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The Roles of Initiating Truncal Mutations in Human Cancers: The Order of Mutations and Tumor Cell Type Matters

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Summary

We propose that initiating truncal mutations play a special role in tumor formation by both enhancing the survival of the initiating cancer cell and by selecting for secondary mutations that contribute to tumor progression, and that these mutations often act in a tissue-preferred fashion. Here, we explain why inherited mutations often have different tissue specificities compared to spontaneous mutations in the same gene. Initiating truncal mutations make excellent neo-antigens for immunotherapy, and understanding why one mutation selects for a second mutation in a particular tissue type could one day aid in the design of gene-targeted combination therapies.

Keywords

tumor evolution; initiating truncal mutations; inherited mutations; mutational order; tissue-specificity

Perspective

Most cancers develop over time, accumulating a series of mutations that combine to produce a malignant tumor. As a consequence, most cancers in humans occur later in life, with a steep increase in their frequencies approximated by the binomial equation $I(t)=kt^{r-1}$, where I is the incidence as a function of time, k is a constant that depends upon the life span of the species, t is time and r is a function of the number of mutations it takes to form the cancer (Armitage and Doll, 1954). If the first mutation in the series is inherited, the curve giving the

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Author Contributions

AJL, NAJ and NGC discussed the ideas presented here and wrote the paper.

Declaration of Interests

AJL is a founder and member of the board of directors of PMV Pharmaceuticals, which develops small molecules that are structural correctors for different mutant p53 alleles. He is also a member of the board of Adaptive Biotechnologies, which develops diagnostics for measuring minimal residual disease and identifies B and T cell receptor sequences.

NAJ and NGC declare no competing interests.

age of the onset of tumors in a population shifts to the left or younger ages, indicating the critical role of r or mutation number. As mutations accumulate, they frequently occur in different functional pathways that regulate genomic stability, metabolic processes, cell cycle regulation, epigenetic stability, immunological detection, etc., in a list that has come to be recognized as the hallmarks of cancer (Weinberg and Hanahan, 2000, 2011).

The first recognition that the order of mutations occurring in a cancerous precursor cell was an important variable and even determined the nature of the subsequent mutations that gave rise to the cancer was appreciated by Bert Vogelstein and colleagues in a series of papers studying colorectal cancer (CRC) in humans. Vogelstein's laboratory took advantage of the fact that this cancer develops in stages over time, starting with benign small-sized polyps, which, with further time, develop into larger benign polyps and eventually malignant colorectal carcinomas (Nigro et al, 1989; Baker et al, 1990; Baker et al, 1990). By taking samples from these different stages using colonoscopy and sequencing the DNA, they appreciated that CRC develops through the stepwise selection of genetic changes. The initiating or gatekeeping mutation was primarily biallelic loss of *APC*, which occurs in >80% of cases, or at a lower frequency the b-catenin gene (*CTNNB1*) in the same WNT signaling pathway. Subsequent activating mutations in *KRAS* were identified in early to intermediate adenoma stage tumors, while loss of function mutations in *SMAD4* were identified in large adenomas. Finally, the loss of p53 functions resulted in a malignant adenocarcinoma. Large-scale CRC genome sequencing studies also identified numerous infrequently mutated genes in CRC, in addition to the commonly mutated *APC*, *KRAS*, *SMAD4* and *TP53* genes (Cancer Genome Atlas Network, 2012; Seshargiri et al., 2012; Giannakis et al., 2016). The existence of large numbers of infrequently mutated genes has made it a challenge to sort out driver mutations from passenger mutations for CRC. These studies demonstrated that a common set of genes, in a common set of pathways, harbored mutations that caused this cancer. More interestingly, the order in which the major genes driving CRC were mutated appeared to be fixed, with *APC* first, *RAS* second, *SMAD4* third and *TP53* last. Based upon these findings, *APC* was termed a gatekeeper gene and, more recently, it has come to be called a truncal initiating mutation, recognizing it as the base of an evolutionary tree of cancer formation (see below). When it became possible to create three-dimensional organoids in cell culture that reproduced the development of colorectal cancer in vivo, these same mutations could be created in normal organoids in different orders and combinations, experiments that again demonstrated the same required order of these mutant genes in vitro (Matano et al, 2015). These results were also consistent with the fact that individuals that inherited an *APC* mutation developed colorectal tumors at earlier times and greater frequencies than individuals with spontaneous mutations in *APC* (Kinzler and Vogelstein, 1996, Cell). They were also consistent with studies showing that individuals that inherit *TP53* mutations, rarely, if ever, develop colorectal cancers over their lifetime, but do develop lots of other tumor tissue types (Levine et al, 2016).

