribution in which the cumulative number of mutations at a given allelic frequency is inversely proportional to the allelic frequency: $M(f) \propto 1/f$ [6, 7]. Given recent reports of worse clinical outcomes for purportedly neutral myeloma tumors [9], as well as potential implications for early intervention and other therapeutic considerations, we were motivated to comprehensively characterize the selective forces responsible for shaping the evolutionary trajectory of multiple myeloma.

To quantify the prevalence of neutrality in multiple myeloma, we analyzed the CoMMpass data (version IA13, Multiple Myeloma Research Foundation Personalized

Medicine Initiative). Copy number profiles were extracted from low-coverage, long-insert whole genome sequencing (WGS) data and the catalog of single nucleotide variants (SNV) was generated with all variants called by at least two of the three variant callers Seurat, Mutect, and Strelka. For 752 patients with available WGS at baseline, we then utilized the *neutralitytestr* R package – which calculates the fit of the tumor's mutational profile and variant frequency to a neutral model via an R^2 value (github.com/marcjwilliams1/neutralitytest). Samples were eligible for analysis if they met criteria dictated by the original neutral evolution study such that they contained ≥12 subclonal mutations within diploid regions with coverage depth ≥10 and at least 3 supporting reads. SNVs were limited to the range of variant allelic frequency (VAF) between 0.12 and 0.24 to select only subclonal mutations per Williams et al. [7]. Application of these criteria left 431 samples (57.3%) eligible for the proposed analysis. Utilization of the *neutralitytestr* algorithm then yielded 114 (26.5%) neutral and 317 (73.5%) non-neutral tumors. In pairwise association analysis, neutrality was not significantly associated with any known drivers [10], canonical translocations, nor sample purity nor coverage after false discovery rate correction (**Supplementary Table 1**). To determine whether neutrality affected clinical outcome, we then ran univariate analysis for neutral vs non-neutral tumors and found no significant difference in disease progression (p=0.528) or overall survival (p=0.796). This was further confirmed using a cox multivariate model including age, stage (ISS), treatment effect (bortezomib, carfilzomib, and lenalidomide) and high-risk features of deletion or mutation of *TP53*, 1q gain, and MAF translocations (**Supplementary Fig. 1**).

Neutral evolution has been hotly debated since its first description [11-13], albeit not in multiple myeloma due to lack of representation in the TCGA. Rather than comment on theorem, we note issues with current methods for assigning neutrality in multiple myeloma. One concern is the use of VAF to surmise subclonality without correction for sample purity and without reconstruction of each patient's tumor phylogeny. To gauge the impact of this shortcoming, we used *phyloWGS* with the full catalogue of SNVs and copy number data to reconstruct phylogenetic trees for each patient. We could then subdivide mutations into clonal and subclonal fractions by setting a cancer cell fraction (CCF) threshold of 0.9; below which SNVs could reliably be designated subclonal in this population per the observed density of CCFs [14] (**Supplementary Fig. 2**). We then filtered SNVs in line with neutrality analysis criteria and evaluated the level of selection among subclonal mutations using dN/dS ratios with the *dndscv* package [15]. This well-established method of inferring the presence of selection measures the ratio of nonsynonymous to synonymous mutations (normalized by their mutation likelihoods). Performing driver discovery on all subclonal variants in the 114 neutral tumors, corrected and uncorrected for CCF, revealed the presence of 9 and 10 driver mutated genes, respectively, at $q<0.05$ (**Fig. 1A**; **Supplementary Fig. 3**). Then, considering only the CCF-based subclonal mutations for the entire cohort, we again ran *neutralitytestr* and for 655 eligible samples, only 12 (1.8%) were called neutral (see example in **Supplementary Fig. 4**). The existence of strongly selected mutated driver genes and the absence of neutrality using CCF-subclonal variants casts doubt on the accuracy of this algorithm for defining neutrality in multiple myeloma.

