Review

Targeting Clonal Mutations with Synthetic Microbes

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Review

Targeting Clonal Mutations with Synthetic Microbes

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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. Clonally mutated proteins can potentially be targeted by inhibitors or E3 ligase small molecule glues, but developing new small molecule drugs for each patient is not feasible currently. Achilles Therapeutics is currently the only company specifically targeting clonal mutations on a patient-by-patient basis. 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.

Keywords: multiregion sequencing; multisample sequencing; cell-free circulating tumor DNA; clonal mutations; achilles therapeutics; overcome

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 leukemia [1]. Additionally, immunotherapies such as CAR T-cells have been developed that target T and B cell malignancies [2].

Immunotherapies, including CAR T-cell therapy, have failed to cure most types of solid tumors, despite many years of work by many research groups [3,4]. This is due in part to an immunosuppressive microenvironment in many solid tumors.

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 melanoma [5]. 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 oncolysis [6]. It may also spread more easily through such lesions due to tight endothelial cell-to-cell junctions [7]. 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 immunogenic [8].

Three other oncolytic viruses have been approved for clinical usage against solid tumors in other areas of the world: Rigvir, Oncorine, and Delytact [9]. 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 [10]. Like T-VEC, all three of these vectors appear to exert their oncolytic effects primarily by potentiating the anti-tumor immune response [11–13].

Finally, there is one FDA-approved bacterial vector that is used to treat non-muscle invasive bladder cancer, Bacillus Calmette–Guérin (BCG) [14]. 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

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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 [15].

Regardless, in most instances, the aforementioned oncolytic 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.

Unattenuated oncolytic vectors can be targeted to cell surface markers like immunotherapies [16]. Unfortunately, the issue with targeting a limited number of cell surface markers is that it can lead to escape variants [17].

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 [18], appear to indicate that many or most patients have at least one clonal mutation in their cancers [19–24].

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 [25] or circulating cell-free tumor DNA in the blood or cerebrospinal fluid [26–31]. Although it is possible to determine clonal mutations, targeting these mutations is not very facile at present.

Clonally mutated proteins can be targeted by inhibitors or E3 ligase small molecule glues [32,33]. However, inhibiting or degrading many proteins in a given cancer cell would not necessarily be cytotoxic. Without a direct link to cytotoxicity, escape variants could evolve more readily. Also, even if a small molecule can be identified rapidly enough for one of a patient's clonally mutated proteins through screening and/or rational design, a favorable biodistribution and lack of side effects cannot be ensured. Depending on the screening method, cell membrane permeability may also not be ensured - and could be an issue that is not easily surmounted.

Antibodies against clonally mutated proteins could be generated rapidly, i.e., in ~two weeks, using OrthoRep [34]. However, antibodies are only effective if the patient has a clonal mutation in a cell surface protein and all of the patient's cancer cells express the mutated protein. Also, they have low tumor penetrance, and the tumor microenvironment is often immunosuppressive.

Charles Swanton, Chief Investigator of the TRACERx study, co-founded a company called Achilles Therapeutics in 2016; it is currently the only company targeting clonal mutations on a patient-by-patient basis. However, they are leveraging an immunotherapy tactic to do so, specifically tumor-derived T cells [35]. 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 [36]. 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) [37,38].

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" [39–44],xxxvii,xxxviii. By having such broad tropism, they will be able to enter cancer cells, even when certain cell surface receptors are absent or down regulated. They will also enter noncancerous cells, but these cells will not have clonal mutations, so the microbe will not replicate inside of them, and will eventually be eliminated by the

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cell or can be induced to "self-destruct" after treatment. The switches in this context are RNA or protein modules that can sense and respond to target molecules. In the basal state, they are inactive. Upon detection of a target molecule, they activate. Moreover, many hyper-virulence modules could be triggered by clonal mutation detection [45–48]. Finally, if necessary, a toxic protein with a bystander effect can also be induced via small molecule after sufficient colonization/destruction of the tumors.

Somewhat similar strategies have been proposed before with oncolytic viruses, but replication was not made dependent on mutation detection. Instead, viral replication has been made dependent on the high level activity of certain promoters or expression of certain miRNAs [49–51]. One example is a telomerase promoter-specific oncolytic adenovirusxlix. Unfortunately, adult stem cells also express telomerase, and 10-15% of cancers utilize alternative lengthening of telomeres [52]. Moreover, high promoter activity and miRNA expression may not be clonal for a given patient. Also, unlike direct detection of a mutated RNA or protein molecule, cancer cell escape variants may be more likely; subclonal mutations in some of the patient's cancer cells could interfere with high level promoter activity or expression of various miRNAs.

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 [53,54]. 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 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.