Until fairly recently it was generally assumed that cancer evolves through a linear stepwise selection of genetic changes. This thinking was radically altered in 2012 when Gerlinger et al. (2012) showed that clear cell renal carcinoma evolved through branching tumor evolution, similar to Darwin's iconic evolutionary tree. Subsequent work by many laboratories has now shown that many, if not most, solid cancers evolve through branching

evolution. Unlike linear tumor evolution, branching tumor evolution creates extensive intra- and inter-tumor genetic heterogeneity and is the likely reason for the extensive heterogeneity seen in CRC. This work led to the notion of truncal mutations, which are mutations that are present at the trunk of the cancer evolutionary tree.

This body of work suggested that the nature of the first mutation in a cancer was important for several reasons. First, it guided, or more likely selected for subsequent mutations in an order that led to the rapid and optimal development of a tumor. Second, by selecting for mutations in four distinct signaling pathways [WNT, RAS, TGF-b (SMAD4), p53], four different functional aspects of tumor development, including metabolic processes and the cell cycle, the extracellular matrix and signaling, and genomic instability, were all altered, but in a specific order and sets of combinations. This hypothesis, that the order in which cancer driver mutations develop in a cell can influence the timing and phenotype of a tumor, helps to explain why an inherited *APC* mutation commonly results in a colorectal cancer, but an inherited *TP53* mutation does not, even though 93% of individuals with *TP53* mutations will develop one to twenty tumors over their lifetime (Levine et al, 2016).

Rather clear proof that the order of addition of the four major mutations driving CRC is important for the timing of CRC development was confirmed using mouse models (Takeda H. et al. 2015). These models used *Sleeping Beauty* (SB) transposon-based insertional mutagenesis to induce gastrointestinal (GI) tract tumors in wild-type mice, or mice that carried heterozygous inactivating mutations in *Apc*, *Smad4* (to activate TGF-b signaling) or *Trp53*, or an activating mutation in *Kras*. The value of these mouse models was that it was possible to change the nature of the initiating truncal mutations at will. Not surprisingly, due to SB mutagenesis, all of the mice developed GI tumors. Strikingly, the mean survival times of the mice reflected exactly the order in which the sensitizing mutations act in human CRC, with *Apc* mutant mice dying first, *Kras* mutant mice second, *Smad4* mutant mice third, *Trp53* mutant mice fourth and wild-type mice last. In addition, >80% of the tumors in wild-type, *Apc*, *Kras* and *Trp53* mutant mice had inactivating SB insertions in *Apc*. This is consistent with *APC*'s gatekeeping or initiating/truncal role in human CRC. Even when gastrointestinal tumors were initiated by mutations in genes other than *Apc*, there was still a strong selection for inactivation of *Apc* and increased WNT signaling.

There was, however, one mouse model where *Apc* was not highly mutated during tumor formation and that was the *Smad4* mutant mouse model. In these mice, only about one third of the tumors had inactivating SB insertions in *Apc*. These findings suggested that another gene in the WNT signaling pathway might be providing the initiating or truncal functions normally provided by *Apc* in *Smad4* mutant mice. This hypothesis turned out to be correct, as nearly two thirds of *Smad4* mutant tumors had activating SB insertions in R-spondin 1 (*Rspo1*) or *Rspo2*. The four R-spondin ligands (RSPO1–4) act as enhancers of WNT signaling. They bind to their cognate receptors LGR4, LGR5 and LGR6 via their furin-like 2 domain, and to the E3 ubiquitin ligases ring finger protein 43 (RNF43) and zinc and ring finger protein 3 (ZNRF3) via their furin-like 1 domain. This tripartite interaction prevents WNT receptor degradation mediated by RNF43 or ZNRF3. Similarly, the ~10% of human colorectal cancers that lack *APC* mutations carry chromosomal translocations involving *RSPO2* or *RSPO3* and also show decreased or mutant *SMAD4* (Cancer Genome Atlas

