CancerLogic

Mutations, Clones, Singularity, Stem Cells and Strategy

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Contents

(For a non-technical introduction, see The Nature of Cancer. Note also a companion article,

*CancerChallenge, that calls for an international dedicated Institute to realise the approaches analysed in **this** paper and makes specific proposals about organisation and finance*.)

Abstract

It has been well understood for more than sixty years that cancer is a disease of multiple mutations: the search for how those mutations produce their effects has given us our detailed knowledge of cellular controls. For some ten years we have known that in a typical cancer there are thousands of mutations scattered over the genome rather than just a handful, creating new difficulty and complexity but also simplifying concepts enormously because we now have in principle a means of exactly identifying the cells of each individual cancer - suddenly, driver mutations, oncogenes and stem cells seem less important.

Cancers are clonal. The existence of stem cells or 'selfies' complicates the issue but there must be a mutation set defining an original malignant clone and all sub-clones, the McDMS. If means exist to attack all cells bearing that set then there is a real prospect of eliminating the entire family of malignant clones rather than merely modifying the behaviour of cancer cells.

Though most mutations are in non-coding regions, some neo-antigens are produced. This offers the prospect of specific agents and vaccines specific against the individual cancer rather than cancers generally. Such agents and vaccines should be directed against targets of the malignant-clone-defining mutation set. Sufficient selectivity, binding strength and

effectiveness may require the presence of two or more binding entities in a single molecule (co-body, heteropolyvalent antibody, etc.) or T-cell.

Clearly such vaccines or binding agents of this new class have to be made extremely quickly, for the individual patient. Both RNA vaccines and co-bodies offer this possibility, but for that reason alone they cannot conform to present-day regulatory processes for medicines. So an entirely new approach to regulation is required, to allow the necessary degree of individuality of treatment; but also required is concentration of massive resources.

Prologue.

Of the mutations in malignant cells a few are known to be active in causation, progression or maintenance of the phenotype. This essay emphasises the others, the unsung majority, what they may be able to tell us and how they can help in our quest for the Holy Grail of cancer research, that is to say Cure, not only understanding.

There are many cancer theories, some seemingly incompatible with what is described here and with each other. To secure a firm foundation for the key argument, explanation is given about the numbers of mutations present in cancers, heterogeneity, clonal evolution, stem-cells, oncogenes, pre-malignant clones and immunity, but 'metabolic' theories are omitted. The section 'A Strategy for Cancer Treatment' develops ideas about multi-target therapy, the potential for exploiting neo-antigens, and also what will be needed by way of changes in regulation and attitudes if our goals are to be achieved. Though there is no need to recite here the argument of a readily-accessible review (Boyde, 2009), its main themes re-appear in this Prologue in the form of assertions for which support will be provided later. Throughout, the treatment is thematic not exhaustive and references are introductory: nevertheless, the approach is intended to be serious and rigorous, to be appraised as such.

In cancer there is random, clonal accumulation of somatic mutations in cells that were already replicating and growing; leading eventually to loss of the normal control of cell growth, division and migration. Those mutations are conventionally divided into driver mutations (which are known to be directly involved in the malfunction of control) and passengers. Because it is not usually possible to distinguish the extent of a gene's involvement in control, the boundary between these categories is hazy: for that and other reasons no general statement can be made about the number of driver mutations required to support a fully malignant phenotype; in a very few special cases one or two may suffice, usually more. After initiation a cancer evolves further, adding more mutations which influence developments under selection pressure from the tumour's local environment within the patient's body, including whatever the immune system tries to do and any medical treatment that is given.

It is now known that the total number of mutations in a typical cancer is very large indeed, to be counted in thousands rather than dozens (Stratton, Campbell & Futreal, 2009) and they are scattered more or less randomly over the patient's DNA, from which we may deduce as highly probable, subject to caveats, that

i] every mature cancer is truly unique in respect of mutational composition, though also

ii] every cancer is heterogeneous, with multiple sub-clones, so that

iii] the totality of mutations detectable in a cancer (the 'mutanome') is a summation over the clones sampled and does not represent the situation in any individual cell, whereas

iv] at an early stage in the clonal sequence leading to cancer there was a common mutation set which remains present in that and all succeeding clones, and distinguishes all these clones from normal cells of the body - the malignant-clone-defining mutation set, McDMS (Boyde, 2009).

v] The McDMS offers targets for therapy preferable to other features of the cancer, since

vi] if all cells bearing the McDMS are removed, the cancer would be eliminated, whereas

vii] if any are allowed to remain re-growth of the cancer is likely to occur.

viii] The number of mutations in the McDMS, though less than the overall number in a cancer, is nevertheless sufficiently large that the McDMS is itself unique.

ix] At least a few of the passenger mutations including those in the McDMS will give rise to products that are neo-antigens or in some other way recognizable as different from normal. Whatever else is done or not done, thought should be given to attacking such targets.

x] Current descriptions and classifications of cancers (e.g., by tissue of origin or possession of this or that particular abnormality) are of limited practical use, since in reality each individual cancer is uniquely identified and characterized by assemblage of mutations present in the McDMS, not by properties of any other kind.

The Mutations in Cancer; How many? Causative? Random?

