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REVIEW

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CAR-T cell therapy in gastrointestinal tumors and hepatic carcinoma: From bench to bedside

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ABSTRACT

The chimeric antigen receptor (CAR) is a genetically engineered receptor that combines a scFv domain, which specifically recognizes the tumor-specific antigen, with T cell activation domains. CAR-T cell therapies have demonstrated tremendous efficacy against hematologic malignancies in many clinical trials. Recent studies have extended these efforts to the treatment of solid tumors. However, the outcomes of CAR-T cell therapy for solid tumors are not as remarkable as the outcomes have been for hematologic malignancies. A series of hurdles has arisen with respect to CAR-T cell-based immunotherapy, which needs to be overcome to target solid tumors. The major challenge for CAR-T cell therapy in solid tumors is the selection of the appropriate specific antigen to demarcate the tumor from normal tissue. In this review, we discuss the application of CAR-T cells to gastrointestinal and hepatic carcinomas in preclinical and clinical research. Furthermore, we analyze the usefulness of several specific markers in the study of gastrointestinal tumors and hepatic carcinoma.

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Introduction

T cells may act as specific antitumor effector cells whose roles tend to be limited by the major histocompatibility complex (MHC) in the human body. CAR-T cells are genetically engineered by the introduction of desired chimeric antigen receptors (CARs) through viral or non-viral methods. CARs are fusion proteins that incorporate three primary domains: the single chain fragment variable (scFv) domain; the hinge and transmembrane domain; and the intracellular domain. The scFv, which is constructed via the connection of the antibody heavy (VH) and light (VL) chain amino acid sequences with a short peptide linker, is attached to the hinge region, where it acts as an extracellular antigen-binding domain.[1] The hinge and transmembrane domain is the bridge between the scFv and the intracellular domain that plays critical roles in anchoring the entire CAR structure firmly to the T cell membrane and in transferring the activation signal from scFv into T cells.[2] The intracellular domain is mainly evolved in the CD3ζ immunoreceptor tyrosine-based activation motif (ITAM) domain, which is responsible for T cell activation. Through the scFv, which is derived from high-affinity antibodies, CARs can specifically engage a target and trigger downstream signals; these signals then confer enhanced T-cell effector function against tumor cells in an MHC-independent manner.[3,4]

To date, the CAR structure has been evolving over four generations, and the major distinctions among these lie in the presence of different co-stimulatory molecules[5] (Fig. 1). The first-generation CAR consisted of the scFv and only one intracellular signaling domain, typically the CD3ζ chain. However, it was later found that the signaling domain could lead only to the short-term proliferation of T cells and low levels of cytotoxicity, and thus, this CAR failed to display persistent polyclonal amplification and *in vivo* antitumor effects. In terms of the structure of the second-generation CAR, apart from the CD3ζ chain, one co-stimulatory molecule (e.g., CD28 or 4-1BB) was added to the intracellular signaling domain. Once the tumor-associated antigen is recognized by scFv, both CD3ζ and CD28 (or 4-1BB) are activated. Compared with the first-generation CAR, a great improvement was gained in second-generation CAR-T cells in terms of proliferation and in its ability to stably recognize and destroy target cells.[6] Further, two different co-stimulatory molecules accompanied by a CD3ζ chain were assembled in the third-generation CAR. A series of preclinical experiments showed that the third-generation CAR had distinct advantages over the first- and second-generation CARs in the amplification of T cells, survival time *in vivo* and the ability to secrete cytokines. Nevertheless, it is essential to mention that one colon cancer patient with liver and lung metastases died 5 d after treatment with third-generation CAR-T cells. This case was reported by Morgan et al.[7] and shows that risks still exist in clinical trials in the context of the third-generation CAR-T technique. The newly generated fourth-generation CAR

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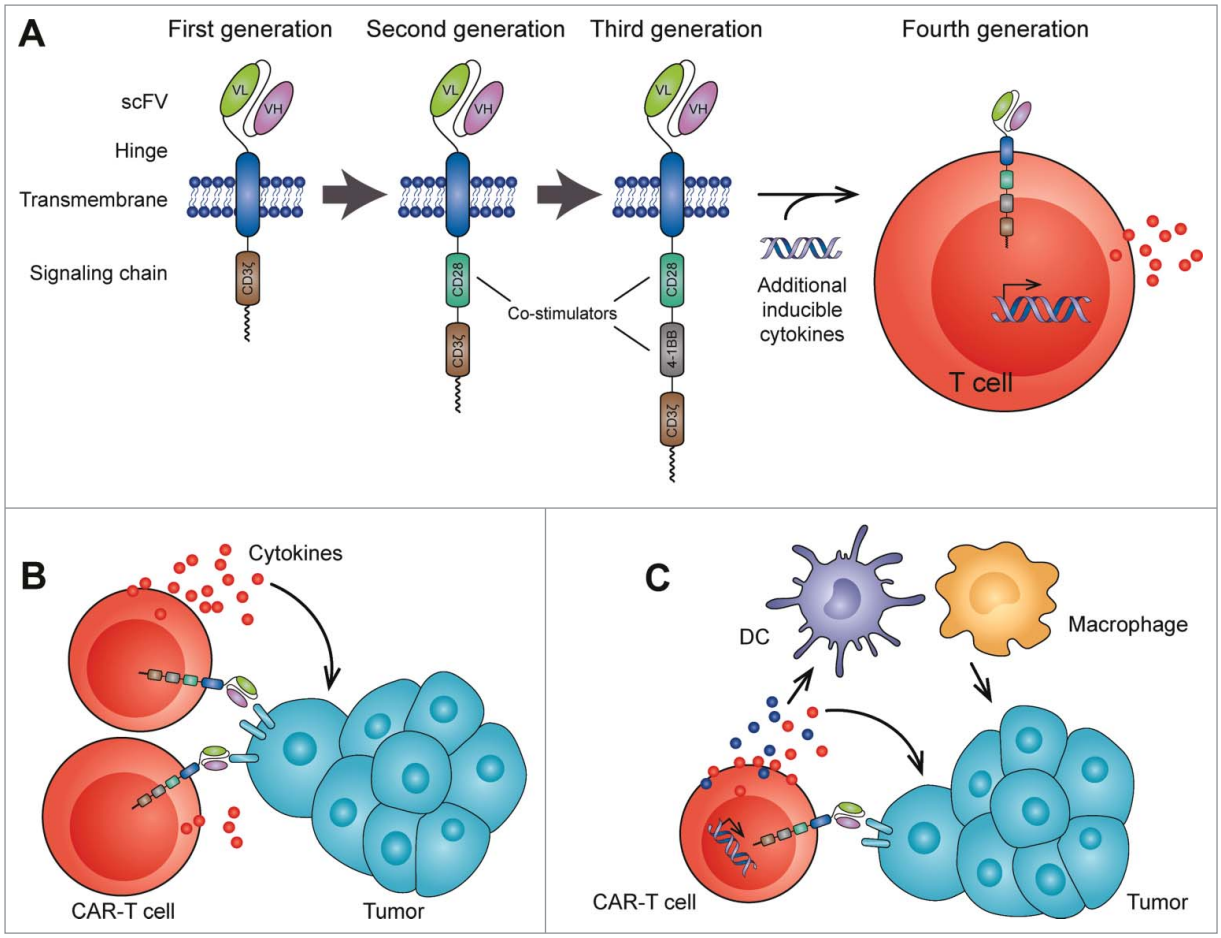

