Perspective

Transcriptome- and genome-targeted cancer immunotherapy

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Abstract

Precision oncology targets cancer cells using antibodies or small-molecule inhibitors directed against a mutated or overexpressed oncogenic *protein*. Targeted drugs are developed individually for each protein at huge costs, and only a small fraction of proteins can be targeted at all. We propose to target cancer cells through their *mutated transcripts and genomes* instead, leveraging their mutations to alert the immune system. Nanoparticles delivered systemically could be loaded with RNA- or DNA-directed Cas mRNA and guide RNAs targeting multiple patient-specific cancer mutations. In transcriptome-targeted immunotherapy, patient-specific guide RNAs would target CRISPR/Cas to cleave the transcripts mutated in the patient's cancer cells. The cleaved transcripts would hybridize with patient-specific 5'-triphosphate RNAs to form blunt-ended dsRNAs, triggering RIG-I and type-I interferon signaling that induces a strong systemic, long-lasting immune response against all cancer cells. This approach could potentially work for all solid cancers and prevent resistance by targeting many mutations jointly.

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The two key challenges for curing advanced-stage cancer

In the last decades great progress has been made in extending the life of patients with metastatic cancer. As yet, however, advanced cancer can usually at best be controlled but rarely healed. The two key challenges are (1) to kill cancer cells while sparing healthy cells and (2) to prevent the emergence of mutations that confer resistance to the treatment.

Chemotherapies with cytotoxic agents,[1] still the standard of care for most cancers, are a blunt weapon. They address the first challenge by killing the fastest dividing cells. Most of these are cancer cells, but other fast dividing cells also die, such as those that generate red and white blood cells, weakening the immune system when it is most desperately needed to keep the cancer in check. The strong adverse effects limit the drugs' doses and with it the efficacy of killing cancer cells.

Targeted therapy[2] is a sharper weapon. It relies on cancer cells requiring continuous mitogenic growth signals.[3] Most cancers supply themselves with these signals by mutations that result in the constitutive activation or overexpression of a kinase in a growth signaling pathway. Targeted therapy blocks this main oncogenic protein with a small-molecule or antibody inhibitor. Second and third generation inhibitors only affect cells that express the specific targeted oncoprotein, resulting in much weaker adverse effects.