An idiosyncrasy of multiple myeloma is early identification of precursors by virtue of a surrogate monoclonal protein. With early diagnosis comes the ability to serially analyze samples across disease phases. Because neutrality requires that malignant transformation begin with emergence of the most recent common ancestor, we next investigated the timing of acquisition of clonal events in plasma cell neoplasms spanning progression. We first examined a published cohort of 35 WGS from 25 patients with newly diagnosed multiple myeloma and precursor disease [2, 4]. 10 cases had paired, serial smoldering multiple myeloma (SMM) and MM samples. Among all samples, 4 were called neutral by *neutralitytestr* using the original parameters (11.4%): 2 SMM and 2 MM. For one neutral SMM case, PD26401a, the subsequent MM sample at progression was called non-neutral. This change was inconsistent with the evolution of the tumor which progressed to MM without emergence of new drivers (i.e. static evolution). For a second "neutral" SMM case, PD26402a, there was clear evidence of selection with eventual dominance at MM progression of a *MYC*-translocated subclone [4]. For one of the newly diagnosed MM tumors classified as neutral (PD26419a), there were sufficient large chromosomal gains to run a molecular time analysis in which the relative order of acquisition of driver events can be measured with respect to chromosomal gains [10]. This case was demonstrated to have clonal gains and complex events across distinct time windows (**Fig. 1B**) in relative contrast to what would be expected for neutral tumors, where the full complement of driver events is assumed to have accumulated by the time of transformation.

As we saw evidence of selection among subclonal mutations and between serial samples in purportedly neutral tumors, we sought further evidence of selection across all

preceding phases of tumor evolution to corroborate the universal presence of Darwinian forces. We applied a molecular time analysis to the MMRF cohort of 114 neutral tumors to examine the relative timing of acquisition of clonal mutations in known myeloma driver genes [10]. We observed driver genes among 15 neutral cases to have been acquired in different time windows, by virtue of their duplication or non-duplication with large gains of chromosomes on which they reside, (**Fig. 1C**) as direct evidence that they were acquired across multiple windows of selection in myelomagenesis.

Finally, we applied the most recent Bayesian model for neutral evolution developed by Williams *et al* to determine if a more refined neutrality algorithm, based on Bayesian statistical inference framework rather than a frequentist approach, would yield similar results (github.com/marcjwilliams1/ApproxBayes.jl) [8]. Importantly, the framework incorporates both selection and neutrality and determines which best fits the sequencing data. Only 11 of 662 eligible tumors (1.7%) fit the updated neutral evolution model and, of note, only 1 of those patients overlapped with the 114 patients called neutral by the earlier iteration of the model. This suggest that though some tumors may independently meet criteria for neutrality (i.e., R^2>0.98), evolutionary trajectories can be better explained by models including positive selection. Similarly, no significant difference in PFS nor OS was apparent in neutral tumors vs. non-neutral tumors.

Independent groups have characterized the progression of plasma cell neoplasms and observed that a significant portion of smoldering myelomas have already undergone clonal and subclonal selection at time of sampling, and that this relative clonal architecture is preserved at time of subsequent progression or sample collection (i.e. static evolution) [4]. Other neoplasms were seen to evolve via the emergence of new drivers under strong

selection that owed to clonal sweeps (i.e. branching/dynamic evolution) [4, 5]. With evidence here of selection at all phases of myelomagenesis and across serial samples, acknowledging potential under-representation of diversity due to inherent under-sampling of spatially heterogeneous sites, these patterns are more likely explained by punctuated evolution [16, 17]; characterized by the emergence of new subclones and selection events or sweeps; with subsequent, alternating expansion under a static model (**Fig. 2**). Though both static and neutral evolution are explained by expansion and retention of a complement of subclones, a key difference is that the neutral model assumes that all drivers are present at malignant transformation while static growth is inclusive of alternating periods of positive selection irrespective of clinical delineation of premalignant and malignant phases. Also worthy of distinction, the static model allows further drift of cancer cell fraction as more advantageous subclones among the complement are preferentially selected. Furthermore, spatial heterogeneity among disparate tumor sites has been multiply demonstrated and neutrality is unable to account for positive selection in early branching for vastly different complements of structural, copy number, and single nucleotide variants from a common ancestor to the seeded locales [18, 19].