Figure 1. The generations of CARs and armored CAR-T cells for improved antitumor therapy. (A) First-generation CARs, including activating receptors, such as CD3ζ; second-generation CARs combine activating and costimulatory signals, such as CD28; third-generation CARs combined two costimulatory and activating signals, such as 4-1BB, etc.; fourth-generation CAR-T cells, also called "TRUCK" cells, are engineered with additional inducible cytokines, which can secrete cytokines upon the activation of CARs. (B) Modified CAR-T cells recognize tumor cells by their tumor-associated antigen in a non-MHC restrictive manner. CAR signaling activates T cells, and the T cells then secrete cytokines, which kill tumor cells and induce them to attack other tumor cells. (C) The fourth-generation CAR-T cells have the additional advantage of activating the innate immune system, which recruits innate immune cells (macrophages or DCs) to attack tumor cells and regulate the tumor microenvironment.

(termed TRUCK T cell) was engineered to express cytokines, particularly IL-12, which regulate the antitumor immunologic microenvironment. Furthermore, IL-15 and GM-CSF also contribute to this strategy.

Methods of CAR-T Cell production

Currently, T cells can be transduced with viral or non-viral vectors that carry the CAR construct.[8] Viral vectors have high gene transfer efficiency, and it takes a relatively short time to amplify the T cells so that the minimum number for a therapeutic dose is obtained. Moreover, the characteristic expression varies among different viral vectors, which allows for multiple choices for basic research and clinical trials.[9] Among viral vectors, retroviral or lentiviral vectors are the most commonly used, but some health risks still exist, such as the potential for an immune response, toxicity, insertional mutagenesis, or some other inducer of tumorigenicity.[10,11] Because non-viral vectors possess the advantages of being non-infectious, providing easy access to large-scale preparation, and having relatively unlimited vector capacity and controllable chemical structure, they have received greater attention from researchers. Transposon-based systems comprise the major class of non-viral vectors and include the *Sleeping Beauty*,[12] *PiggyBac*,[13] and the *Tol2* transposon systems.[14] Recently, RNA-based electroporation of lymphocytes, which is safer and more economical, has become a focus, but this method is less efficient than the lentiviral method. Therefore, after an evaluation of the characteristics of these different methods, we can choose the appropriate technique for CAR-modified T cell production (Fig. 1).

How to manufacture CAR-T cells in clinical practice

With increasing varieties of CAR-T therapies that are applied in various malignancies, the efficient manufacturing of CAR-T cells has become a critical step in clinical practice. The major process in the manufacturing of CAR-T cells involves the following five steps: autologous T cell collection; T cell activation; genetic modification of T cells; CAR-T cell expansion; CAR-T cell formulation; and cryopreservation. Following these five steps, the CAR-T cells produced are infused into a patient for clinical treatment. Moreover, the collection of a sufficient number of T cells for treatment and proper storage of T-cell subsets also involves procedures that require special awareness and different recommended practices. CD3-positive T cells comprise one of the most commonly used subsets,[15-18] and CD3 and

CD28 signals are essential for T cell activation. After genetic modification, the CAR-T cells could reach a therapeutic dose that is sufficient for infusion back to the patient for individualized treatment.

Side effects of CAR-T cell therapy

The most common side-effects in the clinical application of CAR-T cells are off-target effects, which are also known as on-target off-tumor toxicities; these effects result in an autoimmune response against normal tissues that express the targeted antigen. This challenge is primarily observed in solid tumor therapies.