Network, 2012; Seshagiri S. et al, 2012). Having a preexisting mutation in a TGF-β signaling pathway gene therefore appears to have changed the system properties of tumor cells in such a way that mutations in *APC* are no longer the preferred route for WNT pathway activation. Although it is not clear why this occurs, it has been known for a long time that there is extensive cross talk between the TGF-β and WNT pathways (Guo and Wang, 2009). For example, it is known that TGF-b and WNT reciprocally regulate their ligand production, which is critical for establishing extracellular gradients of these morphogens. In the nucleus, SMAD-β-catenin-LEF protein complexes also regulate a number of shared target genes, often in a synergistic manner. Finally, cytoplasmic interactions between components of these pathways have also been observed and are thought to represent new mechanisms for fine-tuning their respective signaling pathways.

Smad4 mutations also predispose to gastric tumors in the upper gastrointestinal tract of mice. Examination of gastric tumors produced by SB in *Smad4* mutant mice showed, once again, that only a minority (16.7%) of tumors had inactivating SB insertions in *Apc* (Takeda H. et al. 2016). In light of this finding, it was surprising that activating mutations in *Rspo1* and *Rspo2* were also not observed in gastric tumors. Instead, insertional mutations were identified in other upstream WNT signaling components, including *Lrp6*, which functions as a coreceptor for WNT signal transduction. These results suggested that *Smad4* mutant gastric tumors had other preferred methods for fine-tuning their WNT signaling pathway and show how much tissue type maters during tumor evolution. These results also have clear therapeutic implications for those who might want to use pathway-targeted drugs to treat cancer.

Thymic lymphomas arising in p53 knockout mice (*Trp53*$^{-/-}$) also show that the order of mutations matters. In these mice, inactivating mutations in *Pten* were found to occur first, followed by amplification of Cyclin D (*Ccnd1*) and *Cdk6* and finally mutations in Ikaros (*Ikzf1*) (Dudgeon et al, 2014). When a T cell is transformed in these mice, it replicates and increases in cell number, and can be identified by its T cell receptor beta-chain sequence. In wild-type C57BL/6 mice, the largest T cell clones (with identical TCR sequences) detected in the thymus represented about 0.01–0.05% of the total thymic T cell population. In *Trp53*$^{-/-}$ mice, clones of this size were observed during embryonic development and up until nine weeks after birth, when T cell clone sizes representing 2–20% of the T cell population were first observed. These expanded clones formed transplantable tumors. Because it took nine weeks after birth to observe a transformation event, it is likely that additional mutations in addition to the *Trp53* inherited mutation must occur to produce a tumor. Consistent with this, when tumors were sequenced, *Pten* deletions, along with *Ccnd1*, *Ccnd2*, *Ccnd3* and *Cdk6* amplifications, and mutations in *Ikzf1*, were observed (Dudgeon et al, 2014).

In any single *Trp53*$^{-/-}$ mouse, multiple independent transformed T cell clones with different TCRs were observed by nine weeks after birth. These clones then competed so that many fewer clones were present at 20 weeks after birth and all the remaining clones had the same four driver mutations. In the multiple different clones of transformed cells from an individual mouse, all *Pten* deletions were identical, but different mice harbored different *Pten* deletions. The simplest explanation for this observation is that the *Pten* deletion occurs first in *Trp53*$^{-/-}$ mice and was selected for early in development before the VDJ

