Next-generation sequencing technologies accelerate advances in T-cell therapy for cancer

Qinan Yin, Jiaxing Tang and Xuekai Zhu

Abstract

Next-generation sequencing has produced a large quantity of DNA or RNA sequences related to the processes occurring within tumors and their microenvironment in a reasonable time and cost. These data have been used to guide the identification of neoantigens and to determine their specific T-cell receptors. Furthermore, adoptive T-cell therapy targeting neoantigens is under development for cancer treatment. In this review, we first provide an overview of sequencing technologies and the updated findings concerning neoantigens related to adoptive T-cell therapy and then summarize the methods and principles underlying the development of next-generation sequencing-based neoantigen-reactive T-cell therapy for cancer.

Key words: next-generation sequencing; neoantigen; T-cell therapy; TILs; TCR-engineered T cells

Introduction

The order of the nucleotides in polynucleotide chains ultimately contains the complex information that determines the hereditary phenotype and biochemical properties of terrestrial life. Therefore, determining the precise order of nucleotide residues in biological research is an integral component of many research applications. Researchers have invested a great deal of time and resources to develop strategies to sequence DNA and RNA molecules over the past 50 years [1]. This review focuses on the basic concepts, advances and general applications of sequencing and how some of these strategies have played a key role in accelerating advances in T-cell therapy for cancer.

History of the development of sequencing technologies

Considering Sanger sequencing as the first-generation sequencing technology, new generations of DNA sequencing technologies have been produced subsequently. The development of next-generation sequencing (NGS) technologies has contributed to this trend by reducing costs and producing massive amounts of sequencing data. Hitherto, four sequencing generations have been defined (Table 1).

The first generation of sequencing technology is based on the chain termination method developed by Sanger and Coulson in 1975. This method involves copying single-stranded DNA with chemically altered bases called dideoxynucleotides, which, when incorporated at the 3' end of the growing chain, terminate the chain selectively at A, C, G or T. The terminated chains are then resolved by gel electrophoresis and autoradiography. This first-generation sequencing technology is characterized by its high accuracy (99.99%) and a read length up to 1000 bp [2], which is considered to be the ‘gold standard’ for clinical research sequencing. However, its high cost and low throughput has had a serious impact on its large-scale application. Its ability to generate long reads at gold-standard accuracy means that the Sanger method is the preferred method for sequencing single genes, sequencing up to 96 samples at a time without barcoding, fragment analysis, NGS confirmation and microbial identification.
Paralleling the development of large-scale dideoxy sequencing efforts, a new technique emerged that set the stage for the first wave of NGS. Markedly different from Sanger sequencing, this technique is based on either ‘sequencing by synthesis’ or ‘sequencing by ligation’ principles, where nucleotides incorporated into a strand of DNA provide a unique signal, which could be either a fluorescent molecule or a form of pH change [3]. These techniques can use massively parallel sequencing to generate thousands of megabases of sequence information. This principle of performing huge numbers of parallel sequencing reactions became defined as second-generation sequencing (SGS), which is currently the most commonly used NGS technology. SGS/NGS profoundly affected biomedical research, including studies on whole-genome sequencing, structural characterization, copy number variation, profiling epigenetic modifications, transcriptome sequencing and identification of infectious agents [4]. SGS/NGS systems are typically represented by 454 Life Sciences, Illumina HiSeq and Applied Biosciences (SOLiD system). Of the three NGS systems, the 454 system has the longest read length (700 base pairs) [5], the Illumina HiSeq has the lowest cost ($0.02/million bases) [5] and the SOLiD system has the highest accuracy (greater than 99.85%) [6]. Although these SGS/NGS technologies have characteristic advantages, there are limitations to the different systems, such as the relatively high error rate in the 454 system [7], and difficulties in sequencing palindromic sequences [8].

