Review

A New Paradigm in Cancer Treatment: Identifying and Targeting Clonal Mutations

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Review

A New Paradigm in Cancer Treatment: Identifying and Targeting Clonal Mutations

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Abstract: Recently concluded, large-scale cancer genomics studies involving multiregion sequencing of primary tumors and paired metastases appear to indicate that many or most cancer patients have one or more "clonal" mutations in their tumors. Clonal mutations are those that are present in all of a patient's cancer cells. Achilles Therapeutics is currently the only company specifically targeting clonal mutations. However, they are doing so with tumor-derived T cells. To address the potential limitations of immunotherapy, I have devised another approach for exploiting clonal mutations, which I call "Oncolytic Vector Efficient Replication Contingent on Omnipresent Mutation Engagement" (OVERCOME). The ideal version of OVERCOME would likely employ a bioengineered facultative intracellular bacterium. The bacterium would initially be attenuated, but (transiently) reverse its attenuation upon clonal mutation detection.

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Outline:

A) The personalized facultative intracellular bacterium, e.g., *Listeria monocytogenes*, will enter a broad variety of cell types. Once inside a given host cell, the bioengineered bacterium will not replicate yet - it will simply send out sensors to look for the target mutation(s). The target mutation(s) will be present in a cancer cell, and the sensors will detect it or them. B) The sensors' response units will cleave a pro-peptide, leading to activation of a two-component regulatory system, e.g., the ComD/ComE system of *Streptococcus mutans* UA159[i]. Activation of the two-component regulatory system will trigger transient vector replication and hyper-virulence. C) The vector will replicate excessively within the cancer cell and potently induce cytotoxicity.

Introduction:

Cancer has plagued multi-cellular organisms since their inception. However, we have only recently begun to develop effective targeted therapies. Most of said therapies have been for blood cancers. Gleevec, the BCR-ABL tyrosine kinase inhibitor, is a prime example of this; it was approved in 2001 for the treatment of chronic myelogenous leukemiaii. Additionally, immunotherapies such as CAR T-cells have been developed that target T and B cell malignanciesiii.

In certain instances, immunotherapies such as anti-PD1 antibodies can help treat melanoma. T-VEC, an FDA-approved oncolytic herpesvirus, is also sometimes effective against melanomaiv. It is somewhat unclear why melanomas respond so well to immunotherapy and T-VEC as opposed to many other types of cancer.

T-VEC may exert its anti-tumor effects mainly by rendering melanoma lesions immunologically "hot", rather than direct oncolysisv. It may also spread more easily through such lesions due to tight endothelial cell-to-cell junctions vi. Thus, melanoma may simply be particularly amenable to immunotherapy. Perhaps this is because it is often caused at least in part by UV damage-mediated DNA mutations, which can be potently immunogenicvii.

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Three other oncolytic viruses have been approved for clinical usage against solid tumors in other areas of the world: Rigvir, Oncorine, and Delytact[viii]. Rigvir is an oncolytic enterovirus approved in Latvia for melanoma, Oncorine is a modified adenovirus that is used to treat head and neck cancer, and Delytact is a herpesvirus used to treat malignant gliomas. Rigvir may not be as efficacious as T-VEC[ix]. Like T-VEC, all three of these vectors appear to exert their oncolytic effects primarily by potentiating the anti-tumor immune response[x,xi,xii].

Finally, there is one FDA-approved bacterial vector that is used to treat non-muscle invasive bladder cancer, Bacillus Calmette–Guérin (BCG)[xiii]. It is a live attenuated strain of *Mycobacterium bovis*. Although it is one of the oldest tumor therapies, its mechanism of action still has not been fully elucidated. As with the aforementioned oncolytic viruses, however, BCG may mainly stimulate an immune response against bladder cancer cells rather than lyse them directly[xiv].

Regardless, in most instances, the aforementioned therapies for solid tumors are not curative. That is largely because they do not target the tumors with sufficient specificity over normal tissue, and so must be attenuated.

Clonal Mutations:

Clonal mutations are defined as mutations that are present in all of a patient's cancer cells. Recently published results from large-scale cancer genomics studies that involve multiregion sequencing of primary tumors and paired metastases, like TRACERx[xv], appear to indicate that many or most patients have at least one clonal mutation in their cancers[xvi,xvii,xviii,xix,xx,xxi].