Sixty years ago it was already a commonplace that cancer is a disease of, and caused by, multiple somatic mutations, the best estimates being four to seven of them: a driving force for study of the subject was concern about radiation following the atomic bombs of 1945: it was implicitly assumed that clonal evolution explained both treatment resistance and progression. Contrarian voices cited causation by infection, chronic irritation, metabolic disruption or loss of key proteins, though none of that formally conflicts with mutation as the main game in town (Burnet, 1957). Loss of confidence in this simple picture sprang from: i) reductionist preference for instances where one or two mutational events seem to be sufficient for initiation of cancer or transformation of cells in culture, ii) the stem-cell hypothesis to help explain therapeutic failures and late relapse, and iii) evidence seemingly incompatible with either [i] or [ii] (and therefore suspect) that there are actually many *more* mutations present in cancer than the early estimates, instead of fewer, indeed several orders of magnitude more.

The presence of mutations does not prove that they are causative and some deny even that they are a necessary feature. For example, Baker (2015) thinks that genetic instability is a consequence rather than a cause of cancer, that there are no mutations at all in some cancers (his references on this point actually provide support for multiple mutations, though less than average in some individual cases), and points out that some carcinogens are not genotoxic (they don't have to be: it is enough if carcinogens make mutations more likely to occur and/or

more likely to persist). If any cancer is found that really has no mutations or only such as can all be accounted for in the ordinary life of a cell, the hypotheses advanced here must be modified or abandoned: until then mutations hold centre stage and we must take account of their presence. It is entirely possible, indeed probable, that genetic instability will enhance the number of mutations without always having any adverse effect, and yet that mutation is the basic causal event: the two ideas are compatible, even mutually supportive.

Knudson (1971) (who died a few weeks before this was written) is often cited with approval for his '2-hit' statistical theory of retinoblastoma ('approval' may be taken as indicating the popular mindset - a reductionist view as in [i] above - favouring few mutations over many) and indeed it soon became clear that for initiation it is sufficient that both copies of the gene *RB1* are corrupted. Strikingly, however, exhaustive testing of tumours reveals that epigenetic modification of the gene *SYK* is regularly present, usually also certain other genes (Zhang *et al*, 2012), so that even in this much-quoted type example it is not sufficient for malignancy that the function of just the two key allelic genes is lost; additional changes are required; further, we must accept so-called epigenetic changes as the equal of somatic DNA mutations - which is reasonable if, as seems to be the case, they affect phenotype to just the same extent and are passed down consistently from one (somatic) generation to the next.

So, certain specific mutations can definitely cause cancer (these are typically such as affect genes governing the cell cycle and/or growth) and cell transformation in culture can be caused also by a single critical mutation. It does not follow that this is always the case, nor that singleton initiating mutations are committed to remaining singletons. In cells with full malignant phenotype additional mutations are always found and we should never confuse cell culture with the whole organism nor xenografts with clinical tumours, no matter how much has been learned from research using these models.

It was proposed over 40 years ago that sporadically-occurring cancer is due to mutations resulting specifically from errors in DNA-copying at mitosis and that cancer displays a 'mutator phenotype' (Loeb, Bielas, & Beckman, 2008). Reports first appearing in the 1990s then suggested that the number of mutations in cancers might be very large, not two or four or seven but hundreds or thousands (Stoler *et al*, 1999, Boland & Ricciardiello, 1999), and this led to a distinction between drivers and passenger mutations - the latter often taken to convey lack of importance (which seems reasonable enough if the only objective is to exploit the pathophysiology of the cancer cell). However, this implication was not present when 'passenger' was first used in the cancer field (Ilyas & Tomlinson, 1996), nor when it was introduced in a genetics context (Suárez-López & Ortin, 1994). Here as in all writing about cancer research it is well to consider carefully the experimental background to the words used, and the prejudices of authors. A number of mutations may be cited as if it were the total present when only coding sequences were considered (Castle *et al*, 2012) or only 'cancer genes' (Lennerz *et al*, 2005): 'genomic' may be used of exons only or some other similar restriction. 'Genetic instability' has been denied because only a few tens of extra mutations appeared in cultured retinoblastoma cells (Zhang *et al*, 2012) (whereas 'stable' should mean no change at all). Reservations have been expressed (Bodmer 2008) about the phrase 'mutator phenotype', yet cancers typically contain so many mutations that explanations on

the basis of normal mutation rates (Tomlinson, Novelli & Bodmer, 1996) stretch credibility. The number of mutations varies with type of cancer and in a few special cases is less than the norm - retinoblastoma is mentioned above and leukaemias later, with regard to stem cell theory - but in general the number is huge, which is the most important and least regarded discovery in cancer research of the last two decades.

A very large proportion of the mutations found in cancers by DNA sequencing are either in non-coding regions (some 97% of the total nuclear DNA) or wholly or nearly silent, producing no change or no significant change in any peptide sequence. It has been too easily assumed that such mutations are of no importance and that study should be directed only at the drivers known to affect cellular metabolism and controls; unnecessarily limiting enquiry. Any port in a storm, any handle on cancer; any peculiarity may be what offers us our chance.