rearrangements took place, and that the clone then expanded and was selected for in the precursor T cell population of an individual mouse. *Pten* mutations happen independently in different mice so that tumors from different mice have a different *Pten* deletion, but in any one mouse's tumors with diverse TCR clones, all have the same *Pten* mutation. The *Trp53* mutation is associated with genomic instability and the *Pten* deletion selects clones that enhance the PI3K/RAS pathway and lead to greater glucose utilization. These expanded clones then undergo VDJ rearrangements followed by amplification of one or more *Ccnd* and *Cdk6* genes, enhancing cell cycle replication. Finally, an *Ikzf1* mutation interrupts T cell development and double positive $CD4^{+}/8^{+}$ progenitor T cells are then produced as the transformed cell product. Much like mouse models of colon cancer, where *Apc* is the initiating truncal mutation, here, the *Trp53* mutation is the first mutation, followed in a fixed order by additional mutations in *Pten*, *Ccnd1/2/3*, *Cdk6*, and *Ikzf1*. Once the initial truncal mutation is fixed in a T cell clone, the simplest explanation for an ordered set of further mutations is that the prior mutation selects for the next mutation, providing enhanced cell division or survival.

There are a number of other examples in humans where initial truncal mutations are observed as the first mutations that may lead to the development of a cancer. Clonal expansion of hematopoiesis has been reported in precursors of the myeloid series of cells as humans get older. From the ages of 60 (1/10 people) to 80 (7/10 people) years, increased levels of myeloid cells have been observed in the blood in individuals and these lineages have been associated with mutations in epigenetic regulators (*DNMT3A*, *TET2*) and the p53 pathway (*TP53*, *PPMID*, *ATM*, *CHEK2*) (Bowman et al, 2018). Evidence from lineage tracing experiments demonstrate that some of these initial truncal mutations in pre-leukemic cells occur in myeloid stem cells that then acquire additional mutations (*KRAS*/*NRAS*, *FLT3*), which can lead to acute myelogenous leukemias (AML) (Corces-Zimmerman et al, 2014). Gammaglobulinopathies (Kyle et al, 2018), composed of enlarged numbers of B cell clones with the same B cell receptors in an individual, and smoldering myelomas (Kyle et al, 2007), are two other examples of cells harboring possible truncal initial mutations, where benign clones are waiting for an additional mutation and where selection will lead to the expansion of clones and malignant transitions.

Similarly, evidence from lineage tracing experiments in human AML has demonstrated that mutations associated with the pre-leukemic phase of the disease occur in hematopoietic stem cells. These mutations occur in "landscaping" genes (*ASXL1*, *DNMT3*, *IDH1*, *ISH2* and *IKZF1*) involved in global chromatin changes, such as DNA methylation, histone modification, chromatin looping, and impact the epigenome (Corces-Zimmerman et al, 2014), while genes mutated at the transition between pre-leukemia and leukemia (*KRAS*/*NRAS* and *FLT3*) increase proliferation or inhibit differentiation and occur in multipotent progenitor cells.

These experimental observations demonstrate that several different truncal mutations (*DNMT3A* and *TET2*, or *PPMID*, *TP53*, *ATM*, *CHEK2*) in a stem or progenitor cell in the myeloid series, for example, can produce the same phenotype, benign hyperplasia. Indeed, changes in either of two different pathways (epigenetic or p53) can produce this phenotype. This demonstrates that in some cases, several different truncal or initiating mutations can

give rise to enhanced fitness in a particular developmental lineage. It should be noted, however, that the great majority of individuals carried a mutation in only one of these genes (*DNMT3A*) and mutations in even one allele of this gene was sufficient to increase levels of myeloid cells in the blood. It is clear that these mutations are not inherited in this population and that the presence of these mutations in the myeloid series are associated with increased aging as well as prior radiation exposures and chemotherapy, smoking, or exposures to mutagens. These diverse mutations or even combinations of these mutations do lay the groundwork for the selection of additional mutations (*KRAS*, *NRAS*, *FLT3*) that can result in AML. AMLs with *TP53* mutations make up ~25% of AML leukemia and have different properties, progressions, responses to treatments (Levine, 2017) and outcomes compared to the other 75% of the AMLs without *TP53* mutations. This is a good example of how different truncal mutations can produce similar benign progeny but the development of a neoplasia from these precursors result in very different cancers. Even though several different genes can function as a truncal or initiating mutation, the resultant cancers produced by the selection of additional mutations are influenced by the nature of the truncal mutation. Rather clearly, the nature of the truncal gene, the order in which the mutations occur and the developmental lineage in which the mutations occur, all influence the nature of the cancer produced.

