Review

Chimeric antigen receptor T-cell immunotherapy: What pathologists need to know?

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Abstract: Chimeric antigen receptor T-cell (CAR-T) immunotherapy genetically modifies patients' own T cells to specifically target cancer antigens on cell surface. CAR-T uses a single chain chimeric antigen receptor and functions in a manner independent of major histocompatibility complex and antigen processing/presentation. CAR-T has shown dramatic improvements in disease free survival in clinical trials on CD19-positive B-cell malignancies, especially B lymphoblastic leukemia/lymphoma. Because of the revolutionary nature of CAR-T, it is expected to become mainstream anti-cancer therapy in the near future. Pathologists have an essential role in CAR-T related patient care and need to be aware of and prepared for the prime time of CAR-T immunotherapy.

Keywords: Chimeric antigen receptor, cancer immunotherapy, B cells, leukemia, lymphoma

Introduction

Cancer is the second top killer in the United States (US) and is responsible for approximately 25% of total death in the US. Nearly 590,000 deaths due to cancer are expected in 2015 [1]. In addition to the standard adjuvant therapies of cancer, new disciplines of oncotherapy have quickly emerged in the last two decades. One of the fastest progressing fields is cancer immunotherapy, which was named as the "Breakthrough of the Year for 2013" by *Science* magazine [2].

Immunotherapy is the treatment of diseases by finely tuning the immune system, such as inducing, enhancing, or suppressing immune responses. Cancer immunotherapy is to utilize the immune system to treat cancer. Cancer immunotherapy can be divided into several distinct subtypes based on various technology used (Table 1). This review focuses on one subtype of adoptive T-cell therapy: chimeric antigen receptor (CAR) T-cell immunotherapy.

Adoptive cell transfer (ACT) is the transfer of immune cells into a patient's body with or without genetic modification. ACT has three forms: 1) tumor-infiltrating lymphocyte (TIL) therapy, which has been used for melanoma only; 2) T-cell receptor (TCR) therapy, which has been tested on melanoma, and synovial sarcoma; and 3) CAR T-cell (CAR-T) therapy, for which the most successful studies up-to-date are on leukemias and lymphomas, especially CD19+ B lymphoblastic leukemia or B-cell acute lymphoblastic leukemia (B-ALL). CAR-T targets cell surface antigen in a major histocompatibility complex (MHC)-independent manner and has shown great potentials in multiple clinical trials. CAR-T is believed to bring a *bona fide* breakthrough in cancer therapy.

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Table 1: Types of cancer immunotherapy.

Types	Mechanisms	Examples
Antibody therapy	Antibody-dependent cell-mediated cytotoxicity	Rituximab (anti-CD20) Vemurafenib (inhibiting mutated BRAF)
Cytokine therapy	Modulating immune system to provoke immune responses	Interleukin 2 Interferon α
Adoptive cell transfer	Endogenous (such as in TILs) or engineered (such as in CAR-T or TCR) T cells with increased specificity in cancer targeting are expanded *ex vivo* and infused back into patient's body	TILs (for melanoma) CAR-T (for B-ALL) TCR (for melanoma, synovial sarcoma)
Immune checkpoint blockade	Blocks the inhibitory immune checkpoints and allows lymphocytes to destroy cancer cells	Nivolumab (anti-PD-1) Ipilimumab (binds to CTLA-4)
Therapeutic vaccine	Engineering patient's own antigen presenting cells to express cancer antigen	Sipuleucel-T for metastatic prostate cancer
Oncolytic viruses	Engineered virus with increased cancer killing	T-VEC (talimogene laherparepvec) for melanoma

Note: CAR-T: Chimeric Antigen Receptor T immunotherapy; CTLA-4: cytotoxic T lymphocyte-associated antigen 4; PD-1: Programmed cell death protein 1; TCR: T-cell receptor immunotherapy; TILs: Tumor infiltrating lymphocytes.

Brief History of CAR-T

The concept-establishing paper was from Dr. Zelig Eshhar's group in 1989 [3], which demonstrated that expressing "chimeric T-cell receptor" in cytotoxic T-cell hybridoma provides antibody-like specificity and induces T-cell activation in a "non-major histocompatibility complex-restricted manner". The so-called "chimeric T-cell receptor" is composed of immunoglobulin variable domains and T cell receptor constant domains. Another finding essential for the working design of CAR-T is the observation that single peptide designs with the cytoplasmic portion of CD3 zeta (CD3 ζ) chain largely recapitulate the functions of T-cell receptor, which is a multi-unit complex [4–6].

The first clinical trials of CAR-T are for HIV infection [7], from which the safety of CAR-T method was confirmed. For cancer therapy, the early versions, the so-called "first-generation" CAR-T, was tested on metastatic ovarian cancer and renal cancer [8, 9]. However, these studies showed suboptimal T cell persistence and no significant antitumor effects.