In an earlier clinical trial, liver toxicity was observed in patients with infusions of autologous T cells that were transduced with a CAR-targeting carboxy-anhydrase-IX (CAIX).[19] This toxicity was attributed to the immune recognition of CAIX, which is also expressed in normal bile duct epithelium. However, later studies demonstrated that on-target toxicity could be prevented after the infusion of first-generation CARs with CAIX mAb pre-treatment at the lowest T cell effective dosage.[20,21] Moreover, fatal toxicity was reported to be associated with on-target off-tumor recognition of ERBB2/HER2.[7] The anti-HER2 CAR-T cells can target lung and heart tissues where low levels of ERBB2/HER2 are expressed, but this event is followed by a cytokine storm and pulmonary toxicity.

In addition, neurological toxicity was reported in trials where serum cytokine levels were increased.[22] Neurological toxicity is associated with the headaches, confusion, hallucinations, dysphasia, apraxia, dysmetria, and seizures. After the administration of a high dose of IL-2, a global encephalopathy may be induced in patients with solid tumor malignancies[23]; however, the detailed mechanism of neurological toxicity remains poorly understood.

Another major factor that threatens the clinical safety of CAR-T therapy is cytokine release syndrome (CRS), which can be triggered by the infusion of a large number of highly active CAR-T cells into the body. CRS is a potentially fatal complication that involves the release of increasing numbers of pro-inflammatory cytokines from immune cells into the circulation, including IL-6, IFNγ, and TNF-α. This release leads to clinical manifestations such as high fever, hypotension, and organ failure.[24-27] The severity of CRS correlates with tumor burden and with the infusion number of CAR-T cells.[28,29]

Interestingly, however, CRS occurs frequently in patients in trials for hematological malignancies, but is relatively rare in patients with solid tumors, which is likely due to the hurdles that CAR-T cells face in the treatment of solid tumors.[25,27,30] It is noted that CRS and neurological toxicity are commonly observed in the same groups of patients, which has led to increasing speculation that these two conditions might partially overlap.[31]

Tumor lysis syndrome (TLS), which usually results from massive and abrupt release of bioactive molecules following the rapid lysis of malignant cells, is another potentially fatal complication of CAR-T cell therapy.[32] The delivery of these molecules from the intracellular to the extracellular space could change the microenvironment and therefore disrupt normal physiological processes.

Furthermore, the integration of vectors including retroviral and lentiviral vectors that are used to insert the CAR gene into the T cells might be a potential safety risk that could cause insertional mutagenesis, as shown in gene therapy studies of primary immunodeficiency.[33]

How to improve the safety of CAR-T therapy

To achieve the maximum therapeutic effects with minimum side effects, the manufacturing of CAR-T cells needs to be improved. The crucial point of CAR-T cell therapy is the effective distinction of both normal and tumor cells. Currently, however, a few CARs are sufficiently tumor-specific. Therefore, it is essential to choose appropriate target antigens and improve tumor selectivity to avoid off-target effects. The specificity of only one individual tumor antigen is limited, and, thus, dual CAR targeting technology provides an alternative optimization method based on T cells that are modified with two separate CARs: a CD3ζ signal coupled with a co-stimulatory signal.[34] Dual CAR-T cells can be activated only by tumor cells that express both CAR antigens.[35-37] When dual CAR-T cells encounter normal cells, their antigen activation signals are weak, and they are unable to trigger co-stimulatory signals. This process results in a lack of T cell activation signals and thus their inability to damage normal cells.[38]

Furthermore, the modulation of the affinity of CAR-T cells is a promising strategy to improve the outcome of CAR-T therapy. A series of studies have demonstrated that CARs with low-affinity scFv recognition could increase the specificity of CAR-T cells for a target that is overexpressed in tumors compared with the same target that is expressed at its physiological level in normal tissue.[39,40]

Another strategy is antigen-specific inhibitory CAR (iCAR) technology.[41] The core idea of iCAR is the installation of a "brake" into the highly active CAR-T cells; this brake exerts inhibitory activities against T cells via the PD-1 or CTLA-4 intracellular regions.[42] The iCAR can restrain the T cell activation signals from the CAR, which results in the deactivation of these genetically modified T cells and their subsequent failure to attack normal cells; thus, avoiding on-target off-tumor effects,[43] it can also avoid the CRS effects in certain conditions.

In addition, a popular method to relieve side effects involves the introduction of a "suicide gene" in transduced cells. The gene products exhibit cytotoxicity with or without exposure to any drugs. Antigen receptor expression is also inhibited, which leads to the selective clearance of engineered cells, and finally, the potential toxicity of CAR-T cells is avoided. Currently, the most commonly used suicide genes include the herpes simplex virus thymidine kinase (HSV-TK) gene[44] and the inducible caspase 9 (iCasp9) gene.[45,46] However, the use of a suicide gene is a "double-edged sword". HSV-TK is potentially immunogenic, which might result in an undesired elimination of transferred T cells.[47,48] iCasp9 can manipulate the intracellular caspase pathway and induce the apoptosis of transferred T cells.[46,49,50]

In contrast to the engineering of stable CAR-T cells, some investigators have induced the transient expression of CARs via the transfection of T cells with RNA.[51,52] CRS can be relieved by blocking the cytokine receptor.[17] With proper adjustment of the transfection dose, this approach could mitigate the toxicity

of CARs; moreover, inevitably, the treatment effectiveness of CAR-T therapy would be partially weakened. Therefore, the method by which fatal complications can be minimized under the premise of a guarantee of efficacy becomes the ultimate goal of the effective improvement of CAR-T therapy.