Similar observations have also been recently reported for esophageal epithelium (Martincorena et al, 2018). In this study, DNA sequencing was used to look for mutations in normal esophageal epithelium of nine donors with an age range of 20 to 75 years. While somatic mutations increased with age, strong positive selection of clones carrying mutations in 14 different genes normally associated with cancer was also observed. In middle-aged (30 – 50 years) and older (60 – 90 years) donors, clones with cancer-associated mutations covered much of the epithelium, with *NOTCH1* and *TP53* mutations affecting 12 to 80% and 2 to 37% of the cells, respectively. Here, again, a number of different truncal or initiating mutations could promote clonal expansion of stem or progenitor cells, but two genes, *TP53* and *NOTCH1*, were the most common truncal mutations.

A rather dramatic impact upon clinical features, the responses to therapy, the biology of stem and progenitor cells and even clonal evolution, has also been observed in myeloproliferative neoplasms, depending on whether a mutation in *TET2* occurs before a mutation in *JAK2* or vice versa. Rather clearly, the order in which these two mutations occur influences the evolution and even the optimal treatment of these tumors (Ortmann et al, 2015; Kent and Green, 2017). When the genetic (*JAK2*) or the epigenetic (*TET2*) mutation is the initial truncal mutation, they each have very different impacts upon the gene expression of the progenitor cell, so that the second mutation results in quite different properties of the cancer. In this case, cancers with the same driver mutations behave differently, depending upon the order of their occurrence in a transformed clone.

Recent studies have also demonstrated that loss of chromosome 3p and gain of 5q are early events in clear cell renal cancers. Loss of 3p is the critical event for >90% of patients. The deleted region always encompasses four tumor suppressor genes that are frequent targets for inactivating mutations on the other chromosomal copy: *VHL* (point mutations in 60%−70% of patients; epigenetic silencing in a further 5%−10%), *BMPR1A* (40%), *BAP1* (10%) and

SETD2 (10%). In this example, secondary mutations are strongly selected due to the nature of the initial 3p deletion event. These events are predicted to occur by chromothripsis during childhood or adolescence, and 30–50 years before kidney cancer is diagnosed (Mitchell et al, 2018; Turajlic et al, 2018). Modeling of early clonal dynamics predict that clonal expansion after 3p loss is not large and only encompasses a few hundred cells with the potential to initiate a future kidney cancer if a mutation in the wild type *VHL* allele is acquired.

Indeed, over a lifetime, a large number of initiating truncal mutations are likely to have occurred and become fixed in a wide variety of tissues. While these mutations may produce local clonal expansions, the cells in most cases remain as benign progenitor cells. A particularly clear example of this is a study where ultra deep sequencing of 74 cancer genes was performed on 234 biopsies of sun-exposed normal skin taken from the eyelids of four individuals (Martincorena et al, 2015). The mutational burden of sun-exposed skin was calculated to be 2–6 mutations per megabase per cell. Positively selected cancer driver mutations in genes like *NOTCH1* were detected in 18–32% of the normal skin cells, while variability in clonal expansions and driver mutations were observed between individuals. Sun-exposed skin, with normal epidermal functions and characteristics, upon aging and exposure to mutagens, appears to be composed of a clonal patchwork of cells harboring initiating truncal mutations, some of which will predispose to skin cancers, such as squamous cell carcinomas, later in life. Based upon all the evidence to date, it is clear that initiating truncal mutations that will lead to cancers rapidly are tissue type-dependent and likely even tissue progenitor type-dependent.

