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Immunotherapy of hepatocellular carcinoma using chimeric antigen receptors and bispecific antibodies

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Abstract

Hepatocellular carcinoma (HCC) is one of the most lethal cancers worldwide with an overall survival rate of less than 15% in developed countries. Despite attempts at new therapeutic strategies, the majority of patients succumb to this cancer. Buttressed by the highly successful clinical impact in melanoma, immunotherapy is gaining momentum as the next treatment modality for many human cancers. Chimeric antigen receptors (CAR) contain the antigen binding moieties of a monoclonal antibody and the co-stimulatory and signaling domains associated with effector receptor signaling. Bispecific antibodies (BsAb) combine the binding specificities of two different monoclonal antibodies, one activating a receptor on a killer effector cell, while the other engaging a tumor-associated antigen to initiate tumor cytotoxicity. In this review, we survey the HCC targets for which CARs and bispecific antibodies have been generated. The pros and cons of these targets for T-cell and Natural Killer cell based immunotherapy will be discussed.

Keywords

Chimeric Antigen Receptor; Bispecific Antibody; Hepatocellular Carcinoma; Treatment

1. Introduction

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is the fifth most common cancer and the second most common cause of cancer deaths worldwide. HCC developed mostly in livers with chronic inflammation. The main causal factors for the latter are chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. However, other factors such as excessive alcohol consumption, non-alcoholic fatty liver disease, obesity, diabetes, aflatoxin, and smoking also play important roles in the pathogenesis of this neoplasm [12]. Despite advances in treatment, the five-year survival rate of patients with

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Contributors

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Conflict of interest

MKSCC and NK Cheung have financial interest in Y-mabs Therapeutics Inc.

HCC remains poor averaging 5–15% [12]. Although viral vaccines [69] and effective anti-hepatitis drugs [75] in recent years have greatly reduced the incidence and the severity of chronic infection in developed countries, for the rest of the world, these benchmarks will take decades to be realized. In the meantime, finding an effective therapeutic for HCC remains an unmet need.

1.1 Immunobiology of liver and liver cancer

Through the portal blood flow from intestine to heart, bacterial products, toxins and antigens continually challenge the liver parenchyma. To effectively neutralize these environmental threats, the liver contains a rich source of innate immune cell including macrophages, natural killer (NK) cells, NKT cells, and γδT cells. Among these, NK cells are a unique population comprising about 30–50% of liver lymphocytes in healthy individuals and up to 90% in liver malignancies [39]. Compared to the peripheral blood NK cells, liver NK cells are more cytotoxic against HCC. In fact, interleukin-2 (IL2)-stimulated liver NK cells express tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), whose receptor is highly expressed on poorly differentiated HCC [52]. Although this local population of NK cells generally have low CD16 expression [17], a limitation for most natural anti-tumor IgG1 antibodies, a BsAb targeting CD16 could potentially overcome this limitation. Since NK cells are less restricted by the immunosuppressive tumor microenvironment intended for T cells, their activation using bispecific antibodies against HCC is appealing.

The role of Lymphocytes in defense against HCC is well known. In fact, there is a positive correlation between T and NK cell infiltration into the tumor site and higher survival rate in HCC patients [24, 53]. However, immunosuppressive tumor microenvironment undermines lymphocyte function. Myeloid derived suppressor cells [41], mesenchymal stem cells [117], regulatory T cells [108], cancer-associated fibroblasts [3], tumor associated macrophages [30], and programmed cell death protein 1 $(PD\text{-}1)^{hi}$ regulatory B cell [111] suppress immune cells and promote HCC progression. Furthermore, overexpression of inhibitory receptors including PD-1 and T cell immunoglobulin and mucin-domain containing-3 (TIM-3) on circulating or tumor-infiltrating T cells was associated with poor clinical outcomes [64, 100]. In addition, PD-1 ligand (PD-L1) or B7-H3 expressed on HCC cells can induce T cell apoptosis or inhibit T cell functions [100, 102]. Hence, modulation of immunosuppressive cells and molecules is an active area of investigation [54, 64, 107].

A Phase I/II clinical trial to evaluate the safety and efficacy of nivolumab, a fully human IgG4 PD-1 blocking monoclonal antibody, was completed on 41 patients with HCC. Patients were treated for up to two years with nivolumab (0.1 – 10 mg/kg intravenously) [31]. Among 39 patients whose response was evaluable, 2 complete responses, 7 partial responses, 18 stable diseases, and 12 progressive diseases were reported. 71% patients experienced drug-related adverse effects (17% grade 3/4) including rash and elevated serum levels of aspartate aminotransferase, alanine aminotransferase, and amylase. Overall survival rate was 72% after six months. In a recent case report, a 75-years old male patient with metastatic HCC unresponsive to sorafenib was treated with pembrolizumab, a humanized IgG4 PD-1 blocking monoclonal antibody. After six cycles of treatment (each cycle with 2 mg/kg every three weeks), the patient’s tumor mass and blood alpha fetoprotein was

markedly reduced [107]. In murine models of HCC, it was shown that sorafenib treatment increases intratumoral hypoxia leading to increased expression of stromal cell-derived 1 alpha (SDF1α), PD-1 ligand (PDL1), and accumulation of immunosuppressive cells [20]. Combination of sorafenib, murine PD-1 blocking antibody, and SDF1α receptor inhibitor provided the most potent tumor growth delay [21].

