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Commentary

# Targeting hepatocellular carcinoma heterogeneity with FAP and GPC3-specific tandem CAR-T cells

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A recent study published in *Molecular Therapy Oncology* by Zhou et al. reported a chimeric antigen receptor (CAR)-T design simultaneously targeting fibroblast activation protein (FAP) and glypican-3 (GPC3). It was found to elicit an effective immune response in the treatment of hepatocellular carcinoma (HCC), hold the potential to reduce antigen escape, and resist cancer heterogeneity.[1]

CAR-modified T cell immunotherapy is a powerful treatment for hematological malignancies. Inspired by the success of CD19- and B-cell maturation antigen (BCMA)-targeted CAR-T cells, much attention has been devoted to extending this therapy to the treatment of solid tumors but with limited progress.[2]

Antigen escape is an important CAR-T cell therapy resistance mechanism for broadly hematological malignancies and solid tumors. The complexities and nature of tumors with antigen loss/downregulation and cancer heterogeneity present challenges for antigen targeting and recognition in CAR-T therapy. Many efforts have been made to improve the precise recognition of CAR-T cells by fine-tuning the affinity of antigen recognition domains and optimizing CAR molecules. Among these approaches, a promising and effective strategy is targeting multiple tumor-associated antigens simultaneously. This includes i) two separate single-targeted CAR-T cell products co-infusion; ii) T cells co-express distinct single-chain variable fragments (scFvs) in tandem (Tandem CAR); and iii) T cells co-express separate CARs (dual CAR or trivalent CAR) for cognate antigen recognition.[3] These optimized CAR designs expanded antigen coverage and triggered enhanced immune responses when CAR-T cells encountered one of multiple antigens. This effectively reduces antigen escape and prevents tumor relapse in cancer immunotherapy.

The efficacy of adoptive therapy was limited by the immunosuppressive tumor microenvironment (TME). Cancer-associated fibroblasts (CAFs) serve as key components of the TME and play a pivotal role in remolding the TME and promoting tumor progression.[4] FAP is an important biomarker on CAFs. FAP-targeted CAF depletion can disrupt the structural integrity of the tumor extracellular matrix and enhance the susceptibility of tumor cells to immunotherapy. This makes FAP an attractive molecule for targeting tumor extracellular stroma. In a recent study in *Molecular Therapy Oncology*,[1] Zhou et al. observed a significant elevation of FAP and GPC3 in primary HCC samples, and the high expression of FAP and GPC3 was correlated with poor prognosis for patients with HCC. Previous studies have demonstrated the anti-hepatoma potential of GPC3-targeted CAR-T cells in preclinical and clinical trials.[5,6] Since HCC is characterized by intratumor heterogeneity and divergent clonal lineages within and among primary and recurrent tumors,[7] the authors hypothesized that targeting HCC using a bispecific CAR target FAP and GPC3 (FAP-GPC3 CAR) can overcome cancer heterogeneity, prevent antigen escape and obtain enhanced anti-tumor efficacy for HCC (Figure 1). The tandem CAR was constructed with an anti-FAP scFv and subsequently linked with anti-GPC3 scFv by a G4S linker, followed by CD28 co-stimulatory signaling and CD3ζ domains. The efficacy of FAP-GPC3 CAR-T cells was evaluated *in vitro* through co-culturing CAR-T cells with the HCC cell line HepG2, SNU387, or SNU398, which exhibit non-uniform FAP and GPC3 expression. These co-culture assays demonstrated that both single-target CAR-T cells and FAP-GPC3 CAR-T cells effectively recognized these cancer cells and were activated by them. Compared with FAP CAR-T cells or GPC3 CAR-T cells, FAP-GPC3 CAR-T cells have heterogeneous inflammatory cytokine secretion (including interferon [IFN]-γ, granzyme B [GZMB], tumor necrosis factor-alpha [TNF-α], and interleukin [IL]-2) when engaged with HepG2, SNU387, and SNU398 cells. Meanwhile, comparable or even stronger tumor-specific cytotoxicity of FAP-GPC3 CAR-T cells was observed against those tumor cells. The ability of CAR-T cells to kill tumors and prevent cancer escape was further evaluated in HCC cell line-derived xenograft (CDX) models and patient-derived xenograft (PDX) models. The results consistently showed that, compared to single-target CAR-T cells, FAP-GPC3 CAR-T cells showed similar anti-tumor efficacy in SNU398 tumor treatment and were stronger in treating HepG2 and PDX models. The survival time was prolonged after administration of FAP- or GPC3 CAR-T cells and further extended in mice treated with FAP-GPC3 CAR-T cells. Thus, Zhou and colleagues have proposed a feasible targeting strategy for CAR-T therapy to improve the immune response and overcome antigen heterogeneity in treating HCC.

The implementation of multiple targeting strategies has been shown to effectively enhance the recognition of CAR-T cells to heterogeneous cancer antigens. In Zhou's study, FAP-GPC3 CAR-T cells exhibited powerful anti-tumor efficacy against HCC, while the persistence of FAP-GPC3 CAR-T cells needed to be improved to support its clinical translation, as the success of CAR-T therapy pivots on not only robust