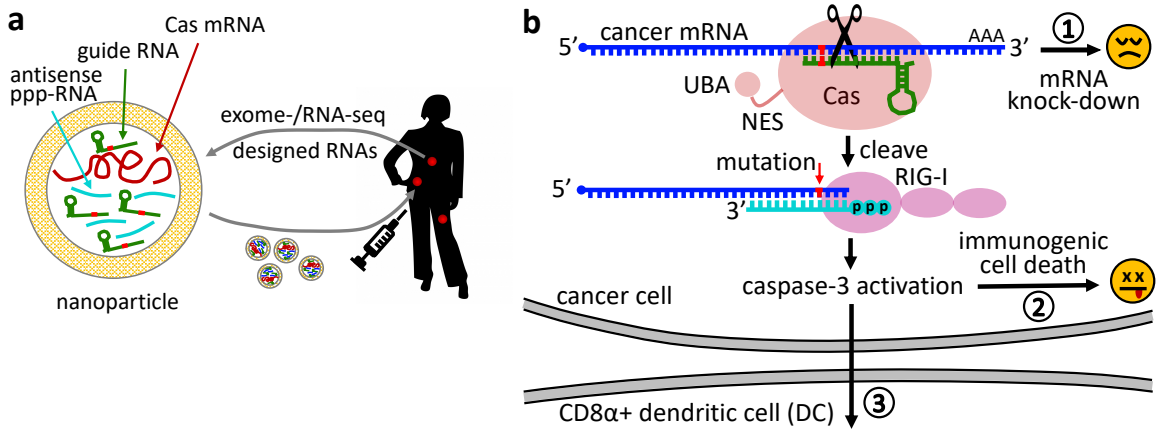

Figure 1: Transcriptome-targeted immunotherapy attacks cancer cells in three complementary ways. (①–③) **a** Nanoparticles functionalized for preferential uptake by cancer cells are injected systemically (or locally). Particles are loaded with RNA-directed Cas mRNA (e.g. CasRx[16] or Cas13a[17]), guide RNAs to target transcripts mutated in the cancer cells, and anti-sense RNAs with a triphosphate (ppp) moiety. **b** In cancer cells, Cas protein is expressed from its mRNA, binds guide RNA, and its endonuclease is activated when the guide RNA matches the target RNA sequence with a cancer-specific mutation. A nuclear export signal (NES) prevents off-target double-strand DNA cuts in healthy cells. A ubiquitin-associated domain (UBA) protects Cas from proteasomal degradation.[18] ① A large number of transcripts carrying cancer mutations can be degraded, resulting in knock-down[16,17] of mutant oncoproteins and essential proteins specifically in cancer cells. ② The anti-sense RNAs, equipped with 5'-ppp caps, hybridize with the upstream fragments of cleaved mRNAs, forming structures that mimic viral double-stranded RNAs. These get recognized by RIG-I and induce RIG-I signaling and caspase-3-mediated immunogenic cell death.[19] ③ RIG-I signaling attracts CD8α^+ dendritic cells that phagocytose the dying cancer cell and activate a long-lasting, systemic immune reaction and immune memory, which could result in complete and permanent remission even if only a fraction of tumor cells were hit by nanoparticles.

However, both approaches and particularly targeted therapy fail quite miserably with regard to the second challenge. Since only a single target protein is addressed, cancer cells find escape mutations resulting in progression usually within less than a year. Also, because a new drug has to be developed for a cost of around a billion dollars for each new protein target,[4] targeted medication is so far only available for a fraction of patients and at enormous costs.

The central role of the immune system

The success of *immune checkpoint therapy*[5] demonstrated that the second challenge, long-term prevention of cancer recurrence, is not insurmountable if the immune system is enlisted in the fight. Unfortunately, long-term remission has so far only been observed in cancers with high mutational burden and even then only in a small fraction of patients. Still, systematic achievement of long-term remission represents an enormous breakthrough that galvanized the field and convinced researchers and clinicians of the potency of the immune system to subdue metastatic cancer. It has spawned a multitude of clinical studies that combine checkpoint therapy with chemotherapy,[6] targeted therapy,[7] radiation therapy,[8] oncolytic viruses,[9] or mRNA vaccines.[10,11]

Evidence had been accumulating that chemo- and radiotherapy can only control advanced cancer over a longer time if they activate the immune system against the cancer.[12,13] A mechanism linking the two is *immunogenic cell death*:[14] phagocytosis of dying cancer cells by dendritic cells, their movement to the lymph node where they present cancer neoantigens to CD4$^+$ and CD8$^+$ T cells, followed by T cell activation, proliferation, and cancer remission. A striking manifestation of the immune system's involvement is the abscopal effect:[8] Radiation applied locally to one tumor can result in remission of distant tumors. An analogous systemic effect is observed when injecting oncolytic viruses into tumors.[9,15]

Transcriptome- and genome-targeted immunotherapy

Cancers harbor between a few and many hundreds of shared nonsynonymous mutations in their exomes, and the most common cancers (lung, colon, prostate, breast, ovarian, liver, bladder) except pancreas have between 30 and 300 on average.[20] Out of these, perhaps one third will be well expressed in cancer cells, leaving on average between 10 to 100 mutant proteins.

The two key ideas of transcriptome- and genome-targeted immunotherapy are (1) to address the specificity challenge by distinguishing cancerous from healthy cells on the basis of these cancer-specific mutations – including passenger mutations – on the transcriptome and genome rather than on the protein level, and (2) to address the resistance challenge by targeting many mutations at once and stimulating a vigorous adaptive response through RIG-I signaling and neoantigen presentation of cancer cells.