1.2 Antibody-based T cell-dependent immunotherapy

Compelled by the striking recent clinical results of T-cell based therapies, cancer immunotherapy was named as the breakthrough of the year in 2013 [25]. Chimeric antigen receptors (CAR) and bispecific antibodies are two powerful extensions of this approach. CARs were originally developed by fusing the antigen-binding moiety of an antibody to the transmembrane and cytoplasmic domains of CD3ζ [34] T cells equipped with these CARs had in vitro cytotoxicity, but their in vivo persistence and function were suboptimal. Therefore, second and third generation CARs were developed by addition of one or two costimulatory domains (e.g. CD28, 4-1BB, OX40), respectively to the intracellular domain of the first generation CARs (fig. 1) [77]. Bispecific antibodies (BsAb) combine the specificities of two monoclonal antibodies in a single molecule. These bispecific reagents can neutralize the effect of two tumor-associated-antigens. More commonly, they activate effector cells and bring them to engage cancer cells to execute their cytotoxic functions [77]. CAR technology has been tested against the target CD19 (in human cancers such as ALL, CLL), GD2 (neuroblastoma), CD22 (ALL), mesothelin (mesothelioma), and HER2 (sarcoma) [4, 8, 38, 45, 76, 90], and IL13R (glioblastoma) [13] with overwhelming success in CD19(+) leukemia, but only select solid tumors. Several factors might be responsible for the inferior efficacy of CART cells in solid versus hematological malignancies including target antigen heterogeneity, poor trafficking and penetration of therapeutic cells, and the hostile tumor microenvironment (hypoxia, acidosis, nutrient depletion, tumor-derived immunosuppressive molecules, various immunosuppressive cells including tumor-associated macrophages, regulatory T cells, and myeloid-derived suppressor cells) [85]. BsAb was successfully proven for CD19 (ALL), leading to the FDA approval of blinatumomab for Philadelphia chromosome (ph)-negative relapsed/refractory B-cell precursor ALL (BLINCYTO^{TM}) in 2016. BsAb targeting CD33, CD52, HER2, CD20, GD2, GPC3, and CD123 are in the clinical pipeline. It is timely to review preclinical and clinical studies where these T-cell based therapeutic strategies have been applied to HCC. Table 1 summarizes the BsAb and CAR targets of HCC. Table 2 summarizes clinical trials involving anti-HCC BsAb and CARs. Table 3 and 4 summarize the characteristics of BsAbs and CARs used against HCC targets.

1.3 Glypican-3 (GPC3)

GPC3 (also known as GTR2-2, intestinal protein OCI-5, and MXR7) is a heparan sulfate proteoglycan expressed as a 70kDa precursor protein. Upon cleavage by furin, GPC3 is divided into an N-terminal 40kDa and a C-terminal 30kDa fragment. The latter, which contains two heparin sulfate chains, is attached to the cell membrane via a glycosylphosphatidylinositol anchor [49].

It has been shown that GPC3 is expressed on the majority of hepatocellular carcinoma (HCC) and hepatoblatsoma cases but not or to a lower degree on normal liver tissue [51, 57, 67, 84]. Interestingly, GPC3 expression could be used as an HCC precancerous marker in cirrhotic livers since it is associated with dysplasia in cirrhotic livers [74]. Furthermore, overexpression of GPC3 in HCC is associated with poor prognostic indicators including poor tumor differentiation, higher TNM score, and tumor invasion into blood vessels [65].

1.3.1 Glypican-3 CAR—The first GPC3 CAR was generated by fusing the scFv of an anti-GPC3 antibody to the CD8α hinge and transmembrane regions followed by the intracellular signaling domain of the CD3ζ. This first-generation CAR does not have the co-stimulatory domains. Therefore a third-generation CAR composed of the anti-GPC3 scFv, CD8α hinge, CD28 transmembrane and intracellular signaling domains, 4-1BB, and CD3ζ was produced. Although human T cells transduced with concentrated lentiviral vectors containing either of the CAR constructs specifically lysed HCC cell lines, the third-generation CAR T cells produced higher levels of interleukin-2 and IFNγ, which was positively correlated with the level of GPC3 expression on target cells. The presence of soluble GPC3 protein, which has been reported in the serum of some HCC patients, only mildly (10%) inhibited the cytotoxicity of CAR T cells. T cells equipped with the third-generation CAR could suppress the growth of established orthotopic xenografts in immunodeficient mice [40].