Application of CAR-T therapy in patients with gastrointestinal cancer and hepatic carcinoma

Gastrointestinal cancer and hepatic carcinoma share the characteristics of a poor clinical outcome and high mortality, which are still prevalent today and are difficult to improve. However, superior methods of cancer treatment are currently being developed. Over the past decade, some studies began to shift attention to the application of CAR-T treatment to gastrointestinal cancers and hepatic carcinoma. Although a series of obstacles of CAR-T-based immunotherapy remain in the targeting of solid tumors (Box1),[30,53] to date, more than 50 preclinical and clinical studies have been associated with the application of CAR-T cells in gastrointestinal tumors and hepatic carcinoma (Table 1). Nine antigens are used as targets in CAR-T therapy for gastrointestinal tumors and hepatic carcinoma (Table 1). Moreover, the application of specific biomarkers also has some potential value (Table 2).

Gastric carcinoma

Gastric carcinoma (stomach cancer) originates from the mucosal epithelial cells located in the superficial layer of the gastric wall. This cancer can occur in various regions of the stomach, including the pyloric antrum, the gastric cardia, and the gastric body. Several tumor markers have been discovered and investigated extensively in preclinical studies. Current clinical trials that involve the application of CAR-T cells in patients with gastric cancer target HER2, CEA, MUC1, or EpCAM (Table 1).

The proto-oncogene *HER2*, also known as *ErbB2*, plays an important role in the pathogenesis and clinical development of gastric and gastroesophageal cancers and other tumor types.[54,55] Amplification of the *HER2* gene and the overexpression of its protein product (p185-protein) have been associated with more than 30% of tumors, whereas negative expression of p185-protein has been shown in normal tissues.[56,57] Therefore, HER2 could serve as an ideal target for antitumor therapy using CAR-T, and a series of preclinical studies have been conducted with HER2-specific CAR-T cells.[58]

Carcinoembryonic antigen (CEA) and a set of other closely related glycoproteins are generally expressed in gastric, pancreatic, colorectal, and hepatocellular cancers. A preclinical study found that CEA-specific CAR-T cells could contribute to the infiltration of tumors by T cells, the delay of tumor growth, and the extension of the survival of mice with gastric cancer.[59]

Mucin-1, which is a transmembrane glycoprotein encoded by the *MUC1* gene, is widely and highly expressed in gastric cancer, among others. Compared with normal cells, the Mucin-1 that is expressed on the surface of tumor cells displays different glycosylation patterns. As a result, the overexpression of Mucin-1 and its abnormal glycosylation are ideal targets for immunotherapy. Wilkie et al.[60] constructed MUC1-specific CAR-T cells and found that they were able to effectively attack MUC1-positive tumor cells. Further, they constructed dual anti-ERBB2 and anti-MUC1 CAR-T cells that could effectively eradicate ERBB2-positive tumor cells and regulate the immunological microenvironment.[36]

Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein that is overexpressed in various cancers. It has been found that altered expression of EpCAM is associated with aggressive biologic behavior in gastric cancer[61] and is considered a potential cancer stem cell marker. Deng et al.[62] reported that EpCAM-specific CAR-T cells could exert significant antitumor activity against prostate cancer.

The ligand B7H6, which targets the NK cell activation receptor NKp30, is expressed in many human tumors *in situ*, including gastrointestinal interstitial tumors, but almost never in the normal tissues. The second-generation $B7H6^{+}$ CAR-T cells was shown to reduce tumor burden in mice with ovarian cancer.[63] In addition, Zheng et al.[64] reported the positive expression of actin-related protein 2/3 (Arp2/3) in the tumor cells of patients with gastric cancer and the ability of Arp2/3 to promote the invasion and metastasis of gastric carcinoma cells.

An immunohistochemical study showed that several proteins could serve as potential therapeutic targets in CAR-T therapy. Neuropilin-1 (NRP-1) is a transmembrane glycoprotein that is involved in cancer growth and metastasis. Li et al.[65] found that NRP-1 expression in gastric cancer tissues was higher than that in normal gastric mucosa and correlated with tumor differentiation and the stage of invasion. Desmocollin 2 (DSC2) is one of the three known desmocollin proteins and is expressed in 28% of gastric cancers.[66] Xu et al.[67] reported that Anion exchanger 1 (AE1), a transmembrane glycoprotein, was expressed in gastric carcinoma but not in normal gastric tissue. AE1 expression is also significantly associated with the development of gastric cancer, and thus, AE1 might serve as an alternative target of CAR-T cells.

Colorectal carcinoma

Colorectal carcinoma (CRC) is one of the most common and deadly malignancies. It is clear that multiple tumor-associated antigens are significantly overexpressed in patients with CRC.

In a preclinical study, CEA was investigated as part of CAR-T therapy as a tumor-specific target. CEA-specific CAR-T cells were found to enhance the antitumor immunity in mice with colon cancer[68] and in human CEA^{+} colon cancer cells.[59] In addition, a high level of IFNγ can be secreted by CEA-specific CAR-T cells and suppress tumor contact.

In clinical trials, five targets are being investigated in CRC, including CEA, HER2, MUC1, CD133, and EGFR (Table 1). CD133 is a highly conserved transmembrane glycoprotein that is associated with various human malignancies such as colorectal, liver, and pancreatic carcinomas. Like EPCAM, CD133 has been proposed as a cancer stem cell marker, and it has been shown that CD133-specific CAR-T cells could eradicate glioblastoma multiforme stem cells both *in vitro* and *in vivo*.[69] Similar to HER2, the epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that belongs to the ErbB family and serves as an indicator of tumor development. In clinical trials, EGFR-targeted CAR-T cells could eradicate EGFR-positive tumor cells in patients with non-small-cell lung cancer.[70]

Table 1. Application of CAR-T cells to gastrointestinal tumors and hepatic carcinoma.