Clonal mutations would be ideal targets for personalized therapy. Some tumors are in anatomical locales that are difficult or dangerous to biopsy, however. A non-invasive option for identifying a patient's mutational spectrum, which is becoming increasingly feasible in terms of clinical application, would be to analyze circulating tumor cells[xxii] or circulating cell-free tumor DNA in the blood or cerebrospinal fluid[xxiii,xxiv,xxv,xxvi,xxvii,xxviii]. Although it is possible to determine clonal mutations, targeting these mutations is not very facile at present.

Dr. Charles Swanton, Chief Investigator of the TRACERx study, co-founded a company called Achilles Therapeutics in 2016; it is currently the only company specifically targeting clonal mutations. However, they are leveraging an immunotherapy tactic to do so, specifically tumor-derived T cells[xxix]. From a mechanistic perspective, immunotherapy may not be the best way to exploit clonal mutations. Firstly, many mutations affect intracellular antigens. While MHC class I complexes can display intracellular peptides derived from mutated proteins, 40-90% of human cancers downregulate said complexes[xxx]. Secondly, even if a mutant protein is on the cell's surface, some of the patient's cancer cells may evolve to downregulate the production of that mutant protein. The latter point applies to the display of peptides derived from mutant intracellular proteins via MHC class I complexes as well.

Recently, I devised an approach for exploiting clonal mutations in solid tumors at least that can theoretically circumvent these issues, which I call "Oncolytic Vector Efficient Replication Contingent on Omnipresent Mutation Engagement" (OVERCOME)[xxxi,xxxii].

Overcome:

The general idea of OVERCOME is to use an oncolytic virus or intracellular bacterium with the broadest possible tropism that is either programmed not to replicate or attenuated until it detects one or more clonal mutations via molecular "switches"[xxxiii,xxxiv,xxxv,xxxvi,xxxvii,xxxviii,xxxi,xxxii]. Moreover, many hyper-virulence modules could be triggered by clonal mutation detection[xxxix,xl,xli,xlii]. Somewhat similar strategies have been proposed before with oncolytic viruses, but replication was not made dependent on mutation detection[xliii].

Crucially, with such a vector, clonally mutated genes can be forcibly upregulated via expressed or secreted transcriptional activators to essentially ensure a detection signal. As direct RNA export from bacteria is currently not very well-understood, a bacterial vector could secrete a multitude of transcriptional activator like effector (TALE)- or zinc finger (ZF)-activators instead of CRISPR-based activators[xliv,xlv]. However, these transcriptional activators would also be expressed or secreted in infected noncancerous cells, which might be problematic even just within the time it takes for

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treatment. Thus, a negative feedback circuit may be of use; in addition to switches that target the mutated part of the upregulated transcript or protein, it might be ideal to also express switches that detect it at one or more non-mutated sites. When the latter switches activate, further secretion of the TALE- or ZF-activators would be halted.

Large mutations in promoters can also be targeted by transcriptionally upregulating their gene product, but smaller mutations in these locales may be less easily exploited. These regions and other clonally mutated intergenic regions could also theoretically be targeted by DNA-binding switches[xlvi,xlvii]. However, if the DNA is targeted, an enzymatic cascade may be required for sufficiently rapid amplification of the mutation "signal"[xlviii]. Such a cascade might increase vector off-target activity. In the near future, induced transcription of any intergenic region might be possible, which could lead to less off-target activity than an enzymatic cascade-based mechanism.

Ideally, the vector would target all of a patient's clonal mutations simultaneously, transcriptionally upregulate any clonally mutated genes, and conditionally become hyper-virulent in many ways. Such sophisticated bioengineering may require a lot of extra packaging space, however. Given the essentially unlimited packaging space of bacteria, an intracellular bacterium may be the best oncolytic vector in this context.

Various attenuated intracellular bacterial species like *Salmonella* Typhimurium and *Listeria monocytogenes* can be intravenously injected in humans with minimal side effects[xlix,l,li]. Notably, bacteria naturally colonize tumors when injected intravenously[lii]. As stated in my previous works, immunosuppressive drugs like dexamethasone could be administered during treatment to allow for unhindered infection of a patient's tumor or tumors. Moreover, some bacteria at least are able to cross the blood-brain barrier after intravenous injection, which is a very helpful characteristic for treating central nervous system tumors like glioblastoma[liii,liv].