Another group compared first-, second-, and third-generation anti-GPC3 CARs and found that the second- and third-generation constructs outperformed the first- generation CAR in vivo [68]. The choice of co-stimulatory domain might influence CART cell behavior. Anti-GPC3 CARs containing CD28, 4-1BB or both have been tested against HCC [68]. The in vitro cytotoxicity of T cells equipped with a CAR signaling through CD28 was higher than one signaling through 4-1BB. Both CARs eliminated HCC cells in vitro and in mice. Whereas CD28 induced preferential production of Th2 cytokines (IL-4 and IL-10), 4-1BB induced generation of Th1 cytokines (IFNγ and GM-CSF) as well as superior T cell proliferation *in vitro* and *in vivo*. More recently, a fourth generation GPC3- specific CAR was generated containing three co-stimulatory domains (CD27, CD28, and 4-1BB) and the inducible caspase-9 suicide gene in a lentiviral backbone [62]. While this CAR induced efficient lysis of GPC3(+) cell lines *in vitro*, pre-incubation of targets with kinase inhibitor sorafenib improved CART cell cytotoxicity. However, the effect of sorafenib on CART cells was not reported.

Besides HCC, lung squamous cell carcinoma (SCC) also expresses GPC3. T cells expressing a third generation (CD28, and 4-1BB as co-stimulatory molecules) GPC3-specific CAR were able to infiltrate subcutaneously inoculated lung SCC tumors and significantly reduce their size in xenograft models [66].

1.3.2 Glypican-3 BsAb—Recently an FcγR-silenced IgG4-based humanized bispecific antibody bearing monovalent specificities for CD3 and GPC3 was successfully tested in mouse models of HCC (fig 2A). To reduce the chance of cytokine release, corticosteroids were tested without compromising the anti-tumor effect [47]. A phase I clinical trial to assess the safety and efficacy of the antibody is in progress (NCT02748837).

Another bispecific antibody named trivalent IgG-Shaped (TriFab) construct was developed by fusing two anti-GPC3 Fab fragments via a flexible linker to one asymmetric third Fab-sized binding domain [79]. The bivalent Fab arms bind GPC3 with similar affinity of the parent IgG antibody. The third domain was designed to bind another antigen or to carry a cytotoxic payload. No *in vivo* experiment was reported.

1.4 Viral antigens

It is well known that several viruses, called oncoviruses, can induce cancer. Hepatitis B (HBV) and C (HCV) viruses are key HCC risk factors accounting for approximately 80% of HCC cases [32]. These viruses can induce HCC via several mechanisms including insertional mutagenesis (mainly for HBV versus HCV) and accumulation of genetic damage due to chronic inflammation and oxidative stress. Furthermore, direct effects of hepatitis B virus x-protein (HBx) on regulatory non-coding RNAs, as well as its interaction with various signaling pathways such as p53, Wnt, and nuclear factor-κB could also account for HBV carcinogenesis [42, 43, 105].

1.4.1 Viral antigen CAR—A second-generation CAR specific for the S domain of all three envelope proteins (S, M, and L protein, combined as HBsAg) of HBV was generated and tested in immunocompetent HBV transgenic mice. Since HBsAg is expressed on the surface of HBV-replicting cells, it can be targeted by CARs [23]. Adoptively-transferred CAR-transduced murine T cells were able to control HBV infection with only transient liver damage. Besides, the high serum level of circulating HBV antigen did not affect the function of CAR T cells. However, anti-tumor effect of this CAR was not tested [61]. In another study, second-generation CARs (containing CD28 costimulation) specific for HBV S and L antigens enabled T cells to eliminate HBV-infected human hepatocytes and hepatoma cells. CART cells specific for the S antigen (that is expressed at higher levels on infected cells) outperformed those reactive to the L antigen (which is expressed at lower levels on infected cells) in the generation of interferon-γ and cytotoxicity [10].

Other investigators using T cells transduced with a T cell receptor (TCR) specific for the S domain showed that although electroporation of T cells with anti-HBV TCR mRNA could equip nearly 80% of T cells with the transgene, TCR expression was transient and disappeared within 72 hours. In contrast to retrovirally-transduced T cells that were able to completely eliminate the HCC xenografts after a single T cell transfer, multiple injections of RNA-electroporated T cell were necessary to suppress, while not able to eradicate, HCC tumors [58]. Since HBV antigens such as the S antigen are expressed on HCC as well as infected hepatocytes, the risk of collateral damage by CART cells against infected liver could be dose-limiting [14].

HCV E2 glycoprotein (HCV/E2) is the key target for the host immune system during HCV infection and also the most variable HCV protein. In an attempt to control this infection in vitro, a second generation CAR was constructed based on a broadly cross-reactive/cross neutralizing anti HCV/E2 monoclonal antibody. Human T cells retrovirally transduced with this CAR were able to generate anti-viral and proinflammatory cytokines and lyse HCV-infected hepatoma cells [96]. However, whether the CAR-transduced T cells can completely

eliminate the HCV infection in vivo or eradicate HCV-associated liver cancers remains an open question.