In order to estimate the number of mutations in a founding malignant clone (the McDMS) (Boyde, 2009) an unavoidable assumption was made that the distribution of mutations is random over the genome or sufficiently close to that. Actual experiments have now been done showing non-random distribution, varying among other things with protection of particular zones by binding to chromatin and thus also with tissue of origin (Polak *et al*, 2015), but the departure from randomness is probably not enough to make a difference and the original deduction stands as good enough for its limited purpose.

Clonality and Heterogeneity.

Evolution is now a routine laboratory procedure: we observe changes in living cells or even *in vitro* systems, such as phage display, used to optimize antibodies and other specific-binding macromolecules; and they always proceed by stepwise changes in nucleic acids from what was there before, selected for by environmental constraints just as Darwin supposed. The process is clonal. In a population of similar cells growing without constraint the huge surprise would be if this did NOT occur. In any population that has developed clonally, it should be possible to trace the sequence of mutational events by a study of the nucleic acid sequences in the surviving clones: we can expect branching of this clonal 'tree' and the pattern of branching may have something to teach us - though back mutation or loss of a segment of DNA containing a mutation previously acquired might complicate analysis. Descriptions of branching have used the words clone and sub-clone (McGranahan *et al*, 2016) or, as direct alternatives, 'trunk' and 'clone' (Willyard, 2016). Both are of course 'correct' and the discrepancy only apparent, but this instance serves to emphasise the need for care in interpretation. Clonality has been demonstrated in lung cancer DNA circulating in peripheral blood (ctDNA) (Abbosh *et al*, 2017).

Cancers are heterogeneous, which is expected if there has been clonal development. If all clones spring from a common origin we will find this piece of their history written in the nucleic acid sequences of later generations, even if intermediate stages have been lost as uncompetitive. But there is an alternative proposal, that cancers are *inherently* heterogeneous *in origin*; and we should also take note that there is no obstacle to several pre-cancerous

clones developing independently, giving rise to genetically unrelated cancers in a single patient.

Inherent heterogeneity requires that a cancer originates in a group of cells of different phenotypes that are mutually interdependent and remain so as the cancer grows. It may seem unlikely that this would be a universal or even a common way for cancers to arise, but there are certain striking observations tending to support the mutual dependence aspect (Parsons 2011, Polyak & Marusyk, 2014, Cleary *et al,* 2014) and the strongest objection that can be made is the absence of 'Popperian' experiments that might disprove the theory (though that is a near-universal feature of cancer theories). If a mutation should occur in such a system we expect that it will be in one single cell and for that mutation to be transferred to another cell, and incorporated into the nuclear DNA, requires a transfection-like mechanism. For the transfer to occur equally to all relevant cells such a mechanism would need to be extraordinarily efficient - far ahead of anything yet known - so it seems certain that a heterogeneous origin would be reflected in the chromosomal nucleic acid sequences of the plurality of distinct successor clones and therefore detectable by a sufficiently detailed sequencing experiment. Nothing like that has yet been found. A mechanism is indeed known by which nucleic acids (MiRNA) can be transferred between cells, and it is operative in cancers (Anastasiadou & Slack 2014) but is not transfection.

Pre-malignant clones arising independently of each other may account for some of the examples reviewed by Parsons (2011) and there is now clear experimental evidence of the development of such clones in skin exposed to ultraviolet light (Martincorena *et al*, 2015) and among leukaemia precursors (Slush *et al,* 2014) in the latter case enhancing confusion over a supposed contribution of stem cells to heterogeneity (c.f. Shibata & Shen, 2013). It seems more likely that stem cells, by giving rise to differentiated daughter clones all with a common cell-type of origin, would rather restrict than increase the range of variation observable.

Heterogeneity of a quite different kind is that cancers mostly have a prominent stroma which may be the greater part of the tumour mass, distinct from the malignant clone proper and itself often heterogeneous. This phenomenon may account for some other examples discussed by Parsons (2011).

Stem Cells, Selfies and Cancer.

Terminology:

That stem cells exist and participate in normal development and differentiation is beyond doubt and is not reviewed here. To use the phrase 'cancer stem cell' (CSC), however, conveys that there are present in a cancer certain cells of a class that truly resemble tissue

stem cells (whereas there is no reason *a priori* to suppose that events in a cancer should be equated with normal development). We wish to avoid any such presumption until it is proved. The stem-cell property most important to us is full self-renewal: to avoid both premature commitment to the CSC concept and troublesome circumlocution the word 'SELFIE' is used here for cells exhibiting the property of self-renewal, whether or not they are stem cells by any other definition. Similarly, various confusing names are used in the literature for cell types (derived from selfies) that are already committed to at least partial differentiation and have limited potential for replication. Here, with motives similar to above, we write 'DISCIPLES', noting that there may be successive generations of disciples that by analogy with the normal we expect to present progressively more features of differentiation and commitment. An important difference is our acknowledging as a possibility that in cancers unlike in the normal, descendants of a disciple might regain selfie properties.

Reports of clinical research commonly cite the stem-cell theory of cancer as if it were proven fact, accounting fully for resistance to treatment or relapse after apparent success, and standing at least somewhat opposed to clonal-evolution theory. The literature is replete with pictures illustrating the cancer-stem-cell concept, but they are only pictures and prove nothing. Contrariwise there are also dispassionate, sceptical critiques (Clarke & Fuller, 2006, Hill, 2006), and recent research papers often open with a passionate commitment to a simple clonal model which does not easily accommodate stem cells (Slush *et al*, 2014).