The two intracellular bacterial species that are best studied in the context of cancer are *S.* Typhimurium[lv] and *L. monocytogenes*[lvi]. I previously suggested the possible use of *Vibrio natriegens* as a vector because of its rapid replication rate[lvii] and the fact that only two genes are required for extracellular bacterial entry into mammalian cells[lviii], but it does not seem to survive in the cytoplasm of human cells[lix]. A prophage-free strain of *V. natriegens* may be more applicable here[lx]. An important benefit of using a facultative intracellular bacterium like *S.* Typhimurium or *L. monocytogenes* instead of an obligate intracellular bacterium is that it may not need to invade very many cancer cells; activated vectors could transmit the detection signal to nearby intracellular bacteria that have not detected clonal mutations yet or in general - and extracellular bacteria - via AI-1, a membrane-permeable quorum sensing molecule[lxi].

Wide tropism via "zippering" could be imbued via the expression of multiple adhesins that bind ubiquitously expressed cell surface proteins - and perhaps an assortment of invasins[lxii,lxiii,lxiv,lxv]. The *Salmonella* Pathogenicity Island 1 type 3 secretion system would also enable entry into a wide variety of cell types through a "triggering" mechanism[lxvi,lxvii].

In order to avoid xenophagy prior to the detection of one or more clonal mutations, the bacteria could even replicate up to a tolerable copy number inside host cells, restrained via quorum sensing - perhaps with AI-2[lxviii]. An *S.* Typhimurium *sifA* mutant could be used here, which lyses its vesicle. HlyE or listeriolysin O secretion could also help to lyse the vacuole[lxix].

An example of a molecular switch that could target a clonally mutated transcript would involve Pumby modules, which allow for modular recognition of RNA in the same way that TALEs can readily be generated to recognize custom DNA sequences. Dual RNA-binding switches would be used to dock next to one another specifically on the mutated transcript, resulting in split intein splicing and reconstitution of an orthogonal protease[xxxiii].

Alternatively, a new CRISPR-based technique that could be used is "Craspase", an RNA-guided protease. The RNA cleavage capacity of Craspase should be abolished in this context, using a "stay-on" variant[xxxviii]. Crucially, this system could detect clonal point mutations, as less than 4 mismatches in the cognate target RNA 3' end precludes Craspase proteolytic activity[lxx]. If necessary, synthetic mismatches could potentially be used to imbue point mutation specificity, as with "SHERLOCK"[lxxi].

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However, Craspase would require the export or release of RNA into the host cell cytoplasm. There are two options for this. The most straightforward one is as follows. Intracellular copies of the bacterial vector could replicate asymmetrically initially or after reaching quorum sensing levels[lxxii,lxxiii], wherein one or more "stem cell" progeny cells survive and one or more "differentiated" progeny cells lyse to release RNA elements[lxxiv,xxxviii].

A second possibility for a Gram-positive vector, e.g., *L. monocytogenes*, is that Eno or Zea could perhaps be programmed to bind and thus enable secretion of custom RNA molecules like the Craspase gRNA[lxxv,lxxvi].

The facultative intracellular bacterial vector could respond to a clonal mutation through activation of Craspase to cleave a pro-peptide; the resulting peptide could then activate a two-component regulatory system like the ComD/ComE system of *Streptococcus mutans* UA159[i,lxxvii,lxxviii] or a synthetic receptor[lxxix,lxxx].

As opposed to viruses, the restoration of intracellular bacterial replication potential or attenuation reversal may need to be transient in order to avoid systemic infections.

Additionally, for neuron-based cancer, *Toxoplasma gondii* could eventually be helpful[lxxxi].

Finally, it is theoretically possible that some number of patients may have no clonal mutations in their cancers.

In this unlikely scenario, a small set of subclonal mutations that together are present in all of their cancer cells could be targeted.

Conclusions:

It is clear that effective therapies for solid tumors are urgently needed. While immunotherapy has had much success in the realm of blood cancers, it is unclear whether it will end up being similarly efficacious for solid tumors. From a mechanistic standpoint, targeting cell surface antigens certainly seems like a less promising strategy than targeting mutated nucleic acids or proteins from the interior of the cell. Thus, the development of a facultative intracellular bacterial vector that can surmount these mechanistic challenges could be crucial to curing solid tumors.

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