1.4.2 Viral antigen BsAb—To engage T cells to the site of HBx-expressing HCC, an anti-HBx × anti-CD3 BsAb was generated by hybrid-hybridoma technology where anti-HBx and anti-CD3 hybridoma cell lines are fused together. When administered in combination with in vitro-cultured effector cells, the bispecific reagent induced apoptosis and suppressed the growth of HCC xenografts in immunodeficient mice [70]. Other investigators reported a tetravalent BsAb composed of one anti-CD3 and one anti-CD28 scFv connected to two anti-HBs antigen scFv via the IgG1 Fc-domain [11]. To minimize the chance of FcγIIR (CD16)-mediated antibody dependent cell mediated cytotoxicity (ADCC), the Fc domain was mutated. The BsAb mediated activation of T cells and redirected their cytotoxicity to HBs antigen infected hepatocytes *in vitro*.

1.5 Epithelial cell adhesion molecule (EpCAM)

Epithelial cell adhesion molecule (EpCAM, CD326) is a 39–42kDa glycoprotein comprised of a large extracellular domain, a transmembrane anchor, and a short cytoplasmic tail. The extracellular sequence contains a thyroglobulin type-1 and an epidermal growth factor-like domain [86]. EpCAM is expressed on the surface of various epithelial cells and plays important roles in cell proliferation, differentiation and migration [86]. EpCAM was reported as a cancer stem cell (CSC) marker in HCC [104, 116]. Expression of this marker was associated with poorly differentiated HCC and poor prognosis [73, 103, 104].

1.5.1 EpCAM CAR—Although not tested in HCC, anti-EpCAM CARs have been assessed in other solid tumors [6, 26, 94]. Transduction of the clinically-relevant NK-92 cells with IL-15 and an anti-EpCAM CAR resulted in proliferation of CART cells in the absence of exogenous cytokines and redirected their cytotoxicity against breast cancer cells that were resistant to unmodified NK cells [94]. In another study, peripheral blood lymphocytes transduced with an anti- EpCAM CAR preferentially lysed $EpCAM^{hi}$ cells *in vitro* and suppressed the growth of corresponding cells *in vivo* [26]. Peritoneal carcinomatosis can occur in patients with advanced gastrointestinal or gynecological neoplasms. To target peritoneal carcinomatosis, CD28/41BB-containing third generation anti-EpCAM CART cells were generated using lentiviral vectors or mRNA transfection. Whereas a single intraperitoneal injection of 10 million lentivirally-transduced EpCAM specific CART cells dramatically reduced the signal of established peritoneal ovarian cancer cells, frequent injections of mRNA-electroporated T cells was necessary to slow down tumor growth. Temporary expression of CAR on electroporated T cells might provide some safety though at the expense of efficacy. Furthermore, life threatening side effects after CART cell injection might rapidly occur [82] necessitating swift methods for eliminating T cells including the inducible caspase-9 system [27]. EpCAM-specific CART cells are undergoing clinical testing in nasopharyngeal carcinoma (NCT02915445).

1.5.2 EpCAM BsAb—The first bispecific anti-EpCAM antibody tested against HCC was a bispecific T cell engager (BiTE, fig 2B) comprised of an anti-EpCAM scFv fused to an anti-CD3 scFv via a Gly_4Ser linker. While the parent anti-EpCAM monoclonal antibody failed to

suppress tumor growth, the co-administration of peripheral blood mononuclear cells (PBMCs) and BiTE was able to suppress the growth of HCC cell lines in vitro and in vivo. Furthermore, it was shown that overexpression of galectin-1 (Gal-1) on target cells inhibited the BiTE-induced cytotoxicity while Gal-1 knock-down broke the tumor resistance to therapy [123]. Solitomab (AMG110, MT110) is a humanized anti-EpCAM×anti-CD3 BiTE. Co-administration of this BiTE and γδ T cells caused a near-complete lysis of HCC and hepatoblatsoma cell lines in vitro [50]. In addition to liver cancers, anti- EpCAM bispecific antibodies have been tested against some other tumors [9, 33, 36, 37, 87, 88, 97, 98]. Ex vivo, incubation of autologous tumor-associated lymphocytes with EpCAM(+) cancer cells in the presence of Solitomab led to activation and proliferation of effector cells and diminished the number of target cells including uterine serous papillary carcinoma [9], and Uterine and ovarian carcinosarcomas [33, 37]. Furthermore, intraperitoneal administration of catumaxomab, an IgG-based antibody with monovalent specificity against EpCAM and CD3 (fig 2C), led to regression of breast cancer-induced liver metastases [87]. In Europe, catumaxomab has been approved for treatment of malignant ascites in patients with EpCAM(+) carcinomas [98]. It is important to note that EpCAM shedding occurs in some cancer patients. Although controversial, soluble EpCAM can interfere with the function of anti-EpCAM bispecific antibodies in vitro [88, 97]. More studies are needed to clarify if EpCAM shedding compromises the anti-tumor effects of CART or BsAb directed at this antigen.

1.6 Angiogenic factors and BsAb

Vascular endothelial growth factor-A (VEGF-A) and osteopontin are two angiogenic factors with different characteristics. Either of these factors can induce the expression of the other and they can function synergistically [91]. Both factors can induce endothelial cell motility that is essential for angiogenesis; however, VEGF chemotaxis is RAC dependent while osteopontin's effect is independent of RAC activation [91, 101] Whereas osteopontin suppresses lipid raft clustering, VEGF stimulates it [109].