The original, simple, clonal theory of cancer sees a founding malignant clone that mutates further and divides without limit: in each patient one or a few successful clones constitute the bulk of the tumour, growing and spreading to cause disease and death, and each individual cell is capable of perpetuating the tumour. Whatever test is applied, however, it is found that the cells of a cancer differ greatly in their ability to self-replicate. The most abundant cell types are commonly unable to replicate at all (at least in culture) leading to the notion that power to reproduce the tumour in all aspects is reserved to a minority, even a very small minority, of untypical cells. This matches the picture built up over many years of research on tissue stem cells, the only cells competent to reproduce themselves and at the same time provide precursors for the differentiated cells of a tissue. They are supposed to be relatively quiescent, replicating infrequently and to a large extent uninfluenced by events in their surroundings though restrained and controlled by their cellular, micro-environmental 'niche'.

We can agree that cancers might behave a bit like that without adopting all aspects of CSC theory. By whatever means the first malignant cell originates, it is capable of yielding a fully-developed cancer or else we cannot give it that name. Its daughter cells may behave like disciples through a restraining mechanism intrinsic to every cell of a multicellular organism (until that mechanism is destroyed or over-ridden; which is the nature of cancer), meaning that there is some differentiation though not necessarily of a normal kind, loss of the ability to self-replicate and reproduce the tumour in its entirety, yet retention of the ability to grow, divide and form the bulk of it. Perhaps we should accept that the first truly malignant cell must have some properties of a selfie - and yet the greater part of its progeny may be unable to self-replicate, to this extent resembling cells in normal tissues.

Extreme versions of the stem cell theory of cancer (Wicha, Liu & Dontu, 2006) go much further. Here it is supposed that the cancer-initiating selfie comes only from a fundamental tissue stem cell or even a germ-line stem cell, presumably by mutation, and cancers can only begin in such a kind of selfie. Let us call it a selfie-plus. This type of cell is supposed to be not susceptible to attack by radiotherapy or chemotherapy because of its reclusive nature and quiescent phenotype (a handy excuse for treatment failures) and after the bulk of a tumour has been destroyed the cancer can re-form in its entirety from surviving selfies-plus and only them. Proposed is a one-way street [stem cell > selfie-plus (self-renewing) > disciple] a bit like the old central dogma of molecular biology (DNA (self-renewing) > RNA > protein), which is now known to be often wrong.

Evidence to prove theories of this nature is hard to obtain because of the presumptive rarity and refractory behaviour of stem cells. Evidence for the simpler selfie notion is easier to get and is reviewed next, including what may be gleaned from DNA sequence information.

Some of that evidence lies in how cells behave on culture or transplantation and the kind of cell-surface markers they carry, which may include those known from basic stem-cell research and are then found to occur on putative selfies - in itself poor evidence. It has been a regular finding, however, that to culture or transplant a cancer many cells must be moved together to the new environment (unlike with bacterial cultures for example, where one cell is enough). This seems to suggest that only a few cells are capable of independent growth and in this resemble selfies, but also proves to be weak evidence because such independence is not a simple intrinsic property of the cells themselves. In one example using a particular melanoma line apparently only 1 in 100,000 of the cells was capable of producing a tumour upon transplantation: in a different recipient mouse strain this increased to around 1 in 4, making the first figure ridiculous (Dick, 2009, and references therein). Alternative explanations exist, such as the interdependence of cells seen in normal tissues. Stronger evidence for CSCs is provided by experiments in which cells bearing particular markers are enriched in culture and only those fractions are able to seed a colony; but that is a long way from proving the complete CSC theory, and selfies have been seen to arise by something like back-mutation (Dirks, 2010, Chaffer *et al,* 2011, Klevebring *et al,* 2014).

So, the whole CSC concept is again in question (Li & Latera, 2012, Shackleton *et al,* 2009). As one example of confusion, two recent papers favourable to CSC-type theories differ as to their interpretation of the role of *LGR5*+ in self-renewal in colon cancer (Shimokawa *et al*, 2017, de Sousa e Melo *et al*,, 2017) - though the concern was with human and mouse-model tumours respectively. Comments on stem cells as contributing to heterogeneity of cancers appear in the preceding section: it seems more likely they would have exactly the opposite effect.

Can DNA sequence information tell us anything about selfies?

(The sketch used here to explain concepts is as if there were a selfie bearing the McDMS, though there is no necessary connection. Existence of a McDMS is consistent with but not proof of the existence of selfies. We shall see that patterns of branching in the clonal-evolutionary tree might provide some evidence.)

Suppose that a selfie exists that bears all the mutations necessary for cancer but no more. Then at mitosis it yields a selfie identical to itself and a disciple. That disciple and subsequent generations go on to yield cancer clones and acquire further mutations, and we may be able to determine the order in which that process occurs and recognize a branch point in the clonal-evolutionary tree, a point at which the clone bearing the original set (1) plus a series of added mutations (2) now divides into two separate clones each having its own characteristic set of further-added mutations, distinguished here as (3) and (4). The two sub-clones are now identified by the mutations they carry, either 1+2+3 or 1+2+4, so we may call [1+2] a binary branch point, meaning that an event distinguishing these two clones from each other occurred after the completion of set (2) but before anything else. The sub-clones can be recognized as members of the same family because they bear the set (1+2).