Larger mutations in a promoter region could be targeted by multiplexed dCas9 or multiple TALE DNA-binding domains fused to transcriptional activators. In other words, "tiling" could be effected to enhance activation. Similarly, the target transcript could be downregulated in noncancerous cells by virtue of CRISPRi or TALE DNA-binding domains fused to transcriptional inhibitors. The resulting discrepancy in expression levels could then be used as a means of promoting replication of an oncolytic vector solely in a patient's cancer cells. If the discrepancy is not close to a 0-1 Boolean relationship, a synthetic gene circuit could be utilized to set a threshold level [55]. However, smaller mutations in promoters, e.g., point mutations, may be less easily exploited in such a manner.

Instead, smaller mutations in promoters and other clonally mutated intergenic regions could theoretically be targeted directly by DNA-binding switches [56,57]. One example of such a switch would be a dual-module ZF protein-based switch wherein both modules binding to next to each other on a DNA target sequence leads to the reconstitution of an orthogonal proteaselvi, [58],lvii. If mutations in the DNA are directly targeted, an enzymatic cascade may be required for sufficiently rapid amplification of the mutation "signal" [59]. Such a cascade might increase vector off-target activity, however. In the near future, induced transcription of any intergenic region might be possible, which might lead to less off-target activity than an enzymatic cascade-based mechanism. A third option might be to insert a larger transcription factor landing pad or replication-promoting transgene with its own promoter at the mutation site using template-jumping prime editing, for example, which does not require double-strand breaks or a DNA donor template [60].

In 2007, Alexander Varshavsky proposed a method for exploiting homozygous DNA deletions in cancer cells called "deletion-specific targeting" (DST)lvi. OVERCOME can be reversed to utilize DST for clonal homozygous deletions, as well as clonal heterozygous deletions, if replication is delayed initially using a temporal promoter cascade.

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.

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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 [61–63]. Notably, bacteria naturally colonize tumors when injected intravenously [64]. 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 [65,66].

The two intracellular bacterial species that are best studied in the context of cancer are *S.* Typhimurium [67] and *L. monocytogenes* [68]. I previously suggested the possible use of *Vibrio natriegens* as a vector because of its rapid replication rate [69] and the fact that only two genes are required for extracellular bacterial entry into mammalian cells [70], but it does not seem to survive in the cytoplasm of human cells [71]. A prophage-free strain of *V. natriegens* may be more applicable here [72]. 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 [73].

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 [74–77]. The *Salmonella* Pathogenicity Island 1 type 3 secretion system would also enable entry into a wide variety of cell types through a "triggering" mechanism [78,79]. Having broad tropism would help negate the possibility of escape variants. For intravenous injections, it may be necessary to delay the expression of cell entry modules - to allow for initial extravasation in various anatomical locales. This could possibly be achieved with a Deadman switch combined with a small molecule in the solution containing the vector [80].

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 [81]. 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 [82].

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 proteasexxxix.

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" variantxliv. Crucially, this system could detect clonal point mutations, as less than 4 mismatches in the cognate target RNA 3′ end precludes Craspase proteolytic activity [83]. If necessary, synthetic mismatches could potentially be used to imbue point mutation specificity, as with "SHERLOCK" [84].

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 [85,86], wherein one or more "stem cell" progeny cells survive and one or more "differentiated" progeny cells lyse to release RNA elements [87],lxxxii.

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 [88,89].

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-

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component regulatory system like the ComD/ComE system of *Streptococcus mutans* UA159 [90–92] or a synthetic receptor [93,94].

For DST-modified, "reverse" OVERCOME, replication of an intracellular bacterial vector at the end of its temporal promoter cascade would be driven by a pulse of gene expression [95]. To prevent the cascade from initiating outside of host cells, the *actA* promoter could be used to drive the expression of the early gene [96]. It would need to reinitiate its temporal promoter cascade at the end of each "session" of replication.

Additionally, for neuron-based cancer, *Toxoplasma gondii* could eventually be helpful [97].

Finally, it is theoretically possible that some number of patients may have no clonal mutations in their cancers. In this unlikely scenario, a set of subclonal mutations could be targeted that together are present in all of their cancer cells.

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 in the interior of the cell. Again, many cancerous mutations, if not most, affect proteins in the interior of the cell. Some affect non-coding DNA as well. The signal can also be amplified by a vector that gains access to the interior of the cell. A vector with a large amount of packaging space might be necessary to enact OVERCOME in a curative manner. An intracellular bacterium might thus be the best vector for OVERCOME. A facultative intracellular bacterium could transmit the clonal mutation detection signal to other intracellular - as well as extracellular - bacteria in a patient's tumor or tumors via a membrane-permeable small molecule, e.g., AI-1. 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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