Continuous efforts from many research groups were carried out to improve the efficacy of CAR constructs. The "second-generation" CARs were endowed with signaling domains of costimulatory molecules, such as CD28 or CD137 (4-1BB). Anti-CD19 CARs based on second-generation design has shown marked anti-tumor effects on B-cell malignancies. For examples, anti-CD19 CARs were tested on relapsed and refractory B-ALL by several medical centers, all of which showed potent anti-tumor effects with complete remission (CR) rates of 70%-90% [10–12].

Design of Chimeric Antigen Receptor

Chimeric antigen receptor is a single chain peptide chain, composed of an extracellular targeting domain, a hinge domain, a transmembrane domain and intracellular signaling domains [Figure 1]. The targeting domain is based on the sequence of the variable fragments of a monoclonal antibody against the target antigen. The construction method of the

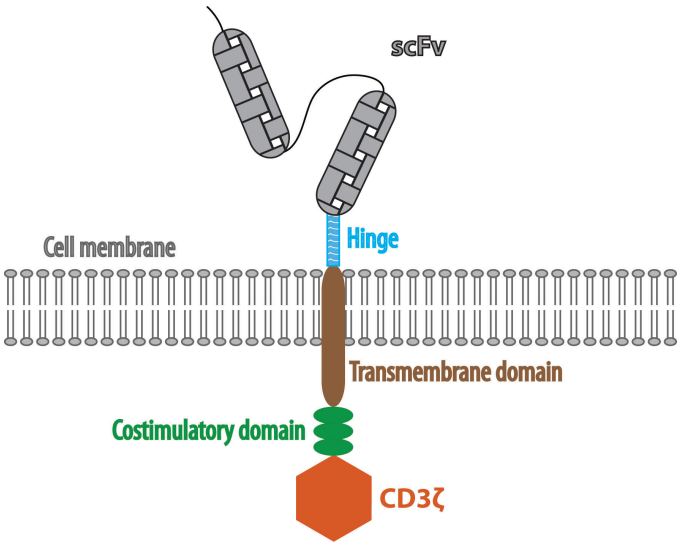

Figure 1: The domain structure of CAR.
Chimeric antigen receptor are composed of: 1) the extracellular targeting domain, derived from single chain variable fragment (scFv) that is composed of light and heavy chain variable domains of a monoclonal antibody with linker sequence in between; 2) the hinge domain; 3) the transmembrane domain; 4) the costimulatory domain(s), the number of which depends on the generations of design; and 5) the CD3 zeta chain (CD3ζ) of the T-cell receptor complex.

targeting domain is called single chain variable fragment (scFv), which fuses the variable domains of heavy and light chains of the antibody with linker peptides. The hinge domain is believed to affect the flexibility of the targeting domain, which is important for target binding [13]. The signaling domain is typically CD3ζ of the T-cell complex. For the second and third generations of CARs, one or two costimulatory domain(s) are added, respectively. The most commonly used costimulatory domains include CD28, CD134 (OX40), and CD137 (4-1BB). It is now clear that co-stimulatory molecule(s) are the main reason of the much improved T-cell persistence observed in clinical trials of newer CAR constructs.

CAR-T works in a MHC-independent manner. Therefore, one design of CAR works for many patients diagnosed with the same disease. Another advantage of CAR-T is its independence of antigen processing and presentation, which is a common mechanism of immune escape by tumors [14].

Target Selection

Selecting a valid target for CAR-T is a critical and, often difficult, task. There are stringent criteria to follow. First of all, the target molecule has to be a surface antigen, and ideally it should ubiquitously express on nearly all tumor cells. To minimize antigen escape, the molecules essential for tumor survival and/or malignant behavior are preferred. To minimize toxicity on normal tissues, it is required to be no off-tumor expression in essential organs (such as brain, heart, etc.) and essential cell types (such as hematopoietic stem cells). Only minimal, if any, off-tumor expression is allowed in non-essential organs.

Using the aforementioned criteria, CD19 is considered an almost perfect candidate for CAR-T, which is one of the main reasons underlying the great success achieved in B-cell malignancies. CD19 is a pan B-cell surface marker, and is ubiquitously expressed at nearly all stages of B-cell development, from immature B-cell precursors (aka. hematogones) to mature B cells and to terminally differentiated plasma cells. As an indispensable marker for B-cell lymphoma panels of flow cytometry, CD19 is detected on nearly all B-cell lymphomas and leukemias. In addition, B cell aplasia, as a side effect of collateral destruction of normal B lymphocytes, is treatable and tolerable.