Cancer	Antigen	Gene transfer vehicle	CAR generation (signaling domain)	Status	Study type	Year (Clinicaltrials.gov identifier or reference)	Sponsor
Gastric cancer	HER2	—	—	Recruiting	Phase 1/2	2016 (NCT02713984)	Southwest Hospital, China
		—	1st and 2nd (CD137)	Recruiting	Phase 1/2	2013 (NCT01935843)	Chinese PLA General Hospital
		—	2nd (CD28)	Active, not recruiting	Phase 1	2009 (NCT00889954)	Baylor College of Medicine
	CEA	—	—	Recruiting	Phase 1	2015 (NCT02349724)	Southwest Hospital, China
		—	2nd (CD28)	Suspended	Phase 2	2012 (NCT01723306)	Roger Williams Medical Center
		Retrovirus	2nd (CD28)	Published	Preclinical	2015[59]	
	MUC1	—	—	Recruiting	Phase 1/2	2015 (NCT02617134)	PersonGen BioTherapeutics (Suzhou) Co., Ltd
	EpCAM	—	—	Recruiting	Phase 1/2	2016 (NCT02725125)	Sinobioway Cell Therapy Co., Ltd.
Colorectal cancer	HER2	—	—	Recruiting	Phase 1/2	2016 (NCT02713984)	Southwest Hospital, China
		—	2nd (CD28)	Active, not recruiting	Phase 1	2009 (NCT00889954)	Baylor College of Medicine
		Retrovirus	3rd (CD28-CD137)	Published	Case report	2010[7]	
	CEA	—	—	Recruiting	Phase 1	2015 (NCT02349724)	Southwest Hospital, China
		—	2nd (CD28)	Terminated	Phase 1	2008 (NCT00673322)	Roger Williams Medical Center
		Retrovirus	—	Terminated	Phase 1	2010 (NCT01212887)	Cancer Research UK
		—	2nd (CD28)	Suspended	Phase 2	2012 (NCT01723306)	Roger Williams Medical Center
		Retrovirus	2nd (CD28)	Published	Preclinical	2014[101]	
		Retrovirus	2nd (CD28)	Published	Preclinical	2015[159]	
	MUC1	—	—	Recruiting	Phase 1/2	2015 (NCT02617134)	PersonGen BioTherapeutics (Suzhou) Co., Ltd
	EGFR	Lentivirus	2nd (CD137)	Recruiting	Phase 1/2	2013 (NCT01869166)	Chinese PLA General Hospital
Pancreatic cancer and pancreatic ductal a denocarcinoma	HER2	—	—	Recruiting	Phase 1/2	2016 (NCT02713984)	Southwest Hospital, China
		Retrovirus	2nd (CD28) and 3rd (CD28-CD137)	Published	Preclinical	2012[86]	
	CEA	—	—	Recruiting	Phase 1	2015 (NCT02349724)	Southwest Hospital, China
		Retrovirus	2nd (CD28)	Published	Preclinical	2012[85]	
	MUC1	—	—	Recruiting	Phase 1/2	2015 (NCT02587689)	PersonGen BioTherapeutics (Suzhou) Co., Ltd.
		Lentivirus	2nd (CD137)	Published	Preclinical	2016[87]	
		Retrovirus	2nd (CD137/ CD28)	Published	Preclinical	2014[102]	
	EGFR	Lentivirus	2nd (CD137)	Recruiting	Phase 1/2	2013 (NCT01869166)	Chinese PLA General Hospital
	PSCA	—	—	Not yet recruiting	Phase 1	2016 (NCT02744287)	Bellicum Pharmaceuticals
		Retrovirus	First	Published	Preclinical	2011[83]	
		Retrovirus	2nd (CD28) and 3rd (CD28-CD137)	Published	Preclinical	2014[84]	
	Mesothelin	RNA	—	Completed	Phase 1	2013 (NCT01897415)	Abramson Cancer Center of the University of Pennsylvania
		Retrovirus		Suspended	Phase 1/2	2012 (NCT01583686)	National Cancer Institute (NCI)
		—	2nd (CD137)	Recruiting	Phase 1	2016 (NCT02706782)	Shanghai GeneChem Co., Ltd.
		Retrovirus	2nd (CD137)	Recruiting	Phase 1	2015 (NCT02580747)	Chinese PLA General Hospital
		Lentivirus	2nd (CD137)	Active, not recruiting	Phase 1	2015 (NCT02465983)	University of Pennsylvania
		Lentivirus	2nd (CD137)	Unknown	Phase 1	2014 (NCT02159716)	Abramson Cancer Center of the University of Pennsylvania
		RNA	2nd (CD137)	Published	Clinical study	2014[80]	
	CD133	Retrovirus	2nd (CD137)	Recruiting	Phase 1	2015 (NCT02541370)	Chinese PLA General Hospital

(*Continued on next page*)

Table 1. (*Continued*)