Transcriptome-targeted immunotherapy achieves this by targeting multiple mutations in the transcriptome using an RNA-directed CRISPR-Cas system. This knocks down the targeted transcripts and induces the formation of highly immunogenic blunt-ended 5'ppp-dsRNA mimicking viral RNA,[19,21] which is a strong trigger for interferon type I signaling[22,23,24] (**Figure 1**).

So far a focus of cancer research has been to use CRISPR-Cas for targeting the genomes of cancer cells through gene knock-outs or insertion of a suicide gene.[25,26]However, besides the need to reach high fractions of genome editing to be effective, the approach brings with it the ubiquitous risk of inducing off-target oncogenic double-strand cuts in healthy cells. Also, introduction of frame shifts and mutations by non-homologous end joining upon double-strand DNA cuts is a stochastic process. Therefore, a large number of neoantigens from frame-shifted peptides that are unique to each cell would be generated, which would severely impede the immune system in learning to recognize specific, recurring neoantigens.

In genome-targeted immunotherapy we use DNA-targeted Cas9 to introduce stop codons by base substitution[27] into genes that are mutated and highly expressed in cancer cells (**Figure 2**). Because the Cas9-variant has only nicking endonuclease activity, off-target double-strand breaks are not an issue. Highly expressed mutated genes would be knocked down specifically in cancer cells, and furthermore the resulting truncated proteins would be unable to properly fold, and would be targeted for proteasomal degradation. If the stop codons are introduced downstream of the mutations, this would result in the presentation of neoantigens by MHC-I on the surface of cancer cells, recognition by $CD8^+$ T cells, and killing of cancer cells.

Strong antigenicity and adjuvanticity

Emerging evidence supports the notion that cancer neoantigens are a major contributor to the recognition of cancer cells by the immune system.[28] In this spirit we suggested recently that the striking efficacy of targeted therapy – albeit mostly short-term – might be explained by blowing the cover of cancer cells by inducing the presentation of neoantigens from the targeted oncoprotein.[29] In cancers with high mutational load such as melanoma and lung cancer, dying cancer cells can supply dendritic cells that phagocytose it with sufficient amounts of cancer-specific neoantigens for training T cells to recognize the cancer cells. In cancers with low mutational load, such leukemias, sarcomas, or pancreatic cancer, we could boost antigenicity by combining transcriptome-targeted immunotherapy with genome-targeted immunotherapy.

For complete cancer remission and long-lasting protection from recurrence, strong antigenicity is not sufficient however. Rather, $CD8^+$ and $CD4^+$ T cells have to be cross-primed in a lymph node with dendritic cells.[30,31] This process starts when a dendritic cell is attracted to a dying cancer cell, phagocytoses it, migrates to the lymph node, and (cross-)presents the cancer antigens on its MHC class I and II complexes.[31] Recognition of its MHC-I-presented neoantigens by $CD8^+$ T cells and its MHC-II-presented neoantigen by $CD4^+$ T cells activates the dendritic cell, which then emits powerful chemokines, promoting the induction of high-affinity effector and memory $CD8^+$ T cells. $CD8^+$ T cells that differentiate without this cross-priming with $CD4^+$ T cells are impaired in their long-term survival and display poor proliferative ability following secondary challenge.[30,31]

RIG-I signaling is a potent adjuvant that enhances the efficiency of cross-priming.[32] It induces apoptosis via caspase-3 activation[33] and triggers interferon type I signaling in neighboring immune cells. The interferon