Suppose that our selfie, identical to the original one, divided again to yield a disciple which gave rise to a second family of clones, bearing the set (1+5), and yet again *ab origo* to yield the family 1+6. We may now call [1] a tertiary branch point because three distinct families of clones (1+2, 1+5 and 1+6) have originated at the branch point [1]. If selfie theory holds true, all related cancerous clones must bear set (1) and there may be several families of cancerous clones that share set (1) - in our example three families - but have no other mutations in common. So according to selfie theory, branch points of order greater than binary may occur (binaries probably also) whereas the simple clonal theory predicts binary branch points only.

At first sight this offers an objective way of testing which of the theories best fits a particular cancer. It will be easily understood, however, that if the experimental method used fails to detect or report some of the mutations actually present or if too few mutations are studied, say in a restricted portion of the genome, this can give rise to a false conclusion with regard to the order of a branch point. For that reason the findings now reviewed should be treated as interesting - but not conclusive even if they were mutually-consistent.

Several studies are of chronic lymphatic leukaemia and of only a small portion of the genome. Gurrieri *et al* (2002) studied intraclonal V_HDJ_H diversification: in the two cases illustrated the first showed apparently 3 tertiary out of 4 branch points; in the second case, one branch point out of 4 was five-fold, the others binary. Volkheimer *et al* (2007) studied immunoglobulin variable regions and give 2 clonal-evolution trees showing, in one case, an apparent five-fold branch point after 6 preceding binaries, in another two tertiary branch points both following a single binary, so the positions of these higher-order branch points do not accord with strict stem-cell theory: however the immediately-preceding clones do look like our selfies, subject to the consideration that we know nothing about mutations elsewhere

in the genome. Campbell *et al* (2008) studied intraclonal diversification at the immunoglobulin heavy-chain locus *IGH*. In the two clonal-evolution trees illustrated there are respectively 7 and 10 branch points, all binary, and overall 'the sub-clones and dominant clone shared a core subset of mutations'. Moving away from leukaemia, Gerlinger *et al* (2012) present genealogical trees for two cases of clear-cell carcinoma of the kidney: samples were taken from different portions of the tumour and metastases: all branch points are binary.

The role of major oncogenes. Cause or consequence, accelerators or epiphenomena.

Gerlinger *et al* (2012) also have something to tell us about the role of some well-known oncogenes.

In patient 1 there was a mutation in the *VHL* gene in the main trunk of the tree (a mutated *VHL* is said to be always present in this cancer) but separate and distinct *SETD2* mutations occurred after the first branch point, as new events in three distinct sub-clones. As if to lend emphasis, these were fundamentally different kinds of mutations so that there is no possibility of an error of interpretation; they must have occurred independently and all of them after the evolutionary trunk had divided. Two of these three sub-clones went on to acquire different mutations in *KDM5C* and only one of those two also a mutation in *mTOR*.

In patient 2 the *VHL* mutation in the trunk was accompanied by one mutation in *PBRM1* whilst *SETD2* mutations occurred independently in the branches (one accompanied by *p53*) and *PTEN* independently in two sub-clones.

Therefore, however important they may be at a later stage in cancer development, the mutations cited, excepting *VHL*, were not involved in the initiation of the cancers, which supports the notion (Boyde, 2009) that many oncogenes including mutated *p53* and *KRAS* - present in a high proportion of cancers and nearly universal in some kinds - are not concerned in originating the characteristic cancer phenotype and more resemble opportunist collaborators, developing and profiting from a situation brought about by the actions of others. It seems likely also that treatment directed against such targets will not prove curative.

The work of Martincorena *et al* (2015), cited earlier, gives another glimpse into this. Studying pre-malignant clones, they looked for individual, specific, putative driver mutations sited in well-known oncogenes and found a startling number in what was still phenotypically normal, non-cancerous skin, raising yet further doubts about the importance of oncogenes and driver mutations. It seems indeed that they are neither necessary nor sufficient for us to recognize a clone as malignant.

Interim conclusions.

There is powerful, direct, experimental evidence for clonal development of malignant clones and sub-clones by successive accumulation of somatic mutations, the total number of which is always large and in most cancers colossal. However the original, simple clonal theory must be modified to take account of the differentiation of certain sub-clones into disciples that have limited replicative potential and are unable themselves, except by way of permissive back-mutation, to develop all the features of a cancer. Other factors that may modify simple clonal succession are i] multiple malignant or pre-malignant clones that arise independently of each other, ii] selfies however they arise, and iii] the mutual influence of malignant and stromal or adventitious cell types within a tumour. There is fairly good evidence for the existence and clinical impact of selfies but none that they are responsible for all aspects of treatment resistance or relapse. Nothing is known about the putative step from tissue stem cell → selfie-plus, which latter cell types may not exist. The evidence so far from clonal-evolution trees is rather opposed to than supportive of the CSC concept as a whole. The McDMS concept is unshaken. Oncogenes and driver mutations may not be all that important.

A Strategy for Cancer Treatment:

i) what happens at present, and the limitations.