Cancer	Antigen	Gene transfer vehicle	CAR generation (signaling domain)	Status	Study type	Year (Clinicaltrials.gov identifier or reference)	Sponsor
Hepatocellular carcinoma and liver metastases	CEA	—	—	Recruiting	Phase 1	2015 (NCT02416466)	Roger Williams Medical Center
		—	2nd (CD28)	Completed	Phase 1	2011 (NCT01373047)	Roger Williams Medical Center
		—	—	Recruiting	Phase 1	2015 (NCT02349724)	Southwest Hospital, China
		Retrovirus	2nd (CD28)	Published	Preclinical	2016[103]	
		Retrovirus	2nd (CD28)	Published	Preclinical	2015[93]	
		Retrovirus	2nd (CD28)	Published	Clinical study	2015[94]	
	MUC1	—	—	Recruiting	Phase 1/2	2015 (NCT02587689)	PersonGen BioTherapeutics (Suzhou) Co., Ltd.
	EpCAM	—	—	Recruiting	Phase 1/2	2016 (NCT02729493)	Sinobioway Cell Therapy Co., Ltd.
	EGFR	Lentivirus	2nd (CD137)	Recruiting	Phase 1/2	2013 (NCT01869166)	Chinese PLA General Hospital
	CD133	Retrovirus	2nd (CD137)	Recruiting	Phase 1	2015 (NCT02541370)	Chinese PLA General Hospital
	GPC3	—	—	Recruiting	Phase 1	2015 (NCT02395250)	RenJi Hospital
		—	—	Recruiting	Phase 1/2	2016 (NCT02723942)	Fuda Cancer Hospital, Guangzhou
		—	2nd (CD137)	Recruiting	Phase 1/2	2016 (NCT02715362)	Shanghai GeneChem Co., Ltd.
		Lentivirus	1st and 3rd (CD28-CD137)	Published	Preclinical	2014[95]	

Abbreviations: CEA, carcinoembryonic antigen; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; GPC3, Glypican-3; MUC1, Mucin-1; PSCA, prostate-specific cancer antigen.

Table 2. Potential tumor-specific antigens for the application of CAR-T in gastrointestinal tumors and hepatic carcinoma.

Cancers	Potential target genes	Expression in gastrointestinal cancer	Expression on adjacent mucosa	Related study (Yes or No)	Outcomes	Year and reference
Gastric cancer	B7H6	++++	−	Yes	B7H6 is expressed in gastrointestinal stromal tumors but not in normal tissues. Second-generation B7H6 CAR T cells can reduce the tumor burden in mice with ovarian cancer.	2015[63]
	ARP2/3	+++	−	No	Tumor cells of patients with gastric cancer are positive for Arp2/3, which induces the prompt invasion and metastasis of gastric carcinoma cells.	2012[104]
	Neuropilin-1	++	+	No	NRP-1 expression in gastric cancer tissues is higher than that in normal gastric mucosa and correlates with tumor differentiation and pathological type.	2016[65]
	DSC2	++	+	No	Gastric cancers are positive for DSC2, which is frequently expressed in GC.	2010[66]
	AE1	+++	−	No	Gastric carcinoma is positive for AE1, but normal gastric tissue is negative. AE1 expression is significantly associated with the development of gastric cancer.	2009[67]
Colorectal cancer	GUCY2C	+++	−	Yes	Second-generation GUCY2C CAR-T cells can effectively inhibit the development of colon tumors and can prolong survival.	2011[73]
	AXIN2	+	−	No	AXIN2 and HNKD can be expressed only in human colon cancer-derived cell lines.	2001[74]
	HNKD	++++	−	No		
	CDH17	++++	+	No	CDH17, a member of the cadherin superfamily, is a membrane-associated glycoprotein.	2014[75]
	CK7	++	−	No	When combined with the detection of CDH17 and CK7, 97% of $CDH17^{+}/CK7^{-}$ tumors are colorectal tumors that originate from the lower gastrointestinal tract.	2013[76]
Pancreatic cancer	HPSE			Yes	HPSE-targeted CAR-T cells promote T cell infiltration in tumors and antitumor activity in matrix-rich solid tumors.	2015[88]
	CD24	+++	−	Yes	CD24-specific CAR-T cells can reduce tumor volume and prolong the survival of mice with pancreatic tumors.	2012[86]
	MUC4	+++	+	No	MUC-4 is a glycoprotein that is often overexpressed in pancreatic adenocarcinomas and has been shown to promote tumor growth and metastasis.	2001[90]2012[89]
	MUC16	+++	+	Yes	MUC-16(ecto) CAR-T cells enhance tumor cytolysis and function in the transfer of IL-12 into the tumor microenvironment, where it participates in tumor elimination in ovarian cancer.	2015[91]
Hepatocellular carcinoma	HBV	+++	−	Yes	CAR-T cells can recognize HBV and inhibit the growth of hepatoma carcinoma cells.	2013[97]
	AFP-L3	++++	−	No	AFP-L3 is uniquely expressed on HCC cells	2001[99]
	SP17	+++	+	No	Sp17 is highly expressed in hepatocellular carcinoma cells. The frequency of Sp17 expression is closely related to the pathologic differentiation of hepatocellular carcinoma.	2013[100]
	FAP	+++		Yes	FAP-specific CAR-T cells are being investigated in a clinical trial for malignant pleural mesothelioma.	NCT01722149

Note: ++++, strongly positive; +++, highly positive; ++, moderately positive; +, weakly positive; −, negative.
Abbreviations: AFP, Alpha Fetoprotein; Arp2/3, actin-related protein 2/3; AE1, anion exchanger 1; CDH17, Cadherin-17; DSC2, Desmocollin 2; FAP: fibroblast activation protein; GUCY2C, guanylyl cyclase C; HBV, hepatitis B virus; HNKD, human naked cuticle; HPSE, heparanase; MUC4, Mucin-4; MUC16, Mucin-16; NRP-1, Neuropilin-1; Sp17, Sperm protein 17.

Guanylyl cyclase C (GUCY2C), which is a protein that is expressed specifically in intestinal epithelial cells, is expressed in colon cancer cells in nearly 100% of cases.[71,72] Snook et al.[73] engineered two generations of GUCY2C-specific CAR-T cells. In preclinical studies, the authors found that these CAR-T cells could effectively inhibit tumor development and prolong the survival of mice. GUCY2C-targeted CAR-T therapy may lead to a more effective immunological treatment for CRC.