VEGF-A gene is comprised of 8 exons that when alternatively-spliced, generate various isoforms with different characteristics. The most common VEGF-A isoform is a secreted 45kDa heparin-binding homodimeric glycoprotein [35]. It has been reported that VEGF mRNA and protein expression is increased in HCC cells [81, 110]. Furthermore, VEGF overexpression was reported in HCC cases with recurrence [119]. Moreover, it was shown that the expression of VEGF was higher in HCC samples overexpressing the CSC markers than in those samples with a lower CSC profile [119].

Osteopontin (also known as secreted phosphoprotein 1 (SPP1), early T-lymphocyte activation 1 (ETA-1), and bone sialoprotein 1 (BSP-1)), is an O-glycosylated phosphoprotein that belongs to the Small integrin binding ligand N-linked glycoprotein family. It is expressed by various cell types including lymphocytes, fibroblasts, endothelial and epithelial cells, macrophages, dendritic cells, and neutrophils. Upon binding to its receptors (CD44 and various integrins) on target cells, osteopontin plays various physiological and pathological roles [7]. In HCC, osteopontin is overexpressed in carcinomas with capsular infiltration and osteopontin-positive cells are positioned in the cancer-stroma interface [16,

44]. Furthermore, osteopontin overexpression was associated with vascular invasion, tumor metastasis, resistance to cisplatin chemotherapy, poor prognosis, and was introduced as an early HCC diagnostic marker and an HCC-stem cell marker [15, 29, 59, 71, 99, 122].

In an attempt to target both VEGF and osteopontin, two bispecific antibodies named dual-variable domain immunoglobulin (DVD-Ig) were generated (Fig 2D) in which the binding moieties of an anti-VEGF antibody bevacizumab, and an anti-osteopontin antibody were fused together in two different orders. Results demonstrated that while the antibody with the VEGF-osteopontin orientation retained its parent antibody affinity, the osteopontin-VEGF format suffered a great loss in the VEGF-domain affinity. The former antibody was able to confer therapeutic efficacy against a human HCC cell line in mouse [60].

1.7 TCR mimics

One of the limitations of antibodies and CARs is that they target cell surface antigens; however, the majority of tumor associated antigens are intracellular. These proteins are processed within the cytoplasm and expressed as major histocompatibility (MHC)-peptide complexes on the cell surface. Alpha-fetoprotein (AFP) is a CSC marker on HCC and is associated with poorer prognosis [113, 115]. To target AFP, an scFv specific to the AFP peptide-MHC (HLA-A*02:01) complex was isolated from a human phage library and incorporated into a second-generation CAR containing the CD28 domain [72]. While intratumoral injection of CART cells had a profound and lasting anti-tumor effect, intravenously-injected CART cells were only able to reduce the growth of subcutaneously-injected HCC xenografts in immunodeficient mice. This led the investigators to propose local delivery of CART cells to the site of tumor via direct injection into the tumor or administration via the hepatic artery. Hepatic artery infusion (HAI) of chemotherapy agents is an established clinical technique. Compared to systemic administration, this method can increase local concentration of chemotherapeutic agents, reduce systemic toxicities, and improve treatment success [55]. In fact, HAI of CART cells specific for carcinoembryonic antigen(+) liver metastasis have been tested in a phase I clinical trial [56].

1.8 Other targets

In an attempt to generate bispecific constructs against liver cancer, antibody-producing cells that secrete antibodies against human hepatoma and a CD3-associated determinant, were fused together. Whereas phytohemagglutinin-activated lymphocytes lysed hepatoma cells in the presence of the bispecific molecule, resting peripheral blood lymphocytes failed to do so [19]. In another study, the Fab or Fab’ fragments of anti-CD3 or anti-CD16 antibodies were chemically attached to the Fab or Fab’ fragments of an anti HCC antibody to generate two bispecific antibodies. Lymphokine-activated killer cells (LAK) or pokeweed mitogen-activated LAK cells (PWM-LAK) were used as effector cells. Whereas 55% of the LAK cells after three days of culture expressed CD16, the phenotype of these cells shifted to CD3+ T cells afterwards. This is why the CD16-containing bispecific antibody mediated cytotoxicity only when it was administered with early LAK cells (day three of culture). On the other hand, CD3-containing bispecific molecule was effective whether early or late LAK cells or PWM-LAK cells were used [95]. Despite the well known immunobiology of natural

killer cells biology of the human liver, the role of CD16(+) Natural Killer and NKT cells in HCC has not been adequately explored.

2. Summary and future directions

T cells are highly proficient killer showing successful clinical results in various human cancers [1, 2, 78, 89, 92, 93]. In HCC, NK cells comprise 90% of lymphocytes [39]. Redirection of these cells against HCC may hold therapeutic potential. While CART cells should migrate to the tumor site upon administration, BsAb can redirect tumor-residing NK cells against cancer cells and eliminate the homing step.

