PTK7-Targeting CAR T-cells for the Treatment of Lung Cancer and other Malignancies

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Research

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Abstract

**Background:** In spite of impressive success in treating hematologic malignancies, adoptive therapy with chimeric antigen receptor modified T cells (CAR T) has not yet been effective in solid tumors, where identification of suitable tumor-specific antigens remains a major obstacle for CAR T-cell therapy due to the “on target off tumor” toxicity. Protein tyrosine kinase 7 (PTK7) is a member of the Wnt-related pseudokinases and identified as a highly expressed antigen enriched in cancer stem cells (CSCs) from multiple solid tumors, including but not limited to triple-negative breast cancer, non-small cell lung cancer, and ovarian cancer, suggesting it may serve as a promising tumor-specific target for CAR T-cell therapy.

**Methods:** In this study, we constructed 3 different PTK7-specific CAR (PTK7-CAR1/2/3) each comprising a humanized PTK7-specific single chain variable fragment (scFv), hinge and transmembrane (TM) regions of the human CD8α molecule, 4-1BB intracellular co-stimulatory domain (BB-ICD), and CD3ζ intracellular domain (CD3ζ-ICD) sequence, and then prepared the CAR T cells by lentivirus mediated transduction of human activated T cells accordingly, and sequentially evaluated their antigen-specific recognition and killing activity in vitro and in vivo.

**Results:** T cells transduced with all 3 PTK7-CAR candidates exhibited antigen-specific cytokine production and potent cytotoxicity against naturally expressing PTK7-positive tumor cells of multiple cancer types without mediating cytotoxicity of a panel of normal primary human cells; meanwhile, in vitro recursive cytotoxicity assays demonstrated that only PTK7-CAR2 modified T cells retained effective through multiple rounds of tumor challenge. Using in vivo xenograft models of lung cancers with different expression level of PTK7, systemic delivery of PTK7-CAR2 modified T cells significantly prevented tumor growth and prolonged overall survival of mice.

**Conclusion:** Altogether, our results support PTK7 as a therapeutic target suitable for CAR T-cell therapy that could be applied for lung cancers and many other solid cancers with PTK7 overexpression.

Background

Chimeric antigen receptor (CAR)-modified T-cell (CAR T-cell) therapy is an innovative immunotherapeutic approach which vigorously rejuvenates the long-term pursuit adoptive cell transfer (ACT) for cancer immunotherapy [1, 2]. Typical synthetic CAR comprises of single-chain variable fragment (scFv) of a monoclonal antibody (mAb), hinge/spacer and transmembrane (TM), and co-stimulatory and activating signaling domains from one or two co-stimulatory molecules and CD3ζ chain of the T cell receptor (TCR) complex respectively [1, 3]. CAR modification confers T cells with “de novo” defined antigen specificities independently of both the natural TCR and major histocompatibility complex (MHC) restriction, which not only overcomes the downregulation of Human Leucocyte Antigen (HLA, human MHC) molecules frequently observed in cancer cells, but also widening the repertoire of actionable targets due to scFv-mediated antigen recognition of non-protein epitopes, thus greatly expanding the potentials of ACT for cancer immunotherapy [1, 3, 4]. CAR T-cell therapy targeting CD19 antigen has achieved a remarkable
therapeutic efficacy in treating relapse or refractory B-cell malignancies, culminating in the regulatory approval of two CAR T-cell products for patients with certain leukemia and lymphoma [1, 2]; in addition, CAR T cells targeting other antigens, such as BCMA and CD22, have also exhibited a promising therapeutic potential in treating some type of intractable leukemia and multiple myeloma [5-7]. These results have demonstrated that CAR T cells can be artificially generated with desirable characteristics to induce durable and complete responses in cancer patients even with highly refractory disease.

Despite great success in treating hematological malignancy, CAR T-cell therapy in solid tumor is still in its infancy with scant objective response seen [4]. Among various factors constraining the efficacy of CAR T-cell therapy in solid tumor, a major obstacle is the lack of appropriate tumor antigens suitable for CAR-T targeting [4, 8]. At present, the majority of CAR T-cell targets in solid tumors are overexpressed tumor-associated antigens (TAA) with lower level expression in normal tissues as compared to tumor tissues, such as HER2, GPC-3, EGFR, mesothelin, PSMA and IL13Ra2, which greatly limits the maximum safety dosage in order to avoid on-target off-tumor side effect, and consequently results in unsatisfactory clinical efficacy [1, 8, 9]. In addition, due to the extreme heterogeneous antigen expression and highly genomic instability in solid tumors, tumor cells are prone to produce antigen-loss variants under the immune selection pressure from CAR T-cell therapy, leading to immune escape [8]. Therefore, the identification of new target antigens that are not easy to generate immune escape is still a key issue for the successful treatment of solid tumors with CAR T cells.