There is a considerable discussion concerning the distinction between the second- and third-generation sequencing technology [9–12]. Third-generation sequencing is characterized by the direct detection of the nucleotide composition of target DNA molecules without any amplification step. In addition, the nucleotide sequences are read at the single molecule level. This technique provides improved accuracy by eliminating the risk of bias introduced during the preparation of sequencing libraries [2]. Sequencing single molecules also allows direct sequencing of RNA molecules; detection of chemically modified bases, such as DNA methylation; and increased read lengths. Longer reads will be useful in de novo sequencing projects and permit experimental phasing [12]. At the time of writing, the most widely used third-generation technology is probably the single molecule real-time (SMRT) platform from Pacific Biosciences (PacBio) [13]. This platform generates read lengths of over a 1000 bases; however, the error rate (11%–15%) is significantly high, and its throughput is currently limited to 0.1 Gb per run [14]. The PacBio system has been applied in de novo sequencing, base modification detection and isoform sequencing of transcriptomes [15]. Another new technology for single molecule sequencing is the Nanopore sequencer from Oxford Nanopore Technologies (ONT). It is based on detecting natural electric or chemical differences between nucleotides and does not require labeling of DNA. This platform is more widely applicable because of its high portability and small size. Meanwhile, advantages such as a longer read length and the lower cost (around $1000 for the whole human genome) could make the ONT platform a routine experimental technique in biomedical laboratories [16].

Fourth-generation sequencing systems permit in situ sequencing in fixed tissue and cells using SGS technologies [17]. Fourth-generation sequencing can be used in certain applications, such as the analysis of cell populations with single-cell resolution. The efficiency of this technique needs to be improved in terms of standardization, cost effectiveness, practicality and full integration into the current sequencing system.

Each sequencing technology has particular strengths and weaknesses. Thus, hybrid sequencing has become a popular approach to fully exploit the advantages of more than one platform to produce data that can be qualified or refined using high-throughput and high-accuracy sequencing.
Typical sequencing technologies used in cancer research

In cancer research, the availability of genome sequences permits real-time decision-making with the potential to affect diagnosis, prognosis and treatment and could lead to the development of personalized medicine. In the fight against cancer, researchers have identified the great potential in harnessing and boosting the efforts of the immune system to attack cancer cells. The immune system plays an integral role throughout the life cycle of many cancers, including preventing initiation, suppressing development and influencing treatment and patient outcome [18, 19]. Therefore, immunotherapy has been accepted as a promising new strategy to fight cancer in which autologous T cells have emerged as a powerful treatment option [20, 21].

Sequencing technologies are changing the pattern of clinical genetic testing. Whole-exome sequencing (WES) is a technique whereby only the sequences of the coding regions of DNA are determined, although it is possible to target any desired region of the genome [22]. The exome contains approximately 85% of disease-causing mutations but only represents 1% of the whole genome [23]. Analysis of paired tumor-normal WES has emerged as an ideal method to discover somatic mutations. For instance, WES was used to identify primary mutations in phaeochromocytoma (PCC) and paraganglioma (PGL) when no mutations were found in the common susceptibility genes for PCC/PGL [24, 25]. Briefly, WES is carried out on tumor cells and matched normal tissue to identify the somatic mutations in the tumor cells. DNA of the host and the tumor cells is extracted and broken down into fragments. The coding DNA fragments are then sequenced using artificial DNA or RNA baits that are complementary to the targeted DNA. After removal of the non-coding sequences, the coding sequences are amplified, sequenced and analyzed using a reference genome. By conceptual translation of the DNA codons, those DNA mutations that result in an altered sequence of amino acids can be identified. WES provides information about mutations that might help to identify tumor-specific neoantigens that can boost T-cell immunity. However, WES has its own limitations, such as missing out regions adjoining the exons, including promoters, enhancers and transcription factor binding sites, unless the probe set is designed to cover them [26].

Although WES is the most comprehensive method to quantify the mutational burden and characterize mutational patterns, it remains an expensive technique, and many researchers need to focus their analysis on specific genomic regions and specific genes to approach disease genetics. NGS can be coupled with DNA capture or enrichment methods to resequencing targeted regions of interest. The targeted NGS panels may also be applied to infer the overall mutational burden at a lower cost [27]. Briefly, genomic DNA is randomly sheared and ligated to universal adapter sequences. Randomly fragmented, denatured genomic DNA is hybridized with oligonucleotide probes. The regions of interest are captured by complementary probes, either on a microarray surface or in solution. The hybrid-selected enriched output library is then eluted and processed for sequencing. Despite providing efficient, cost-effective alternative to WES sequencing, in targeted sequencing methods, unprecedented demands are placed on the upfront methods of sample preparation because of the large ‘appetite’ for templates for the massively parallel sequencers.