In addition to the antigens discussed above, other tumor-associated antigens also deserve attention. Yan et al.[74] found that out of 30 types of human cell lines derived from different tissues, the axin2 and human naked cuticle (HNKD) genes are expressed only in human colon cancer cell lines. A study showed that the expression of CDH17 (cadherin-17), which is a member of the cadherin family, is closely associated with the occurrence and development of epithelial tumors within the

digestive system and plays a critical role in the mediation of calcium-dependent cell–cell junctions. Fan et al.[75] found that 98% of CRCs expressed CDH17, whereas only 3.3% of non-gastrointestinal tumors were positive for this protein. CK7 is a commonly used diagnostic marker for colon cancer.[76] When combined with the detection of CDH17 and CK7, 97% of $CDH17^+$ /$CK7^-$ cells were found to originate from CRC.[77] Consequently, double-antibody CAR-T cells have important implications for CRC therapy.

Pancreatic carcinoma

Pancreatic carcinoma is a digestive tract malignancy with the characteristics of a high-grade malignancy and a short course, and this cancer is difficult to diagnose and treat. A series of clinical trials using CAR-T cells that target different antigens for the treatment of pancreatic cancer have been reported.

The most widely investigated target is mesothelin, which is one of the tumor-associated antigens that is highly expressed in pancreatic cancer and is a target for an endogenous T-cell immune response.[78] In animal experiments, mesothelin-specific CAR-T cells exhibit potent antitumor activity.[79] However, mesothelin is also present on the surface of normal peritoneum, pleura, and pericardium, which may lead to toxicity. To solve this problem, Gregory et al.[80] transiently expressed the mRNA for the T cell receptor CD3ζ and for the tumor necrosis factor receptor superfamily member TNFRSF9 (4-1BB) into autologous T cells; the authors observed the antitumor effects of mesothelin-specific CAR-T cells but observed no corresponding toxicity in patients with metastatic pancreatic cancer and malignant papillary mesothelioma. Furthermore, data from completed preclinical[81] and clinical[82] trials at the University of Pennsylvania confirmed the feasibility, safety, and preliminary effectiveness of this method.

Prostate-specific cancer antigen (PSCA), a glycosylphosphatidylinositol (GPI)-anchored cell surface protein, is upregulated in several major cancers, including prostate, bladder, and pancreatic cancers. However, PSCA is weakly expressed in normal cells, which indicates that it may be an ideal target antigen. Katari et al.[83] engineered second-generation PSCA-specific CAR-T cells, which were later demonstrated to eradicate $PSCA^+$ pancreatic cancer cells without any effect on $PSCA^-$ pancreatic cancer cells *in vitro* and *in vivo*. Moreover, Daniel et al.[84] reported that anti-PSCA CAR-T cells based on the whole human antibody Hal-4.117 could not only delay tumor growth but also reduce tumor volume.

CEA, HER2, MUC1, CD133, and EGFR have already been investigated in preclinical studies and clinical trials (Table 1). Chmielewski et al.[85] engineered CEA^+ CAR-T cells that could recognize and attack tumor cells continuously and significantly reduce the size of pancreatic tumors. It has been reported that 67% of tumor cells in a mouse model were eradicated without any significant damage to other healthy CEA^+ cells. Maliar et al.[86] engineered CD24 and/or Her2-specific CAR-T cells; CD24 is a putative pancreatic cancer stem cell antigen. It has been found that the injection of dual-antibody CAR-T cells into tumors could completely eliminate pancreatic tumors in mice and that the intravenous injection could, to a certain extent, reduce the tumor volume and prolong survival. Anti-MUC1 CAR-T cells have also successfully suppressed tumor growth in a preclinical model of pancreatic cancer.[87]

Ignazio et al.[88] investigated the ability of CAR-T cells cultured *in vitro* to degrade the extracellular matrix (ECM). The authors designed new CAR-T cells that expressed heparanase (HPSE) and exhibited a significantly enhanced ability to degrade the ECM; this design promoted the infiltration of T cells into the tumor and promoted anti-matrix-rich solid tumor activity. Additionally, mucins are a class of proteins related to invasion, metastasis, and other biological behaviors of tumors. Based on its protein core, mucins can be divided into secretory mucins (MUC2, MUC5AC, MUC5B, and MUC6) and transmembrane mucins (MUC1, MUC3A, MUC3B, MUC4, MUC12, and MUC17).[89] Studies have shown that mucins not only serve as diagnostic markers for tumors but can also be used as targets for tumor immunotherapy. MUC4 shows little to no expression in normal pancreas or in pancreas affected by pancreatitis, but is highly expressed in pancreatic tumor tissues, as is MUC16.[90] MUC-16(ecto) CAR-T cells were engineered by Koneru et al.[91] and then used in patients with ovarian cancer. It was found that these CAR-T cells not only enhanced tumor cytolysis but also acted as a delivery agent for further modulation of the tumor microenvironment. MUC-16(ecto) CAR-T cells can secrete IL-12 into the tumor microenvironment, which enhances the endogenous immune response and tumor elimination. In light of current studies and applications of MUC1 to CAR-T cell therapy, it might be presumed that MUC4 and MUC16 may also potentially serve as targets of CAR-T cells for the treatment of pancreatic cancer.