Targeting CSCs is of paramount importance if a cure for tumors is envisaged. Growing evidence points to the origination of HCC from transformation of liver stem and progenitor cells [48]. Several candidate antigens expressed on CSCs have been discovered including CD13 [46], CD24 [63], CD44 [80], CD56 [18], CD90 [114, 120], CD133 [121], DLK1 [112], EpCAM [104, 106, 116], cytokeratin-19 (CK-19) [5], and OV6 [118]. Besides, it was shown that cells expressing two CSC markers, CD133/EpCAM or CD133/CD44, might be better representatives of CSCs and demonstrate superior tumorigenicity in vitro and in vivo [22, 124].

Parallel to the enhancement in effectiveness and potency of immunotherapeutic agents, side effects might also increase, which could jeopardize or slow down their regulatory approval. Therefore the choice of target is critical especially with CAR-modified effector cells which can persist for a long time in vivo or with bispecific antibodies that can recruit polyclonal effector cells. The presence of tumor cells in a vital organ or the unexpected upregulation of even low levels of the target antigen in normal tissues could cause catastrophic side effects. For example, localization of anti-ERBB2 CAR-modified T cells to the pulmonary tissue due to the low expression of ERBB2 on lung epithelial cells culminated in fatal pulmonary edema in a patient with metastatic colon cancer [83]. The lack of dose-limiting adverse effects in early clinical trials does not always guarantee the absence of life-threatening late complications. To reduce the chance of adverse effects mediated by CAR T cells, incorporation of suicide genes, such as the inducible caspase 9, into the CAR constructs would enable rapid destruction of effector cells when needed [28].

Currently, only few centers hold the necessary technology and expertise for development and implementation of CAR-based therapies. This and also the high cost of CAR and bispecific antibody therapeutics, which in most cases are not supported by the insurance companies, would impede widespread commercialization of these therapies. Development of manufacturing processes and technology transfer for widespread generation of affordable therapeutic agents on the one hand and improvement in the insurance companies' coverage for treatment costs on the other hands will enable more patients to benefit from these powerful anti cancer remedies.

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Highlights

1. Hepatocellular carcinoma (HCC), the second most common cause of cancer deaths worldwide.
2. Chimeric antigen receptors (CAR) contain the antigen binding domain of an antibody to redirect effector cells to cancer cells.
3. Bispecific antibodies (BsAb) redirect effector cells toward cancer cells for their killing.
4. HCC targets for which CARs and bispecific antibodies have been generated will be discussed in this paper.

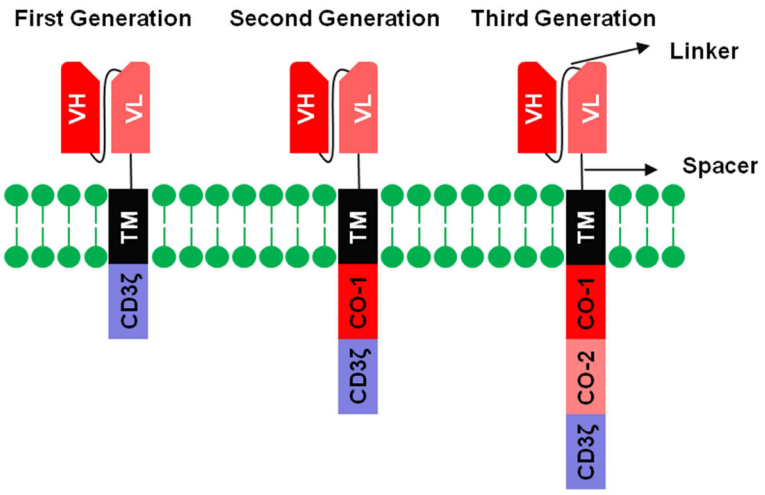

Figure 1.

Structure of chimeric antigen receptors (CAR). CARs are composed of a single-chain fragment variable (scFv, containing the heavy chain variable domain (VH) and the light chain variable domain (VL) of a monoclonal antibody attached together via a flexible linker) linked via a spacer sequence to a transmembrane (TM) domain and to the CD3ζ chain (first-generation CARs). Second- and third-generation CARs additionally contain one or two costimulatory domains, respectively. VH, heavy chain variable domain; VL, light chain variable domain; TM, transmembrane domain; co-1, costimulatory domain 1; co-2, costimulatory domain 2.