NGS has replaced conventional sequencing in many clinical settings over the past decades. NGS-based RNA sequencing (RNA-Seq) has revolutionized gene expression studies by enabling researchers to measure relative expression changes across the whole genome. The technique uses RNA instead of DNA in the analysis and yields both the expression profile and mutational status, including the characterization of splice variants, antisense transcription, fusion genes, allele-specific expression, RNA editing and other forms of sequence variation in the transcriptome [4, 28]. Universal to all RNA-Seq preparation method is the conversion of RNA into cDNA, because most sequencing technologies require DNA libraries. The generation of the cDNA library often varies between platforms [23, 29, 30]; however, they share general basic steps to construct a cDNA library for sequencing. RNAs can be classified into polyadenylation [poly(A)]+ or poly(A)− transcripts according to the presence or absence of a poly(A) tail at their 3’ ends. Typically, the cDNA libraries used for RNA-Seq are prepared from the enrichments of transcripts with a poly(A) tail [31]. For regular poly(A) enriched RNA preparations, RNA is extracted from tissue and mixed with deoxyribonuclease to remove the genomic DNA. After then, the RNA can be filtered with 3’ poly(A) tails to include only mRNA, depleted of the highly abundant ribosomal RNA (rRNA), which typically constitutes over 90% of total RNA in the cell. The RNA with 3’ poly(A) tails is mature, processed, coding sequences. Poly(A) selection ignores noncoding RNA and introduces a 3’ bias [32], which is avoided after removal of rRNA. Poly(A) selection is performed by mixing RNA with poly(T) oligomers covalently attached to a substrate, typically magnetic beads [33, 34]. Alternatively, it is also possible to overcome the limitations of using poly(A) selection by applying methods that deplete rRNA from commercially available kits, such as RiboMinus (Life Technologies) or RiboZero (Epicentre). Thereafter, the RNA is reverse transcribed into cDNA and sequencing adapters are added. After cDNAs have been sequenced using a sequencing platform, the resulting short-read sequences can be analyzed to answer the biological questions of interest.

Properties of neoantigens

Sequencing is frequently used to identify and characterize tumor-specific antigens. Based on the sequencing results and the subsequent validation, the antigens that can be targeted by the immune system become clear.

First, the antigens to be targeted by the immune system should be only expressed in the tumor cells. Otherwise, normal cells expressing the same antigens will also be attacked. At least two types of antigen fit with the tumor-specific expression requirement. One is cancer testis antigens, which are expressed only in cancer and testis cells; however, normally testis does not express the antigen-presenting molecules. Based on the expression pattern, sequencing results can help to identify cancer testis antigens from tumors [35, 36]. The other type of antigen, termed neoantigens, results from mutations that occur in the tumor cells, which have not been presented by thymic epithelial cells; therefore, they have no central tolerance problem. It is possible to induce T cells with high affinity for their T-cell receptors (TCRs) using neoantigens compared with self-antigens.

According to the mutated sequences, neoantigens can be derived from both single nucleotide variation mutations and frameshift mutations caused by insertions and/or deletions, if the encoding sequence for the amino acid is non-synonymous. Both types of mutation have been found extensively in tumor cells and have been analyzed to identify neoantigens [37–39].

According to their influence on tumor behavior, mutations can be divided into two groups. ‘Driver’ mutations promote carcinogenesis and disease progression, while ‘passenger’ mutations are incidental to cancer progression [40]. The correlation of driver
mutations with tumor progression may make them better targets for therapeutic immune responses. Neoantigens derived from driver mutations have been identified in lymphoma [41, 42], gastrointestinal cancer [43] and melanoma [44]. However, neoantigens are frequently passenger mutations [43, 45, 46].

Some neoantigens, such as the histone 3 variant H3.3K27M mutation [47], the MYD88L265P mutation [42] and the KRASG12D hotspot-driver mutation [43, 48], are present in several patients. However, most neoantigens are unique to a single patient [43, 49, 50]. It seems that one single neoantigen can appear in all the metastatic sites within a patient [51]. However, within one tumor entity, the distribution of different mutations is heterogeneous [52, 53].