There have been immense advances over the past 40-50 years, based on excellent basic research which has also taught us all we know about cell controls; though there has been relatively little improvement in treatment for the most common kinds of cancer. New hope comes from harnessing the immune system to act against cancer, notably by the use of immune check-point inhibitors, RNA vaccines and autologous or homologous activated T cells, work that can be reviewed here only insofar as it is relevant to our main topic, which in respect of treatment is how to exploit the uniqueness of each individual cancer.

Currently, a cancer may be treated by one of the following means, or a combination:

Surgical removal of all or part of the tumour

Radiotherapy and/or chemotherapy, damaging cells as they divide

Treatments acting against cancers that rely on hormonal stimulation

A very few treatments specific for cancers in particular tissues, notably the thyroid

Newer kinds of drug treatment directed at cell-growth controls that have broken down

(largely based on new knowledge about driver mutations).

Improvement of the immune system response, by vaccination etc.,

(including by acting against the way that cancers block the immune system).

A hidden assumption is that all the cancer cells will respond equally well, but in fact they differ among themselves so this is an unlikely outcome. As we shall see, there has been a recent approach to immunization against several neo-epitopes at once but otherwise these approaches take no account of the heterogeneity of cancers, yet we may justly say that the individual patient's individual cancer is really many cancers, differing between themselves.

No one of these approaches takes account of cancer evolution - if any cancer cells survive the therapeutic assault the tumour may re-grow with new mutations of such kinds that its cells are now resistant to that particular treatment.

No one of them is better than another in its own right.

None of them is directed at an ideal range of targets, so success has been by the chance of favourable circumstances.

A Strategy for Cancer Treatment: ii) fundamental concept.

It seems obviously best to eliminate all cells of the cancer and leave normal cells unharmed, which may already happen occasionally by surgical extirpation or if killing most of a cancer leads to an immune response which sees off the rest. In the common case neither happens, so we should deliberately fashion treatments that attack all the cells of a cancer or at least all the clones that matter.

A Strategy for Cancer Treatment:

iii) definition of possible targets

A useful target must be some way in which the cancer cell differs from the normal, functionally or structurally or both. With present technology in mind, the obvious and to date most successful means of detecting such differences is by genomic sequencing and this, together with epigenetic profiling, is regarded as the first possible approach to defining targets.

Alternatively or in addition, suppose we had available a library of generic binding molecules such as nucleic acid aptamers, each with fairly broad specificity but none of which bound significantly to normal cells of the body. Then exposing cancer cells to these binders would allow us to detect which ones bound to the cancer and thus what novel kinds of binding site and neoantigens had developed, if any. Such a library of aptamers can be created right now, or it can be done more laboriously with polypeptides.

All other possibilities are lumped together in a third group: they include agents affecting the immune check-points.

A Strategy for Cancer Treatment:

iv) nature of the agents to be used for attacking the defined targets.

At this point in discussion we do not restrict ourselves concerning the physical nature of the agent, interest being only in the kind of effect produced. It may be a small molecule, macromolecule, living cell or anything else whatever.

Excepting radiation, it is a condition of activity that the agent can ‘recognize’ its target, that is, has molecular complementarity of the same kind as an enzyme for its substrate, drug for its receptor or an antibody for the corresponding epitope. Thereafter, modes of action differ:-

Much pharmaceutical research seeks to develop ‘druggable’ small-molecule agents active against cancer by modifying the behaviour of cancer cells, or killing them. It may be that this

way of thinking is unnecessarily limiting - the word ‘druggable’ implies that the objective of research is to find small, stable, easily synthesized, safe, convenient molecules that can be examined for regulatory approval by established procedures.

Simply binding of an agent to the target may be sufficient, by inducing a change in the structural or functional state of the target that causes death of the cell concerned. For this purpose a polyvalent agent may be superior because of improved selectivity and binding strength.

An otherwise passive binding agent may carry a toxic moiety. Radioisotopes have been used for this purpose, presumably as a carry-over of thinking from radiotherapy, but there are realistic alternatives such as ricin and many other cytotoxins. The main objection to radioisotopes is that their lethal effect depends on nuclear disintegration, an event which cannot be timed to occur when desired but only the probability of its occurrence - usually expressed as half-life - meaning that at any particular binding site the effect may not occur when it is needed. Also damage may extend to neighbouring normal tissue.

Binding can induce immune attack, directly because the agent is itself an antibody or some other kind of immune-functioning molecule or cell, or indirectly by arranging that the agent carries a suitable immune-functional unit.

Vaccines, to induce either antibody or T-cell responses.

A Strategy for Cancer Treatment:

v) location of defined targets within the clonal-development map.

That an attackable target exists is no guarantee that it is worth attacking. If a target is present in the cells of only one clone a likely outcome is to favour the other clones present, one of which may be capable of re-seeding the cancer.

In the common case, if several independent clones exist side-by-side it may well be useful to attack all at the same time; so for that reason alone we may expect advantage from the use of multiple treatments, at least sometimes.

Contrariwise, if clones are mutually dependent, killing one will damage the others.

The targets to choose, obviously, if they can be identified, are those derived from the McDMS, because in principle an attack on them, rigorously targeted, is an attack on all cells of the cancer and no other living thing.

vi) multiple agents, combined agents.

Why might we seek deliberately to use agents in combination against a cancer?

- General benevolence

- To act against diverse clones or cell types

- To act more effectively against a single clone by simultaneous attack on two fronts

- Because the combination is more potent in itself.