In summary, though neutral mutations may well exist in some tumors, we show that there is a lack of evidence to support neutral evolution as a dominant evolutionary force in the most extant fraction of multiple myeloma genomes with contemporary methods. Overall, the evolutionary trajectory of multiple myeloma is decidedly Darwinian; shaped by the positive selection and subsequent expansion of diverse clones in virtually all patients. These punctuated episodes are seen to be followed by time windows of static tumor expansion. Serial sequencing data has shown that after the acquisition and

selection of a full complement of drivers, certain tumors can progress statically until clinical detection without the acquisition of new drivers. This finding has particular clinical relevance as many multiple myelomas have a similar genomic landscape as their smoldering counterparts, offering a window into prognostication and strategies for early intervention.

Data availability

Sequence files are available at the European Genome-phenome and dbGaP archive under the Accession codes: EGAD00001003309: 67 WGS data from 30 multiple myeloma patients; phs000748.v1.p1: Whole exome sequencing (WXS) and low coverage/long insert WGS sequencing data from 746 newly diagnosed multiple myeloma patients included in this study (CoMMpass trial; IA 15)

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Author contributions

FM and OL designed and supervised the study, collected and analyzed data and wrote the paper. BD collected and analyzed data and wrote the paper. KHM, VY, ER, MM, MK, GM collected and analyzed the data.

Conflicts of interest

Dr. Landgren's disclosures:

- Grant support: NIH, FDA, MMRF, IMF, LLS, Perelman Family Foundation, Rising Tide Foundation, Amgen, Celgene, Janssen, Takeda, Glenmark, Seattle Genetics, Karyopharm
- Honoraria/ad boards: Adaptive, Amgen, Binding Site, BMS, Celgene, Cellectis, Glenmark, Janssen, Juno, Pfizer
- Independent Data Monitoring Committee (IDMC): Takeda, Merck, Janssen, Theradex

Figure Legends:

Figure 1. Subclonal mutations under selection and clonal event acquisition across multiple time windows in purportedly neutral myeloma tumors. **A)** Subclonal mutations, identified via cancer cell fraction, classified as drivers under positive selection by *dndscv* in neutral MMRF CoMMpass tumors (n=114). Top panel shows number of cases, middle shows boxplots of cancer cell fraction, and bottom panel shows the dN/dS ratio for each of missense or truncating variants with 95% interval of confidence represented as vertical lines. This analysis did not restrict mutations to diploid regions. All q values for dN/dS ratios were <0.05. **B)** Molecular time plot for a newly diagnosed multiple myeloma whole genome demonstrating large clonal copy number alterations acquired across two distinct time windows (horizontal line) demonstrating selection at multiple phases of the evolutionary trajectory. LOH = Loss of heterozygosity. Two gains in chromosome 1 are concurrently acquired in the same window. **C)** Clonal driver mutations in purportedly neutral tumors from the MMRF cohort (n=121) acquired across time windows and phases of disease evolution by virtue of duplication or nonduplication with large duplications of chromosomes on which they reside (15 of 121 samples).

Figure 2. Model for evolutionary trajectory of multiple myeloma. Punctuated evolution (vertical dashed lines) heralds the emergence of new subclones that may sweep to dominance. Static evolution (horizontal dashed lines), wherein a complement of existing subclones expands while under positive selection and drift of cancer cell fraction, alternates between. In this case, the full complement of mutations necessary to confer

malignant potential is present at time of smoldering myeloma diagnosis. The tumor then expands statically until clinical significance.