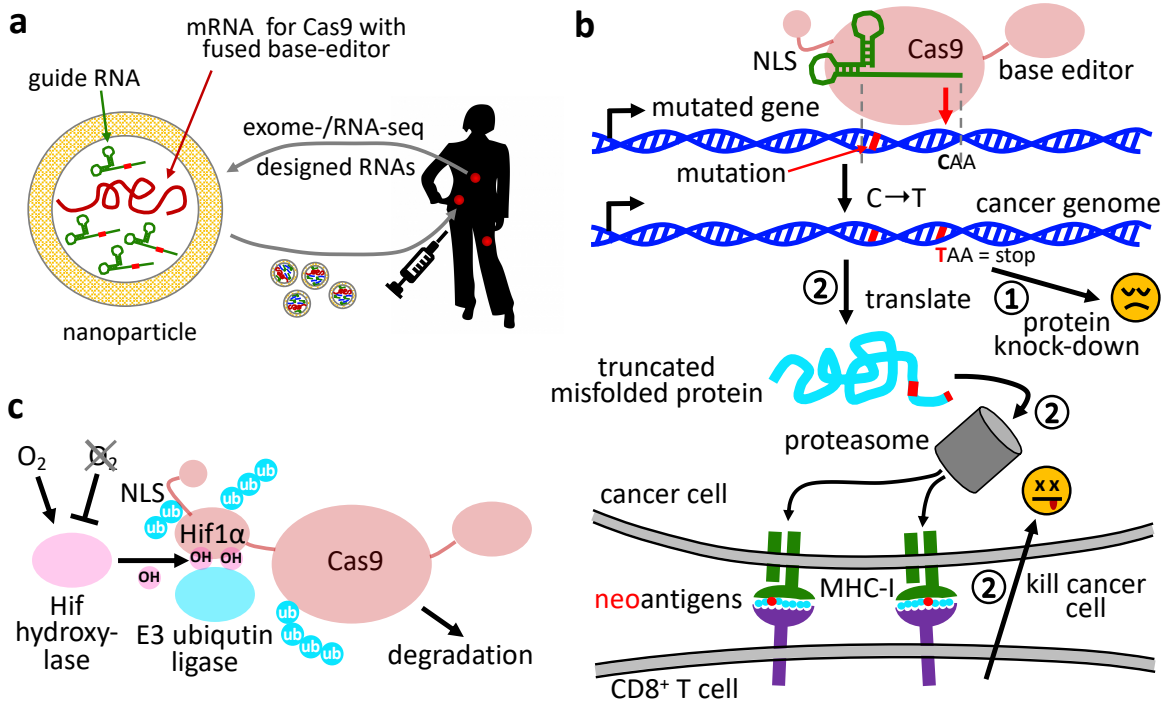

Figure 2: Genome-targeted immunotherapy boosts antigenicity by presentation of neoantigens. a Nanoparticles are loaded with mRNAs encoding a DNA-targeting Cas9 protein that only cuts single DNA strands, fused to a base-editing domain, and guide RNAs to guide Cas9 fusion proteins to mutated genomic sites in the cancer cells. **b** Cas9 protein is expressed from its mRNA, binds a guide RNA, and is imported into the nucleus by means of its nuclear localization signal (NLS), where it introduces stop codons into oncogenes with its base-editing domain.[27] ① The stop codons result in truncated, dysfunctional proteins and hence protein knock-down specifically in cancer cells. ② The truncated proteins will be degraded by the proteasome. Mutations upstream of the introduced stop codon will give rise to neoantigens that will be presented on the surface of the cancer cells on MHC-I complexes, where they can be recognized by patrolling $CD8^+$ T cells. **c** By fusing to Casp9 a domain from hypoxia inducible factor 1α (Hif1α) that recruits E3 ubiquitin ligase under normoxic but not hypoxic conditions, Casp9 will get degraded quickly except in hypoxic tumor cells.

signaling increases the expression of MHC-I in neighboring cancer cells, promotes antigen processing,[6] increases T cell infiltration of tumors, is required by dendritic cells for immune rejection of tumors,[34] and enhance the effects of chemotherapy.[13] The activation of RIG-I signaling by injection of oncolytic viruses into tumors in xenograft mouse models produced systemic tumor regression and long-lasting immunity even in hard-to-treat tumor models.[9,15]