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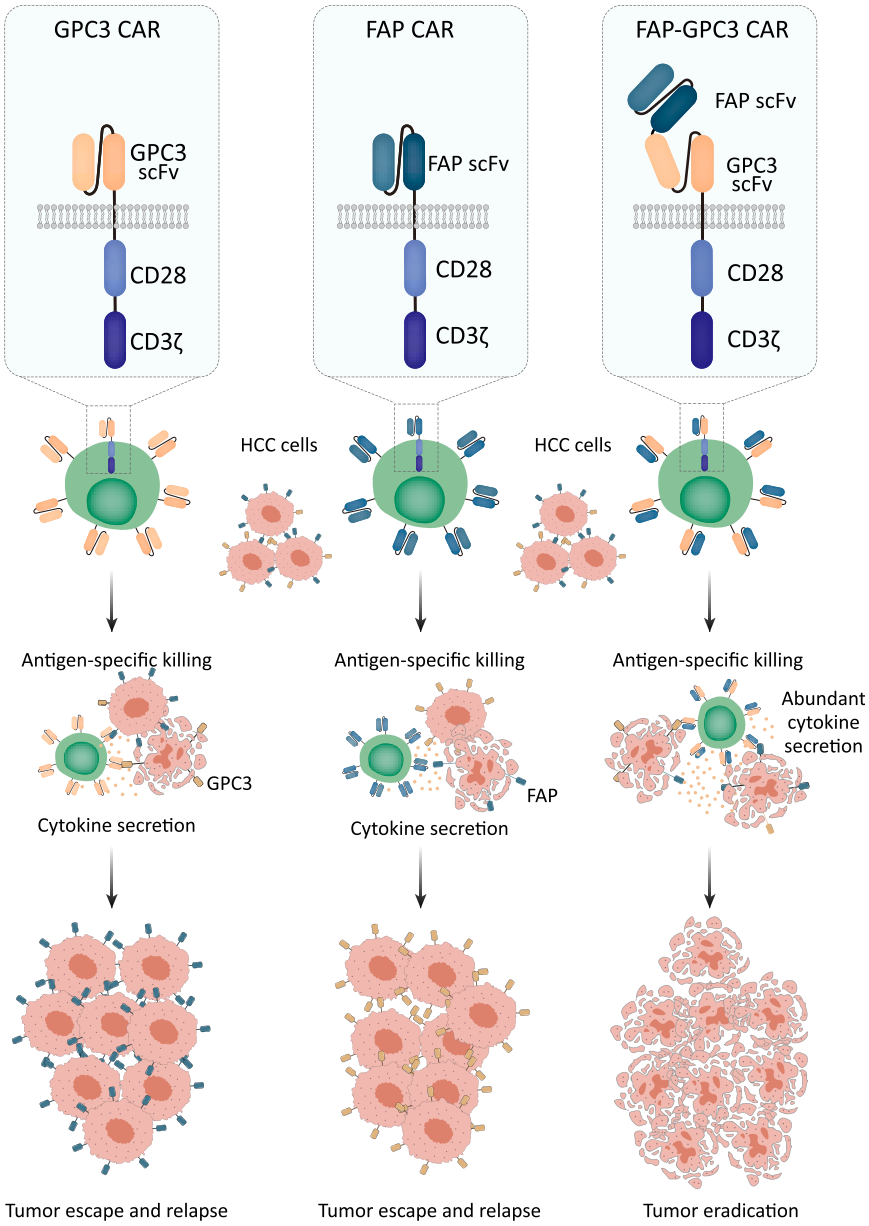


**Figure 1. FAP-GPC3 CAR-T cells hold the potential to enhance tumor-specific killing against HCC tumors and prevent tumor escape and relapse**

The figure shows that both single-target FAP CAR-T and GPC3 CAR-T cells are insufficient to eradicate HCC cancers, partly due to the presence of antigen heterogeneity. In contrast, multiple-target FAP-GPC3 CAR-T cells produce more inflammatory cytokines, exhibit stronger tumor-killing activity, and eventually induce HCC eradication.

cytotoxicity but also sustained persistence over an extended period. In the clinical trials of CD19- or CD22-targeted CAR-T cell therapy, up to 50% of patients with pre-B acute lymphocytic leukemia (ALL) have disease relapse within 12 months after CAR-T cell administration.[8] In these patients, early relapse is often associated with limited CAR-T cell persistence. The determinants of CAR-T cell persistence remain to be fully understood, but decreased memory differentiation and enhanced exhaustion of CAR-T cells should, at least partly, be responsible for poor persistence. Previous experience suggested that CAR signal optimization, such as modulating the co-stimulatory domain (for example, replacing CD28 with 4-1BB or OX40) and cytokine signaling (IL-2, IL-7, or IL-15), serves to enhance the persistence of CAR-T cells.[9] Transcription factors (such as FOXO1[10,11] and c-Jue[12]) signal tuning confers exhaustion resistance activity to CAR-T cells and helps them seek more opportunities in cancer treatment. Another important translational issue for FAP-GPC3 CAR-T therapy is that low levels of FAP expression in bone marrow, adipose tissue, skeletal muscle, pancreas, and other healthy tissues may raise safety concerns for FAP-targeted CAR-T therapy.[13,14] Rosenberg et al. have reported that FAP-targeted CAR-T cells recognized and ablated FAP-positive bone marrow stromal cells (BMSCs), leading to lethal bone toxicity and cachexia in mice.[13] As human multipotent BMSCs also express FAP, a cautious dose escalation strategy will likely be necessary to find a therapeutic window for FAP-GPC3 CAR-T therapy to prevent on-target, off-tumor toxicity.

Overall, Zhou and colleagues developed an effective target strategy to resist the heterogeneity of HCC for CAR-T cell therapy. Expanded antigen coverage of FAP-GPC3 CAR-T cells is crucial for their robust antitumor efficacy and activity to overcome cancer heterogeneity in their study. Such a targeted approach increases the possibility of viable CAR-T therapy for HCC treatment and holds promise for translation.

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## DECLARATION OF INTERESTS

The authors declare no competing interests.

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