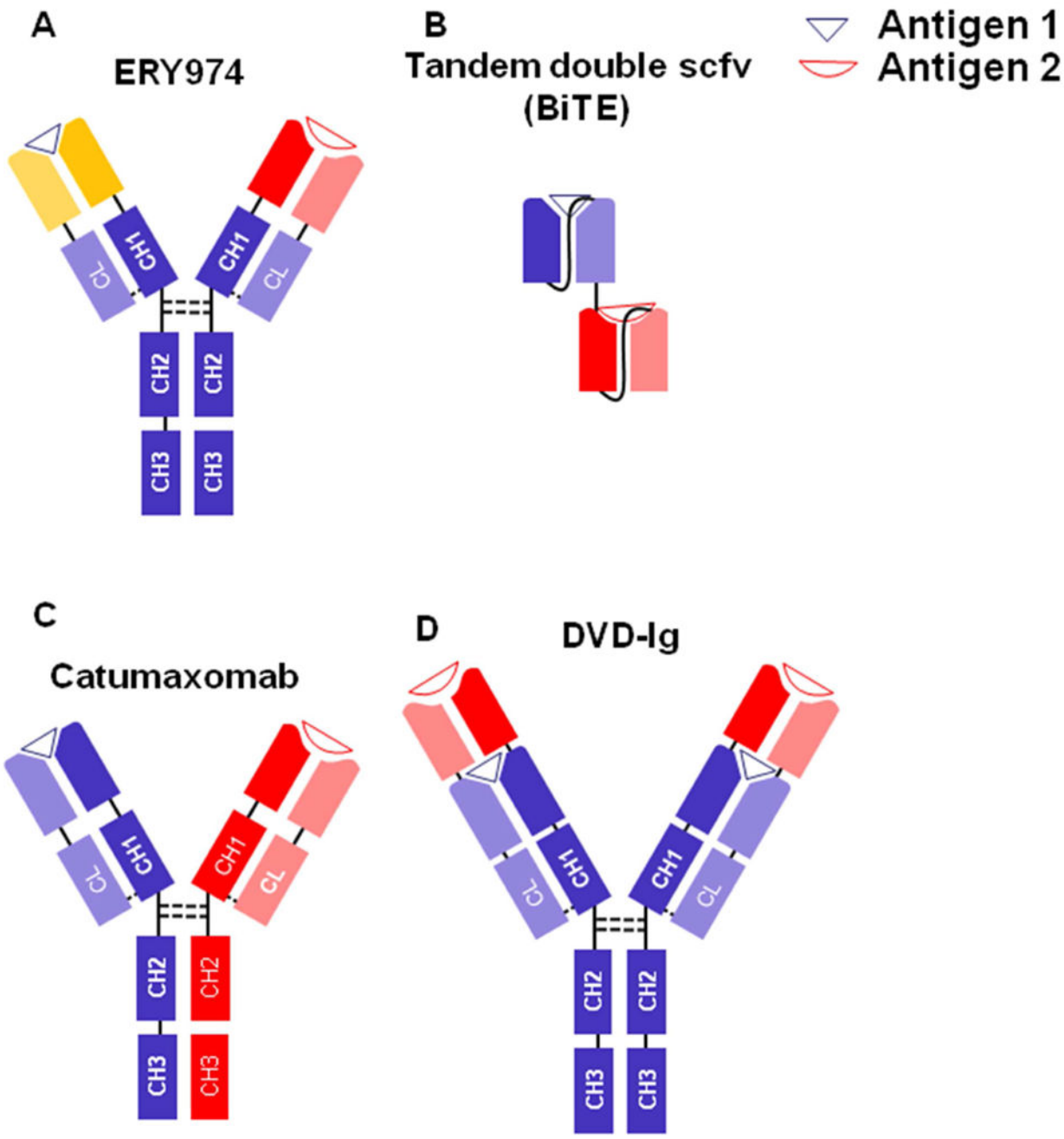

Figure 2.

Structure of BsAbs used against HCC. Heavy chains are depicted in dark colors while light chains are in the same but lighter colors. Linkers are shown by continuous lines and disulfide bonds are demonstrated by dotted lines. (A) ERY974 is an anti-GPC3/CD3 Bispecific Antibody. (B) BiTE consists of two scFv linked together via a flexible linker. (C) Catumaxomab is an anti-EpCAM/CD3 Bispecific Antibody. (D) DVD-Ig is formed by connecting the variable domains of an antibody to the variable domains of an IgG. DVD-Ig, Dual-Variable-Domain Immunoglobulin; VH, variable heavy chain; VL, variable light chain;

CL, constant light chain; CH1-3, constant heavy chains 1 to 3. BiTE, bispecific T cell engager.

Table 1

Advantages and disadvantages of HCC-associated antigens for CAR and antibody development

Marker	Advantages	Disadvantages
GPC3	**1** Expressed on the majority of HCC and hepatoblatsoma samples [32–34, 109]. **2** GPC3 expression on HCC is associated with poor prognosticindicators [37].	**1** Expressed on various tissues including brain, liver, respiratory system, skin, kidney, digestive tract, and testicular germ cells [59, 110]. **2** Not expressed in all HCC samples [109]. **3** Soluble GPC3 protein in the serum of some HCC patients can mildly inhibit the cytotoxicity of anti-GPC3 CAR T cells [38].
Viral antigens	**1** Not expressed on normal tissues (because they are viral antigens).	**1** Not expressed in HCC cases unrelated to chronic viral hepatitis.
EpCAM	**1** Expressed on 56% of HCC samples [57]. **2** Expressed on HCC CSCs [57, 58, 102].	**1** Expressed on normal tissues such as pancreas, colon, kidney tubules, bronchus, thyroid, and parathyroid glands [111]. **2** Not expressed in all HCC samples [57, 112]. **3** Soluble EpCAM might interfere with the function of anti-EpCAM bispecific antibodies in vitro [71].
VEGF-A and osteopontin	**1** Expression of VEGF was higher in HCC samples overexpressing the CSC markers [75]. **2** Overexpression of VEGF was associated with recurrence and reduced overall survival [75]. **3** Osteopontin overexpression was associated with vascular invasion, tumor metastasis, and poor prognosis [79, 82, 84]. **4** Osteopontin promotes a CSC-like phenotype in HCC [81]. **5** Osteopontin overexpression promotes resistance to cisplatin chemotherapy in HCC [83].	**1** Are secreted from cells so T cell responses cannot be redirected against HCC cells. **2** Not expressed in all HCC cases [84].