The first seems to underlie much clinical research in the area and is psychologically attractive but unscientific, rather like the well-meant drive towards 'inter-disciplinary studies' which usually means that no component of the course of study is done right.

If two or more distinct clones must be eliminated to obtain a cure, and they differ sufficiently that one agent is not effective against them all, it is rational to attack each clone separately with the best means available for that particular task. There may even be synergy if destruction of one clone facilitates attack on the others.

But a single agent per clone may not be enough. In spite of much recent propaganda about restricting antibiotics for fear of encouraging bacterial resistance, the concept of using several together against a bacterial or parasitic infection is familiar; it is the correct thing to do if in any difficulty and for *Helicobacter, Mycobacterium tuberculosis* or *Plasmodium falciparum* it is now considered malpractice to do anything else because an organism partially resistant to each of the antibiotics individually may be killed by the combination. This is obviously better for the patient and also minimizes rather than encourages the development of resistant strains in the community, exactly opposite to the view promoted by anti-medical propagandists and cost-cutting governments or insurers. There is clear relevance to cancer.

Different again is the concept of putting together two or several active principles in a single molecule, macromolecule or immune or killer cell. Polyvalent vaccines may be construed as falling within this group but are discussed separately below.

If truly combined, the agents do not act separately but rather it is their combination in itself that conveys effectiveness and that in turn may happen several different ways - a primary thematic division between what we may call bi-functionality, bi-specificity, bivalency and heterobivalency:

An anticancer antibody bearing a toxic or radioactive moiety may be called bifunctional. Such agents have been the subject of many trials - uniformly unsuccessful because they lack any greater selectivity than the original antibody, and the chosen target epitope was of a widely-distributed kind rather than confined to the individual cancer.

Bi-specific antibodies or similar agents bring an effector into proximity to its intended target without necessarily any covalent linkage between carrier molecule and the effector agent. Such are widely used in histopathology and have been pressed into service in therapeutic trials with no greater success than the bifunctional agents.

We may combine two or more binding entities in a single molecule (a ***co-body***) to obtain an agent with enhanced affinity and selectivity for its target (Boyde, 2007a), which can work only if the 'target' bears corresponding, accessible, multiple epitopes. This is no place for a review of immunochemistry but it may help to point out the parallel instances of natural antibodies (which are homopolyvalent and consequently have enhanced affinity and selectivity for the natural homopolyvalent target, as compared with a monovalent Fab fragment derived from that same antibody) and T-lymphocytes, where the receptor itself is monovalent but there are many of them so that the T-cell as a whole is homopolyvalent.

Heteropolyvalency is a step further. It is commonplace for a target molecule or cell to bear several different epitopes and corresponding heteropolyvalent binding agents do occur in Nature (including a few among antibodies) though they seem to be uncommon. Agents of this kind (co-bodies) can be made synthetically.

A Strategy for Cancer Treatment: vii) personalization.

To favour personalized treatment of cancer is rather like being in favour of Christmas. It is a beneficent notion, but we need to know exactly what is meant, and to date that has usually been to identify the particular driver mutations present in the individual's tumour and devise tactics of treatment directed against them, an approach that ignores the established facts that tumours are heterogeneous and (as shown above in discussion of Guerlinger et al (2012)) targets of that nature may be confined to sub-clones; from which follows that other sub-clones may be unaffected and the tumour free to continue. Therefore pessimistic conclusions such as those of Prasad (2016) and others are to be anticipated and yet have no relevance to the real individuality or singularity of a cancer expressed in the McDMS. Repetitiously, let us state here that the personalization reviewed by Prasad and practised by a thousand others amounts only to recognition that a cancer may contain driver mutations having no necessary relationship with the tissue of origin of the tumour, so that treatment can be directed against those targets rather than as crudely determined by histological typology. Reading the literature on personalized treatment will show that the primary interest of researchers and oncologists is on picking out known cancer targets for which relevant drugs are available, not on individuality *per se*. Contrariwise, evidence is emerging, at last, that treatments directed at the McDMS may produce better patient outcomes (McGranahan, *et al,* 2016, Willyard, 2016). It is early days, there is no guarantee of success, but a fair appreciation must be that this is a novel strategic concept rather than mere repetition of well-known tropes.

A Strategy for Cancer Treatment:

viii) neo-antigens and immune-based therapies.

There has been an immense upsurge of interest and activity directed at the immune system in cancer: immune checkpoint inhibition is now such a hot topic that no review need be attempted. It may seem churlish to limit discussion in this way, but for consistency it has to be so; our concentration is upon the features that distinguish an individual cancer or clone from all others, so the particular means used to exploit this 'singularity' are for us of lower priority than singularity itself. In this super-abbreviated sketch, no distinction is made between research on cell lines, animals or patients.

Interpretations of a few years ago might be guyed as follows:- A cancer contains few or no mutations and no neoantigens, so there is no immune response, which explains why vaccines don't work. But in reality cancer is a disease of many mutations, so that glib explanation won't do. The number of mutations is indeed so large that we should expect to find some neoantigens in every cancer: this was predictable, is now confirmed (Lennerz *et al,* 2005, Castle *et al,* 2012, Kreiter *et al* 2015, Tran *et al,* 2015, Ledford, 2016, McGranahan *et al,* 2016) and it seems likely that the numbers detected, and shown to be effective in inducing an active T-cell response, will increase as methods improve. If they attracted little attention in the past it was perhaps because no-one was looking, the methods were inadequate, or both, but most important surely is that there is an efficient evasion mechanism working at least partly through immune checkpoints.