The criteria for target selection are quite stringent and it takes tremendous efforts in finding valid targets. First of all, we have gained our knowledge of biomarker expression from research activities that usually focus on pertinent tissue types only. As a hypothetical example, muscle markers would be studied mainly in (different types of) muscles. It is not always straightforward to find out whether a muscle-related marker express in the brain. Therefore, it is a non-trivial task to examine the expression patterns of any candidate CAR-T targets across all organs

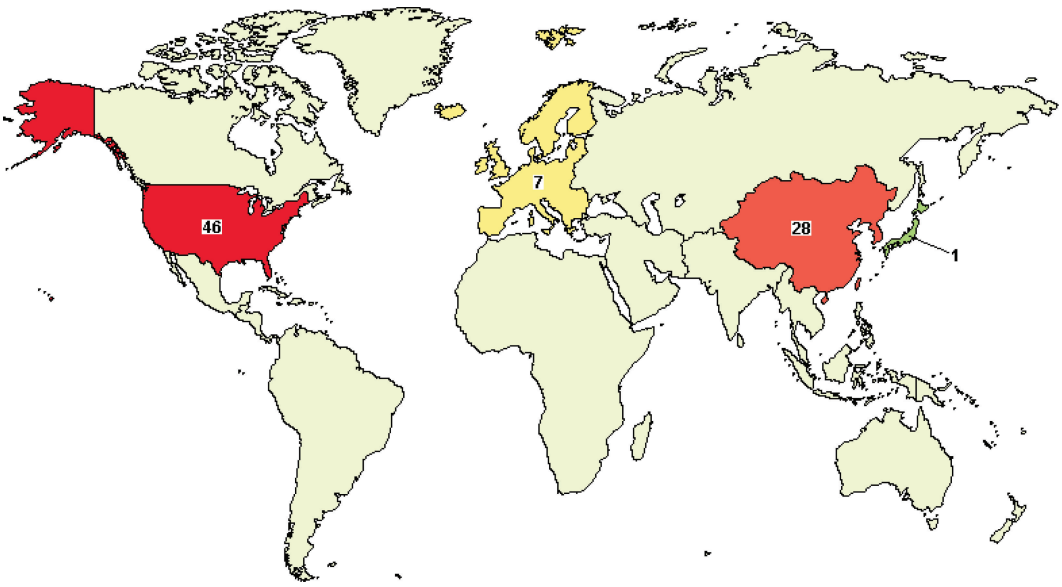

Figure 2: Geographic distribution of chimeric antigen receptor T-cell immunotherapy clinical trials.
Of the 81 open clinical trials, 46 are in the U.S., 28 in East Asia and 7 in Europe. (Source: http://ClinicalTrials.gov Date: February 11, 2016)

and tissue types. In addition, biomarkers may be differentially expressed during various developmental stages, between genders, and among ethnic groups. To ensure the safety of one tumor marker and to establish a successful therapy method, it is extremely important to explore these multi-dimensional factors potentially causing the differential expression of candidate targets.

Unexpected deviation of a target could result in devastating results in immunotherapy. For example, MAGE-A3 was used as the target of TCR immunotherapy for non-small cell lung cancer (NSCLC) in a NCI clinical trial (protocol NCT01273181). Two of the nine patients developed coma and died during the trial, which was contributed to the low level expression of MAGE-A3 in brain tissue discovered afterwards [15]. Even though no such examples exist for CAR-T clinical trials, it is reasonable to expect similar dire consequences from CAR-T therapy under similar situations.

Workflow of Gene Engineered T-cell Production

First of all, the patient's own peripheral blood mononuclear cells (PBMC) are obtained by apheresis. T cells are often enriched and activated, and the *ex vivo* culture systems used are believed to affect the compositions of the final T-cell products, regarding the percentages of naïve, memory, and effector T-cells, which proportionally relate to the replicative potential, and persistence. The ideal compositions of variable subtypes of T cells are still under investigation. Gene transfer of CAR constructs into the T cells is usually done by either retroviral or lentiviral vectors, which are the two most popular methods. Amplification and enrichment of CAR-modified T cells are then performed, followed by formulation before CAR-modified T cells are infused back into the patient's body. The production process needs multiple points of quality control and needs to meet criteria of Good Manufacturing Practices (GMP) guidelines. The production methods available are based on open systems, labor intensive and

needs highly skilled personnel.

Anti-CD19 CAR-T Clinical Trials for B-ALL

B-ALL is a neoplasm of B-cell precursors. The overall survival of ALL is significantly better in pediatric patients (85%) than adult patients (40%) [16]. However, treating relapsed ALL is very challenging for all age groups and the options are limited. On the other hand, certain cytogenetic abnormalities in B-ALL are known to be associated with poor prognosis, such as *BCR-ABL1* translocation, or variable MLL translocations. Even though targeted therapies designed for a specific cytogenetic abnormality was shown to improve survival in B-ALL in the era of imatinib [17], there are no known driver mutations/abnormalities for the majority of B-ALL.