PTK7, also known as colon carcinoma kinase 4 (CCK-4), is a member of the pseudokinase family of receptor tyrosine kinases (RTKs) that has an intracellular catalytically inactive tyrosine kinase-like domain [10, 11]. PTK7 is expressed during embryogenesis but absent from normal vital adult tissues, apart from a subset of immature CD4+ recent thymic emigrants (RTEs) and plasmacytoid dendritic cells (pDCs), and low level expression on some normal tissues [10-12]. Genetic and biochemical studies have demonstrated an involvement of PTK7 in noncanonical Wnt signaling via interacting with Wnt ligands such as ROR2, Wnt5a or Wnt3a [13, 14]. PTK7 is strongly associated with planar cell polarity (PCP) regulation as PTK7-deficient embryos exhibit severe developmental defects in PCP [15, 16]. In addition, evidence is also present for context-dependent roles of PTK7 in the vascular endothelial growth factor (VEGF), semaphorin/plexin, and canonical Wnt signaling pathways [11]. Oncogenic functions of PTK7 have been documented in several hematological and solid tumors [10, 11]. Recent studies showed that PTK7 is overexpressed in triple-negative breast cancer (TNBC), non-small cell lung cancer (NSCLC), ovarian cancer (OVCA), cervical cancer, esophageal squamous cell carcinoma (ESCC), and hepatocellular carcinoma (HCC) and enriched in tumor-initiating cells (TICs) from TNBC, OVCA, and NSCLC patient-derived xenografts (PDXs), and its overexpression is associated with poor survival in NSCLC, cervical cancer, ESCC and HCC [12, 17-22]. Bie J et al. found that PTK7 was dramatically upregulated in the ESCC tissues and cancer stem cells (CSCs)-like cells and its knockdown reduced sphere formation, promoted apoptosis, and suppressed invasive behavior of tumor cells [17]. Chen et al. conducted a large-scale meta-analysis to search the genes specifically overexpressed in lung adenocarcinoma where PTK7 was identified to be the one of overexpressed six genes confirmed by IHC analysis in primary adenocarcinoma samples. Functional investigation revealed that PTK7 knockdown decreased cell viability and increased
apoptosis in lung adenocarcinoma cell lines. More importantly, a PTK7-targeting antibody-drug conjugate (ADC) induced sustained tumor regressions in lung and breast tumor xenograft models [12]; furthermore, recent studies have documented the success and feasibility of PTK7-based tumor-targeting strategies by using PTK7-specific antibodies or aptamers for in vivo imaging or drug delivery [23-25]. These pioneering studies strongly support the potential of the PTK7 as an attractive candidate for CAR T-cell therapy that could be broadly applied.

In this study, we developed an alternative approach of exploiting PTK7 as a target for CAR T-cell therapy. The rationale is based in part upon the hypothesis that PTK7 expression is enriched on TIC/CSCs-like cells and targeting antigens with enriched expression in TIC/CSCs-like cells would achieve a long-term antitumor effect [26]. Given the predicted potential and safety of PTK7 as an immunotherapy target, we sought to develop PTK7-specific CAR T-cell therapy for lung cancer, and to evaluate its efficacy and safety in vitro and in vivo preclinical models.

**Materials And Methods**

**Cell lines**

Human NSCLC cell lines H520, H1975 and H1299, SCLC cell lines H446 and H69, pancreatic cancer cell line BxPC3, breast cancer cell line MDA-DB-468, ovarian cancer cell line OVCAR3, CHO and HEK-293 T cells were purchased from American Type Culture Collection (ATCC) and maintained in DMEM medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 1% penicillin/streptomycin (all from Thermo Fisher Scientific) and all cell lines were cultured at 37 °C in a humidified chamber with 5% CO2. Stably transfected PTK7-CHO cell line was constructed by infecting parental CHO cells with lentiviral supernatants containing PTK7 gene (#HG19399-UT, Sino Biological) and sorting for PTK7 expression by using MoFloTM XDP cell sorting system (Beckman Coulter). These cell lines were also infected with the lentiviral supernatants containing Luciferase-IRES-GFP (GL) and were then sorted for GFP expression to obtain GL-expressing cell lines. Human primary normal epithelial cell lines (Mammary, Small Airway and Renal Epithelial Cells) and human umbilical vein endothelial cells (HUVECs) were obtained from PriCells (Wuhan, China) and cultured according to the supplier's instructions.