Hepatocellular carcinoma and cholangiocarcinoma

Primary gallbladder carcinoma (cholangiocarcinoma) and hepatocellular carcinoma (HCC) are common malignant tumors that are continuing to increase in incidence yearly. Cholangiocarcinoma represents a class of malignant tumors that are derived from epithelial cells. Due to the close anatomical location of the gallbladder and liver, gallbladder carcinoma is clinically similar to HCC in some respects. There are a variety of immune regulation mechanisms in the liver, which can result in either abnormal or pathological conditions, the liver is in an immunosuppressive environment; therefore, tumor cells are easily transferred to the liver, which could be a major obstacle to immunotherapy in cases of HCC.[92] Burga et al.[93] found that anti-CEA CAR-T cells could significantly inhibit the metastasis of CEA^+ liver tumor cells in a mouse model; a similar result was found in clinical trials that involved the hepatic arterial infusion of anti-CEA CAR-T cells in patients with CEA^+ liver metastases.[94] In addition, studies have found that CAR-T cells targeting MUC1 can specifically kill liver cancer cells that express high levels of MUC1, which provides a basis for the use of MUC1-targeted CAR-T cells for immunocyte therapy in the setting of HCC. GPC3, which is a member of the glypican family of heparin sulfate (HS) proteoglycans, can be specifically expressed on the cell surface of liver cancer cells, whereas it is almost never expressed in normal tissues. Gao et al.[95] demonstrated using *in vivo* and *in vitro* experiments that Glypican-3 (GPC3)-targeted CAR-T cells could effectively kill GPC3-positive HCC cells, inhibit tumor growth, and improve the survival of patients with HCC. Proteins in the EGFR family are also expressed in liver cells. Using a human monoclonal

antibody against EGF, Ito et al.[96] found that EGF was expressed in the cytoplasm of liver cancer cells but not in the nucleus or in other locations within cancer cells. This finding suggests that EGF in human HCC tissues is produced by the liver cancer cells themselves, which in turn indicates that EGF may be applied in the CAR-T cell therapy. Therefore, multiple tumor-specific antigens such as CEA, MUC1, GPC3, EGFR, EpCAM, and CD133 are being investigated in clinical trials for HCC (Table 1).

Other tumor-associated antigens have also been investigated in preclinical trials of HCC. From their study on the infusion of CAR-T cells, which specifically expressed hepatitis B virus (HBV), into HBV transgenic mice, Krebs et al.[97] found that these cells were able to quickly translocate to the liver, significantly improve the immunity of mice against HBV, and effectively inhibit HBV replication. However, these cells also caused temporary liver damage, which may have been related to off-target effects of the CAR-T cells. Using the same method and CAR-T cells that specifically express the HBV, CAR-T cells were found to recognize HBV and inhibit the growth of hepatoma cells. In HCC, Alpha-Fetoprotein (AFP) serves as an important biomarker.[98] AFP is synthesized by the fetal liver during fetal development. AFP contains three components including AFP-L1, AFP-L2, and AFP-L3, of which, AFP-L3 is unique to HCC cells. In their study, Khien et al.[99] found that the sensitivity of AFP-L3 in HCC patients was 96.9%, and the specificity was 92.0%, whereas liver cells in cases of non-malignant liver disease did not express AFP-L3. AFP-L3 may serve as an important new target for the treatment of liver cancer by CAR-T cells. Sperm protein 17 (Sp17) has been studied in the diagnosis and differentiation of hepatocellular carcinoma.[100] The frequency of Sp17 expression in hepatocellular carcinoma cells, which is approximately 80%, is associated with the pathologic differentiation of hepatocellular carcinoma. This finding suggests that Sp17 may be a CAR target for the treatment of liver cancer, but this possibility needs further exploration. Moreover, some candidate targets are currently being investigated in clinical trials for CAR-T therapy in liver and other cancers. For example, fibroblast activation protein (FAP)-specific CAR-T cells are being investigated in a clinical trial (NCT01722149) for malignant pleural mesothelioma. FAP is a cell surface glycoprotein that is overexpressed in CRC and is associated with tissue remodeling in liver fibrosis.

Conclusion and prospective studies

From candidate-gene studies to genome-wide association studies, a large number of tumor-specific markers have been identified in various cancers. Over the past decade, CAR-T technology has provided valuable experimental platforms and has opened new avenues for the clinical treatment of cancers. In clinical trials, CAR-T cells that target tumor-specific markers have exhibited ideal therapeutic effects in malignant lymphoma. With regard to solid tumors, however, multiple barriers need to be overcome in the application of CAR-T therapy. For instance, various suppressive immune cells, such as myeloid-derived suppressor cells (MDSCs), Tregs, and macrophages, are present in the microenvironment of solid tumors, where they inhibit the antitumor effects of CAR-T cells.

In this review, we summarized the ongoing clinical trials of CAR-T therapy for gastrointestinal tumors and hepatic carcinoma. Two strategies are studied with respect to the engineering of CARs. First, an ideal CAR should be able to effectively and specifically distinguish tumors from normal tissues based on expression of the target antigen and should be able to rapidly migrate to the tumor tissue. Among the accessible clinical trials, various types of CAR-T cells have been engineered to target HER2 and CEA, which are overexpressed on the surface of cells in solid tumors and are associated with the development and metastasis of tumors. Selection of the appropriate targeted tumor antigens can fundamentally reduce the side effects of CAR-T. The antigen density and affinity on the tumor and in normal tissues should be considered. Second, the co-stimulatory molecules in the CAR structure should be taken into account because of their different roles in T cell expansion and activation. In addition, the sources of T cells, optimal timing, and dosage of CAR-T therapy still require further exploration.

With the rapid development of preclinical and clinical studies, CAR-T therapies for hematologic malignancies have been very successful and have offered us a perspective on solid tumors. Current explorations of CAR-T therapies for the treatment of solid tumors will hopefully also lead to greater success. We are optimistic that the consideration of individual genetic and epigenetic variations will lead to a more personalized application of CAR-T therapies for patients with various cancers.

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