Detection of neoantigens was at first by identifying reactive T-cell lines (Lennerz *et al*, 2005 and references therein), more recently from whole-genome sequence data which are sieved to find putative mutations of a kind expected to make changes in peptide structure (Castle 2012); or by combining these approaches (Tran *et al,* 2015). Then immunization (not necessarily of the patient) may be done by injecting peptides designed to give the desired immune response and synthesized on the basis of the DNA information (Castle *et al,* 2012), or more recently still using as the vaccine a synthetic messenger RNA which may be of sequence chosen to represent, in a single RNA molecule, several epitopes from the chosen mutations (Kreiter *et al,* 2015). Autologous (Rosenberg & Restifo, 2015) or homologous (Strønen *et al,* 2016) activated T-cell lines may be grown up in culture and used directly as the weapon, with or without further genetic engineering, and there may be long-term persistence of these cells in the recipient (Rosenberg & Restifo, 2015).

Such approaches are now usually combined with the use of checkpoint inhibitors and there is some indication of better success from treatment based on neoantigens from the McDMS rather than just anywhere (McGranahan *et al,* 2016). Neoantigens may be lost from later somatic generations because of mutations favoured by immune editing, others added, and it seems that neoantigens underlie the most effective immune response, though that should be no surprise (Verdegaal *et al* 2016). Loss of markers has a long history in cancer research and this phenomenon was discussed as a caveat in relation to the McDMS approach (Boyde, 2009), though how important is unknown.

A Strategy for Cancer Treatment: ix) speed.

A cancer patient must receive effective treatment in a matter of weeks from diagnosis, and yet as emphasized in this article, each cancer, or rather each cancer clone, is unique. A new drug of the regular kind takes many years and billions of dollars from discovery to market so even if one could be developed for a particular, peculiar, unique cancer it cannot possibly be ready in time, even if society could contemplate such a thing as a drug for one individual, no matter how rich. The really new approaches of the past few years, to a strategy for cancer, are the new-generation ***RNA vaccines*** against neo-antigens, and possibly ***also co-bodies*** though there has been essentially no practical development of the latter. By good fortune, each is capable of extremely rapid development for an individual case (Boyde, 2007b, Kreiter *et al,* 2015). In each of these instances, in principle, the active agent can indeed be made ready within weeks - there is no scientific obstacle - given an organization capable of working that fast, which would have to be also a massive and extremely expensive organization. To develop activated T-cells for the same purpose would usually take somewhat longer and is intrinsically a more expensive procedure, even when a laboratory is fully-prepared.

A Strategy for Cancer Treatment:
x) What kind of organisation is required?

Neither governments, nor research funders, nor the pharmaceutical industry as at present constituted can possibly do what is needed because of the commercial interests, other vested interests and rivalries that would have to be neutralised. A dedicated, privately-funded, independent institute might be able to do it, notwithstanding a financial requirement in the order of tens of billions of dollars. The first patients will probably be extremely rich people who in this instance will serve as both contributors and guinea-pigs. Only they will be able to finance this effort; and a huge investment of money, skill, organization and effort is needed before anything can be done for even the first sufferer. So in practical terms this article may be read as a call for enlightened self-interest by a substantial number of those who have the financial capacity to contribute, collectively, as a genuine, disinterested, philanthropic contribution plus the off-chance that they themselves may need treatment and the necessary preparations have been made in time. A name? - the Singular Institute. Its location? - can be anywhere, though the USA or China are the most plausible places; perhaps Hong Kong? The idea is explored further in a companion piece, *CancerChallenge*.

Regulation.

Nothing resembling the present law and regulatory systems can handle these new approaches. Legislation may well be needed, either to exempt the enterprise from regulatory interference or introduce a whole new system capable of handling unanticipated problems (unknown unknowns, which will surely arise) in a completely different way.

Envoi.

In a touching essay Weinberg (2014), the doyen of the cancer cell biology community, reviews the history of his 40-plus years in research and bemoans the fact that decades of apparently ever greater clarity and simplicity concerning cause and treatment of cancer have been succeeded by years of increasing confusion and complexity, stemming from the new findings about numbers of mutations and the rise of stem-cell interpretations. But the stem-cell picture is not what it seems and is perhaps anyway of limited importance in the actual treatment of patients; and the huge number of mutations can be seen also as an immense simplification. Perhaps we don't need to understand cellular control systems or how to repair them, or anyway not so much. Perhaps it will be enough to simply kill all the cancer cells, like the old-fashioned surgeons always wanted to do, and perhaps we will soon have the means, at a price.

Billionaires of the World, Captains of Industry, step forward. The money can only come from you and you will be the first patients! One hundred at a billion dollars each should do the job, even perhaps as little as ten to twenty billion dollars in total and not necessarily in the form of donations because loans could serve equally well. If there is sufficient response, the present writer will gift to the new Institute an interest which might be thought as comparable in scale, namely all rights in co-bodies.

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