**PTK7-CAR construction**

Sequences of 3 humanized mouse anti-human PTK7 antibodies (Hu23, Hu24 and Hu58) were obtained from an US patent (US20150315293A1). The variable region sequences of heavy (VH) and light chain (VL) of these antibodies were used to design scFv with the sequence of VH-G4S Linker-VL. PTK7-CARs containing scFv from Hu23, Hu24 and Hu58 were designated as PTK7-CAR1, PTK7-CAR2, and PTK7-CAR3 respectively. From the 5'-end to 3'-end, each CAR is comprised of the human CD8α signal peptide sequence, PTK7-scFv, hinge and TM regions of the human CD8α molecule, 4-1BB intracellular domain sequence (BB-ICD), and CD3ζ intracellular domain sequence (CD3ζ-ICD) as previously described [27].
Following CAR, a truncated tEGFR sequence is included via T2A ribosomal skipping sequence in the construct to allow for potential enrichment, tracking and depletion if needed of transduced T cells [28]. DNA encoding the CARs was codon-optimized and synthesized by General Biosystems (Anhui, China) with appropriate restriction sites. The CAR sequences were then cloned into third generation self-inactivated lentiviral vector pLVEF derived from pRRLSIN.cPPT.PGK-GFP.WPRE vector (Plasmid #12252, Addgene) with replacing its original human PGK promoter with human EF1α promoter from pWPXLd vector (Plasmid #12258, Addgene). As a negative control, lentiviral vector encoding truncated tEGFR was constructed.

Lentivirus production

High-titer replication-incompetent lentiviruses were produced and concentrated as described previously [29]. Briefly, HEK-293 T cells were transfected with pVSV-G (VSV glycoprotein expression plasmid), pRSV-Rev (Rev expression plasmid), pMDLg/p.RRE (Gag/Pol expression plasmid), and pLVEF transfer plasmid using polyethylenimine (PEI, Sigma). The viral supernatant was harvested at 24 and 48 hours after transfection and concentrated by using Lenti-X Concentrator (Clontech) in accordance with manufacturer’s instructions.

CAR T-cell production

Human PBMCs were obtained from healthy donors under protocols approved by the Institutional Review Board of Harbin Medical University and isolated by density gradient centrifugation over Ficoll-Paque (GE Healthcare). Freshly isolated PBMCs were then activated with anti-human CD3/CD28 Dynabeads (Thermo Fisher Scientific) at a 3:1 ratio for 48 hours followed by two sequential transduction with lentiviruses on RetroNectin-coated non-tissue treated plates and maintained in culture in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific) and recombinant human IL-2 (300 U/mL). Fresh media containing cytokine were replenished every other day to maintain T-cell concentration at 0.5×10^6 cells/mL. Five days after transduction, the CD3/CD28 Dynabeads were removed from the culture by magnetic separation, and CAR T cells were propagated for 14 days in total before using for functional assays. To track T cell numbers over time, viable cells were counted using trypan blue.

Flow cytometry

PTK7 expression on tumor cells was detected by mouse monoclonal anti-PTK7 antibody (clone OTI2E7, Invitrogen) and goat-anti-mouse IgG-phycoerythrin (PE)-conjugated antibody (Jackson ImmunoResearch). CAR expression on 293T cells was detected by APC-conjugated rabbit monoclonal anti-EGFR antibody (Sino Biological) and biotin-conjugated goat-anti-human IgG F(ab’)2 (Jackson ImmunoResearch) and streptavidin-PE (Biolegend). CAR expression on T cells was detected by FITC-conjugated CD4 (clone OKT4), PE-conjugated CD8 (clone SK1, all from Biolegend), and APC-conjugated anti-EGFR antibody. The phenotype and effector molecule expression on T cells were detected with a panel of monoclonal anti-human antibodies as follow: BV510-conjugated CD3 (clone UCHT1), BV421-
conjugated CD4 (clone OKT4), APC-Cy7-conjugated CD8 (clone SK1), APC-conjugated CD45RO (clone UCHL1), PE-conjugated CCR7 (clone G043H7), PE-conjugated TIM-3 (clone F38-2E2), APC-conjugated PD-1 (clone EH12.2H7), and PE-conjugated Granzyme B (clone GB11, all from Biolegend). CAR T cells in spleens from tumor-bearing tumor was detected by BV510-conjugated CD3 and APC-conjugated rabbit anti-EGFR antibody. In most assays, cells were stained with Zombie Aqua™ Fixable Viability Kit (BioLegend) to exclude dead cells from analysis. Flow cytometry data were acquired with a FACSCantoTM system (BD Biosciences) using DIVA software according to the manufacturers’ instructions.