Table 2

Clinical trials involving CARs and bispecific antibodies in HCC

name	format	Phase	Clinical trial number	Sponsor	Study population	status
ERY974	CD3×GPC3 IgG4-based	1	NCT02748837	Chugai Pharmaceutical	Patients with GPC3(+)advanced solid tumors	recruiting
GPC3-CAR	-	1	NCT02932956	Baylor College of Medicine	Patients with elapsed or refractory GPC3(+)solid tumors	Not yet recruiting
GPC3-CAR	-	1	NCT02905188	Baylor College of Medicine	Patients with unresectable, HCC	Not yet recruiting
GPC3-CAR	-	1	NCT02395250	RenJi Hospital	Patients with relapsed or refractory GPC3(+)HCC	recruiting
GPC3-CAR	-	½	NCT02723942	Fuda Cancer Hospital, Guangzhou	Patients with GPC3(+)HCC without extrahepatic metastasis	recruiting
GPC3/mesotheli n/CEA CAR	-	½	NCT02959151	Shanghai GeneChem Co., Ltd	Patients with GPC3(+)HCC	recruiting
MUC1 CAR	-	½	NCT02587689	PersonGen BioTherapeutics (Suzhou) Co., Ltd	Patients with MUC1(+) HCC, NSCLC[1], pancreatic and breast cancer	recruiting
MUC1 CAR	-	1/2	NCT02839954	PersonGen BioTherapeutics (Suzhou) Co., Ltd	Patients with MUC1(+) HCC, NSCLC[1], pancreatic, glioma, colorectal, gastric, and breast cancer	recruiting

[1]Non-small cell lung cancer

Table 3

Characteristics of bispecific antibodies generated against HCC

Name/format		MW(kDa)	Affinity (K_D) nM			EC_{50} pM	clone
			Effector (CD3, etc)	Target (GPC3, etc)			
				Parent clone	Bispecific format		
ERY974 (Humanized IgG4 MAb) [42]		150	-	-	-	-	-
TriFab [43]		150	-	3.4	3.4	-	GC33 (GPC3)
EpCAM×CD3 BiTE [63]		54	-	-	-	1.42 - 96	1H8 (EpCAM)
EpCAM×CD3 BiTE (Solitomab, AMG110, MT110) [113]		55	77	-	16–230	7.9	diL2K (CD3)
EpCAM×CD3 (Catumaxomab) [114]		150	-	0.55	0.56	-	Ho-3 (EpCAM),
VEGF×Osteopontin [85]		200	-	HuA.4.6.1 (1.55) hu1A12 (9.27)	HuA.4.6.1 (1.64) hu1A12 (9.43)	-	HuA.4.6.1 (Bevacizum ab, VEGF), hu1A12 (osteopontin)
(CD3×anti-HCC) DQ-33 [91]		150	-	-	-	-	Hepama-6 (anti-HCC)
[92]	CD3×anti- HCC	100	-	-	-	-	OKT3 (CD3), L-7- 6 (anti- HCC)
	CD16×anti- HCC	100	-	-	-	-	3-G-8 (CD16), L- 7-6 (anti- HCC)

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Table 4

Characteristics of CARs generated against HCC

Reference	Hinge	TM	Costimulatory	Signaling	Epitope	Clone	Virus
[38, 41]	CD8α	CD8α	CD28, 41BB	CD3ξ	C-terminal region of GPC3	GC33	Lenti
[39]	IgG1	CD28	CD28, 41BB	CD3ξ	C-terminal region of GPC3	GC33	Retro
[40]	-	-	CD27, CD28, 41BB	CD3ξ	Conformational epitope (N- and C-terminal) of GPC3	HN3 (Kd 0.6nM)	Lenti
[41]	CD8α	CD28	CD28, 41BB	CD3ξ	C-terminal region of GPC3ξ	GC33	Lenti
[49, 50]	Human IgG1 Fc	CD28	CD28	CD3ξ	conformational epitope in the HBs antigen	C8, and 5a19	Retro
[53]	Human IgG Fc	CD28	CD28	CD3ξ	HCV/E2	e137	Retro
[61]	CD8α	CD28	CD28	CD3ξ	EGF-like domain I of EpCAM	MOC31	Lenti
[62]	CD8	CD28	CD28	CD3ξ	EGF-like domain I of EpCAM	C215	Retro
[88]	-	-	CD28	CD3ξ	$AFP_{158–166}$ peptide presented by HLA-A*02:01	ET1402L1	Lenti