The idea that a truncal initiating mutation can dictate, through selection, a set of subsequent mutations that leads to cancer, provides a reasonable explanation for several observations that have never had adequate solutions in the past. The first of these is that for many different tumor suppressor genes, the types of tumors that are produced when mutations are inherited can be very different from the tumors that have high spontaneous mutation rates in these same genes. For example, inherited *TP53* mutations in Li-Fraumeni patients give rise to medullary blastomas, choroid plexes carcinomas, adrenocortical carcinomas early in life, rhabdomyosarcomas and osteogenic sarcomas at young ages, and liposarcomas and breast cancers at mid-life. Rarely does a Li-Fraumeni patient develop ovarian, colorectal, pancreatic or lung cancer. Yet, spontaneous *TP53* mutations are seen in 100% of ovarian, 80% of colorectal, 75% of pancreatic and 60–75% of lung cancer (Levine et al, 2016). In addition, the breast cancers in Li-Fraumeni female patients are commonly ER-positive, while in the population that does not inherit *TP53* mutations ER-positive breast tumors rarely have *TP53* mutations. Rather, *TP53* mutations are common in triple negative breast cancers, which are rarely observed in Li-Fraumeni patients. Indeed, the excess risk of a Li-Fraumeni patient developing a cancer, compared to the risk in the normal population, is 100–1,000 times higher in ectoderm and mesoderm derived cell types, while only 2–4 times higher in endodermal cell types (Levine et al, 2016). This may be due to the observation that *TP53* does not seem to function as a truncal initiating mutation in endodermal-derived cells [prostate, ovarian, pancreatic, colon and non-small cell lung adenocarcinomas (NSCLC)], while it might be an initiating truncal mutation in some ectodermal and mesodermal tissues. A similar argument can be made for the retinoblastoma gene and protein, which is an

initiating or truncal mutation (inherited gene mutation) in retinal cells and osteoblasts, but not in other tissues, where spontaneous mutations are found at high levels in many tumor types. It is clear from these observations that organ and tissue specificity influence whether mutations are initiating truncal mutations in that cell type. We will need to understand what properties of a tissue or cell type act upon a gene product and a mutation so that it functions as a cancer initiating mutation in one cell type while delaying that path or producing a benign tumor in another cell type.

The concept of an initiating truncal mutation demonstrated by experiments reviewed here not only helps to explain several observations about tumor suppressor genes that appeared enigmatic in the past, but gains new importance with the demonstrated role of the immune system in regulating and controlling cancers. Cancers with multiple mutations clearly arise over time and at least in some tumors, the prior mutation(s) selects for the next mutation in a clone, even though mutations arise in a quasi-random fashion across the genome. That means that most tumors are composed of a mixture of clones with diverse mutations. While the driver mutations are essential for fitness and some combinations eventually dominate a tumor, genetic heterogeneity is always present. $CD8^+$ T cells recognize a subset of antigens that arise from these mutations (neo-antigens) and may kill cancer cells and control tumor growth. The tumor may escape T cell cytotoxicity by expressing proteins that prevent T cell expansion and tumor regulation, such as checkpoint proteins programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). In some cases, every cell in a tumor has an initiating truncal mutation contributing to the growth of the tumor and it can form a neo-antigen recognized by $CD8^+$ T cells. These initial truncal mutations, present in all tumor cells, would provide an excellent neo-antigen for the immune system to react with and kill the tumor. That is not to say that the second, third or fourth neo-antigen to be selected will not also function to bring the immune system into the tumor and result in its control. The goal, however is to determine from the truncal initiating mutation, the nature of the selection pressures exerted by this first mutation so that we can identify the neo-antigens that will be expressed by a tumor. In many cases, a tumor's path to malignancy may be predictable, at least for immunotherapy, from knowing the truncal mutation. We will also need to understand if the immune system responds to an inherited truncal mutation differently than the same spontaneous mutation in a cancer cell because of immune tolerance.