**Cytokine release assays**

Control or PTK7-CAR T cells (1×10^5 cells/100 μl media) were co-cultured with an equal number of target cells for 24 hours, after which cell-free supernatants were harvested for testing IL-2 and IFN-γ secretion by ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Proliferation Assay**

Control or CAR T cells were first labeled with 5 μM fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) according to the manufacturer’s instructions, and then co-cultured with tumor cells at an effector-to-target ratio of 1:1. CFSE dilution was measured on gated T cells on day 3 using flow cytometry.

**In vitro killing assays**

For tumor cell killing assays, GL-expressing target cells (1x10^4 cells/100 μl media) were co-cultured with control or PTK7-CAR T cells at the varying effector-to-target ratios in triplicate wells of white 96-well plates. In some assays, it was conducted in the presence of soluble PTK7 protein (OriGene). Target cell viability was monitored 18 h later by using Bright-Glo™ Luciferase Assay System (Promega) according to the manufacturer’s instructions. The percent lysis (%) was calculated by using the following equation: 1- [bioluminescence value in sample well (target cells + CAR T cells)/maximum bioluminescence value (target cells alone)].

For human primary normal cell killing assays, target cells were first labeled with 5 μM fluorescent dye CFSE according to the manufacturer’s instructions, and then co-cultured with control or PTK7-CAR T cells at the indicated effector-to-target ratios in triplicates. After 18-hour incubation at 37°C, mixed cells were harvested and stained with 7-AAD and then subjected to flow cytometric analysis to quantify remaining live (7-AAD negative) target cells. The cytotoxicity was calculated as 100% - the percentage of alive target cells/alive target cells in control wells without effectors.

**In vitro recursive cytotoxicity assays**

GL-expressing tumor cells (1x10^5 cells/500 μl media) were seeded in 12-well tissue culture plates, after overnight plating, 2.5 × 10^4 (effector-to-target ratio of 1:4) CAR T cells were added to the monolayer of
tumor cells. Three days later when tumor cells had been completely eradicated (round 1), all cells in the well were collected and washed with PBS, resuspended in fresh medium and added to a new plate seeded with tumor cells for 3 days (round 2). This procedure was repeated one more time, if applicable (round 3). At the end of each round, a duplicate well was harvested for counting of residual tumor cells (GFP⁺) and CAR T cells (CD3⁺) and other phenotypic analysis (granzyme B, PD-1, TIM-3) of CAR T cells by flow cytometry.

**In vivo tumor models**

All animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Harbin Medical University. Six- to 8-week-old B-NSG mice (NOD-Prkdcscid Il2rgtm1/Bcgen) were obtained from Biocytogen Co., Ltd (Beijing, China) and maintained on a 12-h light–dark cycle in a temperature-controlled high barrier facility with free access to food and water and treated under specific pathogen-free conditions at the Animal Centre of the Harbin Medical University. The tumor xenograft model was established by subcutaneous (s.c.) inoculation with 3 x 10⁵ H520 or H69 tumor cells suspended in 100µl PBS. After 7 days when the tumor was consistently palpable (50-100 mm³), mice were randomized into 3 groups (3-5 mice per group) and intravenously (i.v.) injected with control or PTK7-CAR T cells suspended in 100µl PBS and repeated once one week later. Mice were weekly monitored for tumor growth by using a calliper for 60 days, and then euthanized by cervical dislocation with blood and tumor harvested for analysis when they seemed moribund or their tumors reached 15 mm in diameter. Tumor volume (V) was calculated according to the following formula: V (mm³) = length x width²/2.

**Immunohistochemistry (IHC)**

Tumor tissues were fixed with formalin and embedded in paraffin. Then, 4-mm-thick sections were deparaffinized with xylene and rehydrated in decreasing concentrations of ethanol. After heat-induced antigen retrieval, slides were then blocked by 3% BSA and stained with rabbit monoclonal anti-human CD3ε antibody (clone SP162, Abcam) or rabbit polyclonal anti-PTK7 antibody (Invitrogen) in the blocking solution overnight at 4°C. Slides were then rinsed with Tris-HCl/0.05% Tween-20 buffer and visualized with a horseradish peroxidase (HRP)-conjugated anti-rabbit EnVision+ Kit (Dako). PBS substituted for the primary antibody was used as the negative control.