Different types of cancer cell harbor different amounts of mutations. Cancer with a high mutation load often is immunogenic and shows a good response to immunotherapy. However, in cancer cells with a low mutation load, neoantigens also occur [54, 55], although they are more difficult to detect.

Therefore, neoantigens have been identified in many types of cancer, including microsatellite instability-colon cancer [36], gastrointestinal cancer [43], triple-negative breast cancer [51], lung cancer [56–60], bladder cancer [61], head and neck cancer [62, 63], ovarian cancer [64], pancreatic cancer [65] and cancers with DNA-mismatch-repair deficiencies [66].

Neoantigen validation

The basis for mutations becoming neoantigens includes two aspects (Figure 1, Table 2). First, the epitope containing the mutation(s) should bind to a major histocompatibility complex (MHC) with sufficient affinity to be presented on the cell surface. Second, the epitope should also interact with a TCR with adequate affinity to stimulate the corresponding T cell.

Based on MHC-binding algorithms, bioinformatics can be used to predict the neoantigen from its mutations [68, 69]. The TCR-binding ability of a neoantigen can also be predicted based on solvent-exposed possibilities, which determine the accessibility to the TCRs [69]. Information on the frequency of neoantigens extracted from an immunopeptidomics database [81] and the expression levels from RNA-Seq data [67] can assist the identification of neoantigens in silico.

Although bioinformatics requires little cost and time, ultimately, a neoantigen must be confirmed experimentally.

The MHC-binding ability of a neoantigen can be checked by mass spectrometry analysis of eluted peptides from MHC molecules on the cell surface [35, 69–73]. The advantage of mass spectrometry is a direct identification of truly presented epitopes. However, the approach may face the sensitivity problem as enough amounts of total proteins are still required for one analysis. And mass spectrometry may fail to detect those epitopes with low frequencies on the cell surface.

An alternative method to check the binding of neoantigens to MHC molecules would be to express the encoding genes with the mutations or pulsing the predicted mutated peptides, and let the antigen-presenting cells (APCs), such as autologous dendritic cells or B cells, select the epitopes that can bind to their MHC molecules [44, 74]. In this approach, one minigene encoding 25 amino acids, in which the mutated amino acid is located in the middle, is designed. A tandem minigene (TMG) encoding 12–24 minigenes is then synthesized and cloned into an expression vector to evaluate a relatively large number of mutations simultaneously. Based on a similar principle, 25-aa-long peptides with the mutation in the middle can be synthesized and combined together for pulsing [74].

If a mutated peptide can be presented on the surface in complex with MHC molecules, the next step would be to check whether the peptide could be recognized by a TCR molecule on the T cell. Two methods have been developed to detect neoantigen-specific T cells in tumors or peripheral blood (PB).

The first method tries to detect neoantigen-specific T cells directly. After prediction of MHC-binding peptides, panels of peptide-MHC multimer containing different mutated peptides can be generated and used to bind the T cells. If T cells can be detected, the corresponding peptide is identified as a neoantigen [35, 75].

The second method is to detect T-cell responses when the mutated peptides are used to stimulate the T cells, either alone or presented on APCs. Cytokines secreted by activated T cells can be analyzed by an enzyme-linked immunoSpot assay [35, 75] or enzyme-linked immunosorbent assay [68, 73, 76] or the activation of markers on T-cells surface, such as PD-1, 4-1BB or OX40, which can be measured by flow cytometry [74, 77].

Determination of neoantigen-specific T cells

It is important to perform a functional analysis of neoantigen-specific T cells after the identification of neoantigens, as they may influence cancer progression.

First, to study neoantigen-specific T cells, they need to be enriched or isolated. Neoantigen-specific T cells have been often found in tumor infiltrating lymphocytes (TILs) [75, 76, 82, 83], suggesting that TILs can be a good source. Using peptide–MHC multimers, 3% or 0.003% of TILs prepared from a patient with melanoma recognized two predicted neoantigens, respectively [83]. PB is another attractive source for neoantigen-specific T cells, as the use of PB avoids the surgical step. For example, 0.002–0.4% T cells were isolated from the PB of a melanoma patient before immune therapy, which could recognize the neoantigens [84]. Isolation of neoantigen-specific T cells can be achieved by flow cytometry based on peptide–MHC multimer staining of T cells from TILs or PB. If neoantigens are not recognized first, neoantigen-specific T cells may be enriched by flow cytometry, according to the surface expression of activation markers, such as CD137 and PD-1 [74].