CD19-targeting CAR-T immunotherapy against B-cell malignancies is the best-tested CAR-T therapy to date. Clinical trials of B-ALL have shown robust responses and spectacular results. Results and details of the B-ALL trials from three medical centers, including Children's Hospital of Philadelphia and University of Pennsylvania (CHOP/UP) [12], Memorial Sloan-Kettering Cancer Center (MSKCC) [10] and National Cancer Institute (NCI) [11] are summarized in Table 2).

The explosive growth of interests and investment in CAR-T immunotherapy are reflected by the steadily increased number of CAR-T clinical trials globally. According to the website of ClinicalTrials.gov, there are 81 CAR-T clinical trials with an "open" status worldwide [Figure 2]. Among the 81 studies, 42 are CD19 CAR-T related trials. Thirteen studies are for other targets of hematopoietic malignancies. Studies in various solid tumors, including gliomas, neuroblastomas, carcinomas, sarcomas, account for another 15 studies. One study is for Type I diabetes mellitus [18].

Complications of CAR-T therapy

Cytokine release syndrome (CRS) is the most common complication associated with CAR-T infusion. CRS is a process of inflammation, characterized by marked increase of cytokine levels, including soluble interleukin-2 receptor α, IL-6, IL-10, and interferon γ. The cytokine profile of CRS is similar to that of hematophagocytic lymphohistiocytosis (HLH). Severe CRS cases show similar clinical and laboratory features as HLH, including marked hyperferritinemia, hypofibrinogenemia, and hepatosplenomegaly [16].

Another complication observed in CAR-T clinical trials is encephalopathy. According to one study, about 20% of the patients had self-limited encephalopathy, resolving in days [12]. The cause of encephalopathy is unclear.

B-cell aplasia and hypogammaglobulinemia is an on-target toxicity expected from successful CD19 CAR-T therapy. In addition, B-cell aplasia is an evidence of CAR T-cell persistence in patients' bodies. The infectious complications associated with B-cell aplasia is managed with immunoglobulin infusions.

Limitations and challenges of CAR-T

With all the spectacular progresses achieved by CAR-T research and clinical trials, the limitations are also obvious. First of all, the price tag is strikingly high with the estimated cost of $300,000 to $500,000 per patient. Secondly, the techniques and expertise are limited to a few academic medical centers only, such as NCI, MSKCC, CHOP, M.D. Anderson, etc. In addition, the production is low-throughput and and laborious. Large-scale production needs the invention of automatic close system. Last but not the least, standardized protocols in production and side effect management are needed.

Table 2: Summary of three CD19 CAR-T clinical trials on B-cell acute lymphoblastic leukemia.

	CHOP/UP	MSKCC	NCI
# of patients	30	16	20
Age group	Children, Adult	Adult	Children, young adult
Types of B-ALL	Relapsed, or refractory	Relapsed	Relapsed, or refractory
Complete Remission	90%	88%	70%
CAR Constructs	CTL019-BB-ζ	MSKCC19-28z	FMC63-CD28z
Vectors	Lentiviral	Retroviral	Retroviral
T cell persistence	up to 2 years	1-3 months	<68 days

Note: B-ALL: B-cell acute lymphoblastic leukemia.

Pathologists' role

Pathologists, as integral members of cancer patient management, have critical roles during the development of CAR-T therapies. In addition to the obvious contributions in diagnosing original diseases and monitoring disease persistence and recurrence, specific methods designed for monitoring CAR-T cell persistence and distribution also reflect the intimate collaboration among hematologists, hematopathologists and molecular pathologists.

In the near future, after the expected approval from U.S. Food and Drug Administration and CAR-T immunotherapy becoming a first-line choice of cancer therapy, a few changes will be anticipated in CAR-T related pathologic practice. First of all, established CAR-T target antigens would be routinely examined after the initial diagnosis, which is needed to evaluate patients' qualification for specific CAR-T therapy. Secondly, special laboratory tests for detecting minimal residual disease in CAR-T treated patients will be used in daily practice. In addition, morphologic features associated with CAR-T immunotherapy, if any, will be actively explored by pathologists and incorporated into pathology textbooks.

Conclusion

CAR-T immunotherapy offers a nearly reachable hope for some types of cancer, such as B-cell malignancies. It still needs tremendous amount of work before we know whether CAR-T can be as effective on solid tumors. Nonetheless, CAR-T shows great potentials in bringing a *bona fide* breakthrough for cancer therapy. Many are eagerly waiting for the era of CAR-T immunotherapy to officially start and to provide the much needed help for many cancer patients. We, the pathologists, need to be aware of our roles in CAR-T immunotherapy and be prepared for the opportunities and challenges ahead.

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