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism software (version 7.0). Differences in groups were determined by two-way ANOVA with Tukey’s multiple comparison test with P<0.05 considered to be a statistically significant. The survival curves were constructed using the Kaplan-Meier method and analyzed by using a log-rank test. All values and error bars represent the mean ± SEM. In the figures, significance of findings was defined as follows: p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001, or ****p < 0.0001.
Results

Generation of PTK7-CAR T cells

To assess the suitability of PTK7 as a target for CAR T cells, we designed 3 CARs (PTK7-CAR1, PTK7-CAR2 and PTK7-CAR3) each containing a scFv derived from 1 of 3 humanized anti-human PTK7 monoclonal antibodies (Fig. 1a). The PTK7-specific scFv was fused to CD8α hinge and transmembrane domain with intracellular 4-1BB (CD137) co-stimulatory and CD3ζ activating signaling domains in tandem. To facilitate the detection of transduced T cells, a truncated EGFR (tEGFR) tag was included via T2A ribosomal skipping sequence. Expression of tEGFR alone was served as a negative control. We synthesized full-length DNA encoding each of the CARs and cloned into a self-inactivating lentiviral vector. 293T cells were infected with CAR-encoding replication-incompetent lentiviruses where CAR and tEGFR displayed a linear co-expression pattern, indicating that tEGFR is a reliable marker for PTK7-CAR expression (Fig.1b). T cells from healthy donors were then transduced with the lentiviruses following anti-CD3/CD28 bead stimulation, and tEGFR expression was determined by FACS analysis 5-7 days after transduction. We observed a high level of tEGFR on each CAR transduced T cells with both CD4+ and CD8+ cells having the similar percentage of tEGFR expression among 3 PTK7-CAR candidates (Fig. 1c). The transduction efficiency of activated T cells was generally greater than 60% (Figure S1a)., and phenotypic analysis showed that PTK7-CAR T cells contained central-memory, effector-memory, and T stem cell memory, without significant differences among 3 candidates (Figure S1b). In addition, no difference in T-cell expansion without antigen stimulation was seen in vitro among control and those candidates (Figure S1c).

PTK7-CAR T cells secrete effector cytokines and proliferate after exposure to PTK7-expressing tumor cells

To test specific recognition by PTK7-CAR T cells, we initially exploited PTK7-negative parental CHO cells and stably transfected PTK7-expressing PTK7-CHO cells (Fig. 2). PTK7-CAR T cells and control T cells of 3 donors were co-cultured with CHO or PTK7-CHO cells, and effector cytokine IFN-γ and IL-2 release in the supernatants were evaluated after 24 hours (Fig. 3a). PTK7-CAR T cells secreted significant amounts of IFN-γ and IL-2 after exposure to PTK7-CHO cells compared with control T cells; however, parental CHO cells did not stimulate PTK7-CAR T cells to produce effector cytokines, indicating that cytokine production requires both the expression of PTK7 on target cells and PTK7-CAR expression on transduced T cells. We confirmed the above findings using a panel of tumor cell lines naturally expressing the varying levels of PTK7 representative of multiple cancer types, including NSCLC (H520, H1975, H1299), SCLC (H446, H69), pancreatic (BxPC3), breast (MDA-DB-468) and ovarian (OVCAR3) cancer (Fig. 2). Similarly, PTK7-CAR T cells produced a large amount of IFN-γ and IL-2 which is positively associated with the expression level of PTK7 on respective tumor cells (Fig. 3a). Notably, PTK7-CAR2 T cells had a trend of producing a higher level of cytokines especially responding to stimulation by tumor cell lines expressing lower level of PTK7 (H1299 and BxPC3 cells).
We also evaluated the antigen-specific proliferation of PTK7-CAR T cells in response to PTK7-expressing cells. T-cell proliferation was dependent on the expression level of PTK7 on target cells, and tumor cells with high level of PTK7 expression induced more vigorous T-cell proliferation than that with lower level of PTK7 expression (Fig. 3b). Again, PTK7-CAR2 T cells exhibited a trend of more potent proliferation when stimulated with tumor cells expressing lower level of PTK7.