The anti-tumor function of enriched neoantigen-reactive T cells has been proved in a preclinical study, in which they were shown to inhibit the growth of autologous patient-derived xenografts in mice [85].

Cloning of neoantigen-specific TCRs

With the advances in sequencing technology, neoantigen-specific TCRs can also be cloned from T cells (Figure 1). If the neoantigen-specific T-cell clones are available, TCR sequences can be obtained from a single clone directly. In most cases, the sample comprises polyclonal T cells, which contain information from mixed TCR chains. Deep sequencing of TCR chains can be achieved first and then the paired sequences can be acquired based on pairSEQ analysis [74, 77].

Recently, single-cell RNA-Seq has been used to acquire paired TCR a/b sequences directly, which simplifies the analysis [78]. When the data are correlated with cells expressing high levels of interferon-gamma and interleukin-2, neoantigen-specific TCRs can be predicted.

Neoantigen-reactive T cells can be found in TILs and autologous PB; apparently, these T cells will supply the resources for cloning neoantigen-specific TCRs [79, 84]. It seems that the specificities of TCRs from PB are similar to those from tumors [79].
Figure 1: Development of NGS-based neoantigen-reactive T-cell therapy for cancer. NGS is used to identify non-synonymous mutations in the resected tumors. Identification of neoantigens is based on analysis of MHC-binding and TCR-binding abilities of the encoding mutant peptides. MHC-binding epitopes can be predicted by bioinformatics, then the high-affinity binders will be used to generate peptide–MHC multimer, if they indeed can bind to their MHC molecules. The mutant epitopes can also be identified directly by mass spectrometry from MHC molecules on the tumor cell surface. An alternative method without the requirement of knowing binding affinity is letting the autologous APCs select the epitopes, which are encoded by non-synonymous mutations. TMGs or long peptides can be used to deliver mutations onto the APCs. When the epitopes can be presented on the APCs or form peptide–MHC tetramers, they will be used for TCR-binding analysis. TILs, T cells from a patient’s blood or T cells from an unrelated donor will be co-cultured with the APCs and checked for T-cell activation, including cytokine detection or analysis of activation markers by flow cytometry. Alternatively, neoantigen-specific T cells can be detected directly by peptide–MHC tetramers. Enriched or isolated neoantigen-specific T cells can be used in the clinical study for adoptive T-cell therapy. The neoantigen-specific TCRs can also be cloned from the above T cells based on NGS and used to generate TCR-engineered T cells for cancer treatment.
PB from unrelated donors can also be a source of neoantigen-specific TCRs [80], which may be superior, as they have not undergone immunoediting during tumor development.

**Development of TCR-engineered T cells**

Although neoantigen-specific T cells exist in TILs, the preparation of these cells can be a challenge, because TILs cannot be expanded from some patients; e.g. 30% of resected melanoma samples fail to produce active and specific TILs for the therapeutic use [86]. In addition, the percentage of neoantigen-specific T cells in autologous PB normally is low. Therefore, an attractive strategy is to introduce neoantigen-specific TCRs into T cells in vitro, which can be autologous blood T cells or naive T cells from a donor’s PB [87]. T cells engineered with neoantigen-specific TCRs increase the steps of manufacture and may cause more regulatory challenges. However, TCR-engineered T cells have more possibilities for genetic manipulation and potentially better efficacy.

Neoantigen-specific TCR-engineered T cells can specifically recognize tumor cells harboring the relevant mutations or autologous APCs presenting the corresponding neoantigens [78–80]. Furthermore, they can significantly suppress the progression of tumors in mice and enhance the survival of tumor-bearing mice [37, 47] (Table 3).