Systemic administration of nanoparticle containing ppp-dsRNA in the KPC mouse model of pancreatic cancer with only two injections led to significantly prolonged survival and low toxicity.[35] Intratumoral injection of ppp-dsRNA nanoparticles into breast cancer tumors decreased tumor growth and metastasis.[36] In human AML xenografts in immune-reconstituted humanized mice, stimulation with 5'ppp-dsRNA sensitized tumors to therapeutic immune checkpoint blockade using anti-PD-1[37] and anti-CTLA-4 antibodies.[38] RIG-I signaling in non-tumor immune cells enables – and in fact is required for – highly efficacious anti-CTLA-4 therapy.[38] These results bode well for the combination of transcriptome-targeted immunotherapy with checkpoint inhibitor therapy.

Challenges

Transcriptome- and genome-targeted immunotherapy would require the adaptation and improvement of technologies that have already been demonstrated in animals or even reached the clinic: (1) Nanoparticles are being developed for enrichment in tumor cells by surface functionalization.[39,40] For example most tumors express matrix metalloproteinases (MMP) at high concentrations to degrade extracellular matrix proteins and invade healthy tissue. By attaching PEG chains via MMP-cleavable peptides to the

nanoparticles, nanoparticles will only be taken up by cells once they reach the tumor tissue and shed their PEG protection.[41] Also, endosomolytic nanoparticles can deliver 5'-ppp-dsRNA into the cytosol, preventing lysosomal degradation.[24] (2) Efficient delivery of mRNA into the cytosol while avoiding non-specific immunostimulation by TLR7 has recently been achieved using modified nucleosides.[42] This technology could be critical to allow systemic delivery of nanoparticles while avoiding immune reactions in healthy cells. (3) Strong, long-lasting mRNA expression in patient tissues and low-cost mass production has been impressively demonstrated by SARS-Cov-2 vaccines from BioNtech and Moderna.[11] (4) Cas9 and Cas13 orthologs have been identified or engineered that can cleave single-stranded RNA and achieve high knockdown efficiencies surpassing RNAi in human cell cultures.[16,17] (5) Delivery of Cas9 mRNAs and guide RNAs in nanoparticles produced highly efficient gene knockout in target tissues in mice and primates.[43,44] (6) The ability to knock down genes using Cas9 fused to a base-editing domain (CRISPR-STOP) has been demonstrated on two human cell lines[27] and in utero.[45] (7) Stabilization of proteins against proteasomal degradation by C-terminal fusion of a UBA domain has been demonstrated in a few studies,[18,46] even on spCas9.[47]

Besides these developments, the mRNAs, guide RNAs, and antisense RNAs need to be optimized for stability and avoidance of non-specific immunostimulation.[11,42] Engineering the antisense RNAs is a particular challenge. They should have high affinity for RIG-I while not interfering with Cas endonuclease activity, which might require a mismatched bubble not too far from the 5' end of the cleavage site.[48]

Because ppp ssRNAs also binds RIG-I, although with 24 times lower affinity than blunt-ended ppp-dsRNA,[21] they might also stimulate RIG-I signaling. Given the right dosage that is unlikely, however, because RIG-I signaling shows a threshold behaviour due to a mechanism analogous to kinetic proofreading,[49] which amplifies the 24-fold preference for 5'-ppp dsRNA ligands, and due to the highly cooperative oligomerization of RIG-I's CARD domains.[50] If immunogenicity of 5'-ppp ssRNA still turns out to be problematic, it could be minimized using modified nucleosides.[42]