**PTK7-CAR T cells specifically kill PTK7-expressing tumor cells and retain effector function upon recursive target exposure**

We next evaluated the specific killing of PTK7-positive tumor cells by PTK7-CAR T cells in both short-term (18 h) and recursive long-term (3 rounds with each round of 3 days) cytotoxicity assays. In the short-term assays, PTK7-CAR T cells exhibited a robust dose-dependent cytotoxicity against PTK7-expressing PTK7-CHO cells and tumor cells but not parental CHO cells (Fig. 4). Noticeably, PTK7-CAR2 T cells demonstrated a comparatively higher degree of cytotoxicity against tumor cells expressing the lower level of PTK7 (H69, BxPC3 and H1299 cells). As PTK7 has be reported to be shed from tumor cells in a soluble form [12], we also evaluated the effect of soluble PTK7 on the cytotoxicity of PTK7-CAR T cells, which showed minimally impacted the tumor killing of these cells (Figure S2). Maintenance of specific cytotoxicity and proliferative response exposure to continuous antigen stimulation has been described to be associated with preferential antitumor activity [30, 31]. To mimic that context in vitro, we performed the recursive long-term cytotoxicity assay where CAR T cells were exposed to recursive target cells at a certain ratio, and tumor cell killing and T cell proliferation served as readouts after each round (Fig. 5a).

We observed that PTK7-CAR2 T cells retained effective through 3 rounds of tumor challenge whereas other 2 PTK7-CAR T cells failed to control tumor cell growth after the first or second round of challenge (Fig. 5b). In parallel, PTK7-CAR2 T cells remained proliferative after each round of challenge (Fig. 5c). PTK7-CAR2 T cells also exhibited superior effector function at the individual cell level as evidenced by higher levels of lytic enzyme granzyme B expression and reduced expression of the exhaustion markers PD-1 and lower percentage of PD-1⁺TIM-3⁺ cells as compared to other 2 PTK7-CAR T cells (Fig. 5d,e,f and Figure S3).

**PTK7-CAR T cells mediate antitumor activity against established lung cancer xenografts**

In view of the in vitro preferential target-specific recognition and cytotoxicity of PTK7-CAR2 T cells as well as the fact that the antibody from which the scFv used by PTK7-CAR2 is derived has been testing in the clinical trial [12], we evaluated the in vivo antitumor activity of this candidate CAR T cells in the xenograft tumor models established from two lung cancer cell lines with distinct antigen expression: H520 and H69 cells with high or moderate level of PTK7 expression respectively as determined by flow cytometry and IHC staining of cell line-derived xenografts (Fig. 2 and Figure S4). NSG mice (n = 3-5/group) were s.c. inoculated with H520 or H69 tumor cells, 7 days later mice started to receive two injection of control or PTK7-CAR2 T cells one week apart, and tumor growth was monitored by measuring tumor size. Three independent experiments with T cells from different donors showed that administration of PTK7-CAR2 T cells greatly inhibited tumor growth and significantly prolonged the overall survival of mice bearing H520
(p<0.0001) and H69 (p<0.001) tumors (Fig. 6a,b), culminating in tumor-free survival of more than half of mice at the end of experiment in both tumor models. In contrast, mice treated with control T cells or PBS developed a rapidly progressive tumor necessitating euthanasia approximately 6 weeks after tumor inoculation, excluding the contribution of allogeneic reactivity to antitumor effect of PTK7-CAR T cells. Accordingly, PTK7-CAR2 T cells exhibited superior initial expansion (day 10 after T-cell infusion) in vivo in the peripheral blood and extended persistence when mice were sacrificed (Fig. 6c and Figure S5). In addition, CD3+ T-cell infiltration in tumor xenografts was determined by IHC staining at the endpoint of the experiment, and mice treated with PTK7-CAR2 T cells exhibited a prominent accumulation of T cells within tumor tissues compared to mice treated with control T cells (Fig. 6d).

Importantly, there was no overt evidence of adverse reaction associated with the infusion of PTK7-CAR2 T cells to mice, as measured by body weight loss and physical signs of toxicity in above animal studies performed (Figure S6).

**PTK7-CAR2 T cells do not mediate detectable on-target off-tumor toxicity**

Low level of PTK7 expression has been documented in the normal epithelial cells from some tissues, including mammary gland, lung, kidney, esophagus, and urinary bladder. Since on-target off-tumor toxicity is a key limiting factor when developing novel CAR T therapies, we roughly address this concern using a panel of primary human normal cell lines with low level expression of PTK7 (Figure S7). Control or PTK7-CAR2 T cells were co-cultured with the primary human normal epithelial cell lines from mammary gland (Mammary Epithelial Cells, MECs), lung (Small Airway Epithelial Cells, SAECs), and kidney (Renal Epithelial Cells, RECs) and human umbilical vein endothelial cells (HUVECs), and cytotoxicity assays were performed. Compared to control T cells, PTK7-CAR2 T cells did not exhibit more potent killing against this limited panel of normal human primary cells, except for low level cytotoxicity of HUVECs that was only observed at the highest effector to target ratio tested (Fig. 7). As not all human tissues with PTK7 expression are represented, these studies are limited but can serve as an initial screen for off-tumor activity.