There are already a number of algorithms that predict neo-antigens from the DNA sequences of a tumor and the normal tissues, the HLA types, and the microbiome (Balachandran et al, 2017), and are tested against the results (overall survival) of checkpoint therapies. It is of some interest that these algorithms can predict neo-antigens in long-term cancer survivors that have not had checkpoint therapies (Luksza et al, 2017). In particular, one of the neo-antigens in the tumor of a long-term survivor was $p53^{E191K}$, a mutation in the *TP53* gene. We must now bring together our knowledge of truncal initiating mutations, selective forces placed upon developing cancers and pathways of mutations that arise in cancers, with the development of neo-antigens and the immune response to a cancer and understand these complexities in every tissue type.

The hypothesis of initiating truncal mutations that expand the clones of stem or progenitor cells helps to explain the observations of intra-tumor heterogeneity and branched evolution in cancers where second or third mutations during tumor development occur in different lineages derived from the expanded clone with the truncal mutation. When the second or third mutations differ in different lineages giving rise to the heterogeneity in a tumor, this suggests random mutations and weak selection pressures for increased fitness. However the observation that convergent evolution of different lineages then occurs in cancers demonstrates an over-riding selection for enhanced fitness of tumor growth (Gerlinger et al, 2012). These competing forces give rise to the observed heterogeneity of different cancer cells in a tumor even though there are often some dominant clones. The demonstration of competing clones or lineages of cancer cells was clearly observed when *Trp53* was the inherited truncal mutation in mice where there was greater diversity of independent T cell thymic lymphomas at nine weeks after birth than at 20 weeks after birth where the fewer clones of cells that remained were often dominated by one or a few clones that had both the truncal mutation (*Trp53*) and a common second (*Pten*), third (*Ccnd1/2/3*, *Cdk6*) and forth (*Ikzf1*) set of mutations (Dudgeon et al, 2014).

When using the mathematics of real numbers, a times b always equals b times a, which is termed commutativity. When the mathematics moves into higher dimensions (matricies), the order of the components being multiplied becomes important and the mathematics becomes non-commutative, so that ab does not equal ba, just as we observe with mutations in some cancers. Perhaps, the use of non-commutative mathematics will shed light upon or even predict the outcomes of cancers or what drug combinations to employ in which tissues, once we know the order of the mutations. Hopefully, this at least will define the problem. The next generation will have to solve it.

Finally the question should be asked whether or not all cancers arise by the appearance of a truncal or initiating mutation in a stem or progenitor cell of a specific tissue that expands the clone producing a benign tumor that selects for additional mutations improving the fitness of these clones. While many examples of this process are discussed here it is difficult to prove this assertion for all cases. It certainly remains possible that given enough time a random order of mutations can produce a cancer. If one is looking for these examples, older patients may be a good source. It is clear that inherited mutations in selected genes that increase the excess risk of cancers in selected tissues are by definition truncal mutations that begin the process of cancers at younger ages. Improvements in single cell DNA sequencing of normal human tissues as a function of the topology of tissue types, age, environmental exposures, etc. have already uncovered expanded clones in normal tissues with potential truncal mutations (Martincorena, 2015, 2018). Improvements in the methodology of detecting and creating the evolutionary trees of mutations that gave rise to a cancer and to the responses of a cancer to treatment may permit a test of the ideas proposed in this perspective. Certainly, we need to isolate and identify the stem and progenitor cells of the body and monitor them as a function of age. The ideas presented here predict that stem cells in the body will over time and division acquire mutations that will give them selective advantages, so that the heterogeneity of stem cell populations should decline with age and selected mutations should take over the population and even expand the population numbers with age. Even if the population is expanded in size it may not function well to produce differentiated cells of

the lineage. The selection is for fitness, division and survival, not necessarily for regeneration of a tissue. Matching initial or truncal mutations with tissue specific stem or progenitor cells will be one avenue for future experiments. As we develop technologies to monitor the changes in stem cell populations with aging we might be able to interfere with the development of cancers prior to their initiation or progression. It is hoped that these ideas will move the field in new directions.

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