**Neoantigen-specific T cells in clinical studies**

The ultimate question would be whether neoantigen-specific T cells are sufficient to cure patients with cancer. Until now, few clinical studies aiming to use neoantigen-specific T cells directly have been reported. A retrospective study showed that transferred TILs containing 95% neoantigen-reactive T cells could mediate tumor regression in one patient with cholangiocarcinoma [82]. Recently, a pioneering work demonstrated that using selected TILs with 75% polyclonal T cells, which targeted a driver mutation, mediated objective regression in one patient with metastatic colorectal cancer [48]. More studies are needed to determine the overall response rate of neoantigen-specific T cells.

Inducing or using neoantigen-specific T cells directly for cancer treatment is an attractive strategy. Increasing data show that neoantigens or neoantigen-specific T cells correlate with the efficacy of cancer immunotherapy, including adoptive T-cell therapy [88]. A high predicted neoantigen load and enriched immune activation signatures are associated with improved progression-free and overall survival in adoptive TIL transfer therapy [88]. Therefore, increasing the percentage of neoantigen-specific T cells in the products transferred into patients may improve clinical efficacy.

Finally, neoantigens are specific for tumor cells, and the current clinical data for the use of neoantigen-reactive T cells have not shown any major toxicity [48]. Therefore, adoptive T-cell therapy specifically targeting neoantigens is probably safe.

Sequencing technologies have inspired the discovery of the influence of neoantigens on T-cell immunity in cancer progression or regression. Studying the changes of neoantigens and neoantigen-reactive T cells in the context of adoptive T-cell therapy shows that neoantigens can become lost and the expansion of T-cell clones can occur after therapy, which means that neoantigen-specific T cells contribute to the killing of tumor cells expressing neoantigens [89]. However, this finding also raises the problem of tumor resistance.

**Challenges for adoptive T-cell therapy targeting neoantigens**

Although a CR was achieved after neoantigen-specific T-cell-based immunotherapy, subsequent tumor recurrence in some patients has been observed [48, 90]. Mechanism analysis

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**Table 2: Development of neoantigen-specific TCR-engineered T cells**

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<td>Prediction by bioinformatics</td>
<td>[35, 69–73]</td>
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<td>Mass spectrometry</td>
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**Table 3: Application of neoantigen-specific TCR-engineered T cells for cancer therapy**

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<tr>
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Future perspectives

In the future, NGS-based neoantigen-specific T-cell therapy should be designed to meet the above challenges. For heterogeneous tumors without shared neoantigens, polyclonal T cells should be used to target a sufficient number of essential antigens. Alternatively, a combination with a neoantigen vaccine may be chosen to boost more diverse T-cell responses [71, 93, 94]. If it is possible to select targets, the optional neoantigens should be those epitopes encoded by driver mutations or shared by all the tumor clones. A recent study showed that the quality of neoantigens is critical to the long-term survival of patients with cancer [95]; therefore, neoantigens that fit the quality model are also good targets for T-cell therapy. Targeting maximal heterozygosity at human leukocyte antigen (HLA)-I loci may be a strategy to enhance the T-cell response and counteract the partial loss of HLA-I molecules [96], because recently, the HLA Class I genotype has been shown to influence cancer response to checkpoint blockade immunotherapy [92]. The diversity of the TCR repertoire should also be considered because immunodetecion occurs [91], or the tumor microenvironment evolves continually in the setting of T-cell immunity [92]. In these cases, donor-derived TCRs may be a better choice.

Finally, using TCR-engineered T cells can solve the problem of manufacture failure of TILs, supply freely designed targeting specificities and give more opportunities for additional modifications of the transferred cells. Gene engineering is a powerful tool to manipulate the functions of neoantigen-specific T cells by knocking out inhibitors or compelling the expression of enhancers. NGS will be involved in the discovery of these regulator genes and in the analysis of foreign genes in the transferred products.

Collectively, new sequencing technologies such as WES, targeted sequencing and RNA-Seq can provide new insights for the understanding and manipulation of T-cell-positive therapeutic outcomes across many human cancers, which will facilitate T-cell transfer therapy for cancer.

Key Points

- Next-generation sequencing supplies databases for the identification of neoantigen in tumors.
- A neoantigen can be validated experimentally based on its affinities of MHC binding and TCR binding.
- Next-generation sequencing can accelerate the determination of neoantigen-specific T cells.
- Neoantigen-specific T cells may contribute to a cure for cancer.

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