**Discussion**

Here, we described the generation and anti-tumor efficacy of second-generation PTK7-targeting CAR T cells with 4-1BB intracellular co-stimulatory signaling domain and demonstrated antigen-specific cytokine production and cytotoxicity against multiple PTK7-positive tool cells and naturally expressing human tumor cells in vitro; more importantly, in vitro recursive tumor challenge assays pointed to a preferred candidate (PTK7-CAR2) out of 3 CAR constructs in term of repetitive target cell killing, CAR T-cell expansion and exhaustion-associated phenotypes which was previously reported to be associated with in vivo antitumor effect of CAR T cells [32]. Using in vivo lung cancer cell line-derived xenograft models, we showed that PTK7-CAR2 T cells significantly inhibited tumor growth and prolonged overall survival of tumor-bearing mice. We did not evaluate PTK7-CARs integrating CD28 co-stimulation as CD28-containing CAR T cells have undesirable increases in T cell exhaustion markers, limited persistence and increased
possibility of recognizing normal cells with very low levels of antigen as previously reported [30, 33]. Although further studies will be needed to evaluate the antitumor efficacy of PTK7-CAR T cells in a more clinically relevant setting such as using PDXs and patient-derived cancer cell lines, our data support PTK7-CAR T cells as a viable therapeutic option for lung cancers and many other solid cancers with PTK7 overexpression given that it is impractical to develop blocking antibodies or small molecule inhibitors as typically done with receptor tyrosine kinases due to PTK7's lack of catalytic activity.

Several reports have identified PTK7 as a potential antigen target in solid tumors. Previous studies documented that PTK7 is overexpressed in multiple types of solid cancer, and more significantly, its expression is enriched in TICs/CSCs from PDXs or cancer cell lines. As TICs/CSCs with unlimited self-renewal capacity and differentiation potential have been broadly considered to be source to tumor recurrence, metastasis, and therapeutic resistance, it is reasonable to hypothesize that a durable antitumor efficacy would be achieved if specifically targeting TIC/CSCs by immunotherapeutic modalities, including CAR T-cell therapy. In fact, CAR T cells targeting several biomarkers of TICs/CSCs, including CD133, CD24, Receptor tyrosine kinase-like orphan receptor 1 (ROR1) or the epithelial cell adhesion molecule (EpCAM) have been developed and exhibited the excellent antitumor effects in preclinical models [34-40]; more importantly, CD133-targeting CAR T cells alone or in combination have demonstrated antitumor activity in treating patients with CD133-positive metastasis malignancies with controllable toxicities in clinical trials [41, 42]. Intriguingly, both PTK7 and ROR1 belong to Wnt ligand binding receptors with important roles in the non-canonical Wnt signaling [10]. ROR1 exhibits high and homogeneous cell surface expression in many epithelial tumors with expression profile similar to PTK7, and targeting ROR1 with CAR T-cell therapy improved survival in xenograft models of ROR1+ human tumors with treating lung and breast cancer in an ongoing clinical trial (NCT02706392) [38, 40]. Thus, our result documenting a potent antitumor effect of PTK7-CAR T cells adds PTK7 to the kind list of ROR1 which, as a member of Wnt signaling related pseudokinases, had a characteristic enriched expression in TIC/CSCs and is suitable as a potential therapeutic target for cancer immunotherapy.