The Cas protein is non-self and so might provoke an immune system attack on healthy cells. This problem is currently tackled in gene therapy and seems to have presented no limitation in a recent impressive demonstration of gene therapy in monkeys.[44] However, in our application Cas is expressed in cells undergoing immunogenic cell death, which makes the proliferation of anti-Cas effector T cells more likely. To minimize immunogenicity of Cas, the C-terminal UBA domain suppresses its proteasomal degradation[18] and thereby the presentation of Cas antigens on MHC-I complexes. Additionally, substituting exposed lysine residues on Cas with arginines excludes ubiquitination and could protect from proteasomal degradation.[51] Furthermore, patients could be desensitized to Cas protein beforehand using mRNA immunotherapy.[42] If immunogenicity still excludes systemic delivery, nanoparticles can be injected directly into tumors.

A major limitation of the genome-targeted immunotherapy approach is that, first, only certain codons can be edited into stop codons. Second, for maximum specificity the cancer-specific mutation must be contained in the seed region upstream of the edited base. Third, most CRISPR-Cas systems require a match with a PAM motif (e.g. GG). These conditions will often not be compatible, limiting the choice of targetable mutations. It might be necessary to pick for each patient from a multitude of available CRISPR-cas systems performing various substitutions[52] the system most suitable for her specific mutations.

Off-target alterations in the genomes of healthy cells are of major concern in gene therapy. CRISPR systems are highly specific when the mismatching base is within the $\sim$10 bp long seed region,[53] hence off-target effects should not be an issue in transcriptome-targeted immunotherapy: First, Cas is kept out of the nucleus, and second, a slight lowering of transcript levels in healthy cells would be inconsequential.

In genome-targeted immunotherapy, although we avoid the risk posed by oncogenic double-strand DNA cuts, healthy cells could suffer knockouts of the genes targeted in cancer cells. However, because we mostly aim for stimulating the immune system by neoantigen generation, we might not need to reach very high editing efficiency. The loss of both copies of a gene in a healthy cell would therefore be rather unlikely. Still, off-target effects will need to be carefully controlled for.

An additional measure of specificity in this regard is to fuse a domain from hypoxia-induced transcription

factor 1 α (Hif1α) to Cas9 that induces fast degradation of Cas9 in normoxic cells[54] (Fig 2c). Because cells in solid cancers are often hypoxic[55] while healthy cells in the absence of tissue injury are not, this would ensure that Cas9 is only activated in the hypoxic tumor environment.

Conclusion

Transcriptome- and genome-targeted immunotherapy have five compelling advantages: First, because mutations can be targeted all in the same way, once they work for one type of cancer they should work for most. So, despite the enormous heterogeneity of cancers,we might combat them using what they have in common: mutations. Second, in contrast to conventional targeted therapy, most cancers should be treatable, because any mutation – driver or passenger – resulting in coding changes in well-expressed proteins is targetable. Third, because we can target many mutations at once and we target cancer cells in several complementary ways, resistance development is less likely. For instance even when some cancer cells find a mutation that disables RIG-I signaling, neighboring cancer cells will provide sufficient RIG-I signals for strong adjuvanticity. Fourth, because of the exquisite specificity of CRISPR-Cas systems and by eschewing the use of dsDNA cuts, healthy cells should be rarely affected by off-target Cas endonuclease activity. Fifth, RIG-I-facilitated T cell cross-priming promises to make even immunologically cold tumors treatable and induce long-term immunity to cancer antigens.[35, 36, 37, 23]

These advantages are strong arguments for tackling the various discussed challenges to make transcriptome- and genome-targeted immunotherapy a reality. While surely not magic bullets, they might make a substantial contribution to vanquish advanced cancer by combining them with promising novel approaches: immunotherapy using checkpoint inhibitors,[38, 37] agonist antibodies and microbiome modulation , mRNA vaccination against cancer neoantigens,[10, 11] targeted therapy[2] including PROTACs, and routine screening and diagnostics using liquid biopsies.

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Conflicts of interests

The author declares to have no competing interests.

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