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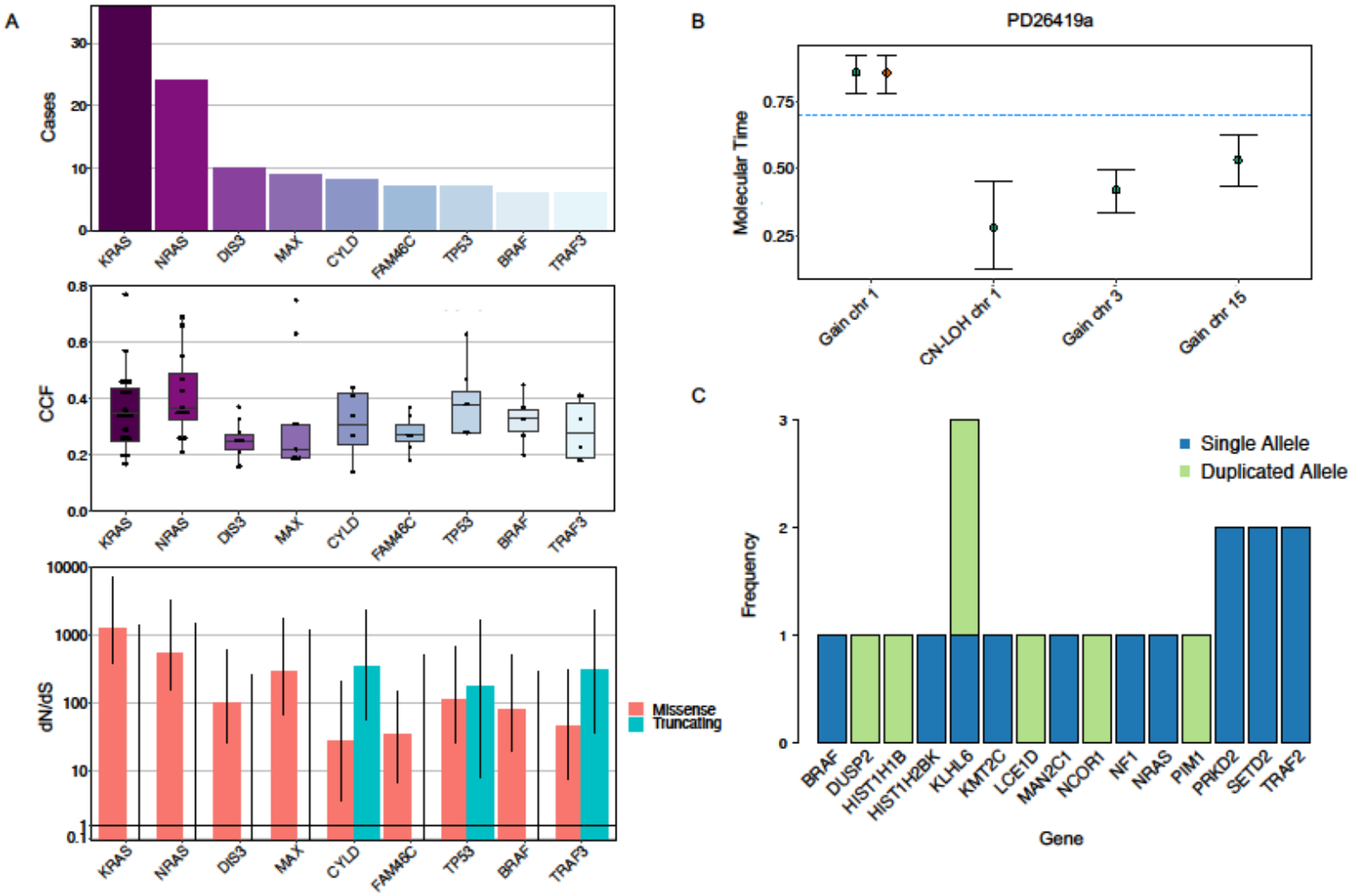
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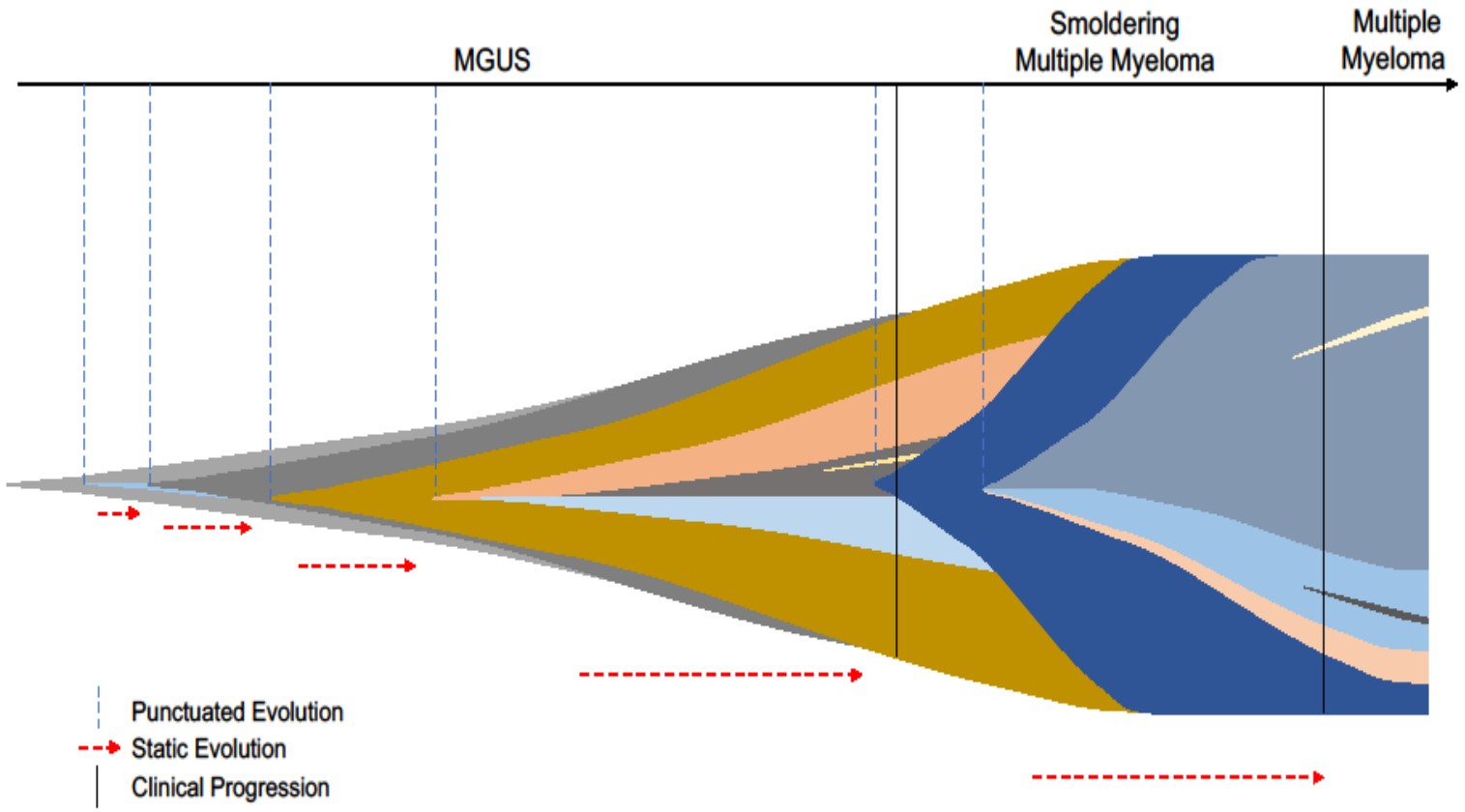
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Smoldering
Multiple Myeloma
Multiple
Myeloma
Punctuated Evolution
Static Evolution
Clinical Progression