On-target off-tumor effect is a major concern when developing CAR T-cell therapy targeting less tolerable TAAs for solid tumors [8]. On-target toxicities have been observed in clinical trials with CAR T cells specific for antigens that are shared on some normal tissues [43, 44], and a critical issue to be addressed is whether targeting PTK7 will be safe. Damelin et al. has shown that PTK7 is absent in vital organs, but detected a low level of expression in esophagus, urinary bladder, kidney, mammary gland, lung, ovary, uterus, and digestive tract with more prominent expression in stromal part [12]; in addition, previous studies detected PTK7 expression in human hematopoietic progenitors committed to myeloid and T lymphoid lineages, illustrating the potential for toxicity to normal cells [45-48]. We evaluated the activity of PTK7-CAR T cells in vitro against a normal cell panel that included mammary, lung, kidney epithelial cells and HUVECs where a low level of lysis against HUVECs was detected only at the highest effector to target ratio tested, which is consistent with comparatively higher PTK7 expression on these cells as determined by FACS. Unfortunately, we cannot evaluate the potential toxicity profiles of PTK-CAR T cells in our in vivo murine tumor models due to non-cross reactivity with murine counterpart of humanized
anti-human PTK7 antibodies used to construct scFv part of our PTK-CARs (US patent US20150315293A1). Positively, Damelin et al. showed that a PTK7-targeting ADC did not exhibit target-dependent toxicity in any of the tissues examined, including those with PTK7 expression [12]. As the scFv we used to generate PTK7-CAR2 is derived from the same antibody (Hu24) of that PTK7-ADC, the nonclinical safety profile of PTK7-ADC in that study provides some evidence of safety and potential toxicity estimate of PTK7-CAR2 T cells in vivo. Cautions still should be taken when translating this PTK7-CAR T cells into clinic considering different mechanisms of action and target recognition sensitivity between ADC and CAR T cells directing the same targets. In addition, we may learn from the experience of targeting ROR1 by CAR T cells as these two molecules have the similar expression profile in both normal and tumor tissues [10, 49]. Although ROR1-CAR T cells (derived from R12- and 2A-scFv) without cross reactivity with murine ROR1 exhibited no evident toxicity in NSG mice tumor model, murine ROR1-specific CAR T cells (derived from R11-scFv) induced lethal bone marrow failure due to recognition of ROR1+ stromal cells which can be rescued by the logic-Gated strategy of CAR construction [38, 40]. Thus, same configuration should be considered when similar results seen in future investigation of PTK7-CAR T-cell’s toxicity profiles; alternatively, tuning scFv affinity and/or concomitantly integrating different co-stimulatory domains may ameliorate the potential concern of on-target off-tumor effect as typical representation for CAR T-cell therapy targeting a range of different antigens including but not limited to HER2, EGFR and CD38 etc. [8, 50, 51]. In sum, further detailed investigations are definitely needed to explore the potential toxicities of PTK7-CAR T cells before translating into clinic by using PTK7-CARs with cross reactivity in mouse and even nonhuman primate models.

Conclusion

Here we describe a PTK7 targeting strategy that is based upon CAR T-cell engineering. This synthetic biology approach overcomes the issues related with PTK7 being pseudokinase unsuited for developing antibody and small-molecule inhibitors as therapeutic agents, and is supported by the effector functions of modified T-cell in order to deliver PTK7-specific cytotoxicity. In summary, the data presented herein serve as an initial step for future clinical development of PTK7-CAR T-cell therapy safely and efficiently treating PTK7-expressing lung cancer and other malignancies.

Abbreviations

CAR: chimeric antigen receptor; CSCs: cancer stem cells; scFv: single chain variable fragment; ACT: adoptive cell therapy; TM: transmembrane; TCR: T cell receptor; MHC: major histocompatibility complex; HLA: Human Leucocyte Antigen; TAA: tumor-associated antigens; CCK-4: colon carcinoma kinase 4; RTKs: receptor tyrosine kinases; PCP: planar cell polarity; VEGF: vascular endothelial growth factor; TNBC: triple-negative breast cancer; NSCLC: non-small cell lung cancer; OVCA: ovarian cancer; ESCC: cervical cancer, esophageal squamous cell carcinoma; HCC: hepatocellular carcinoma; TICs: enriched in tumor-initiating cells; PDXs: patient-derived xenografts; ADC: antibody-drug conjugate; FBS: fetal bovine serum;
HUVECs: human umbilical vein endothelial cells; IHC: Immunohistochemistry; tEGFR: truncated EGFR; ROR1: Receptor tyrosine kinase-like orphan receptor 1; EpCAM: epithelial cell adhesion molecule.

Declarations

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Authors’ contributions

Conception and design of studies: YJ, GR, HW, AG. Acquisition, analysis and interpretation: YJ, GL, LF, YL, ME, LW, HW, AG. Drafting article: YJ, AG. Critical review and discussion: GR, HW. The authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

This study was approved by ethical committees of the Harbin Medical University. Buffy coats or fresh whole blood were obtained from healthy donors under protocols approved by the Institutional Review Board of Harbin Medical University and handled with necessary safety procedures and ethical requirements.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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**Figures**
PTK7-CAR generation, cell-surface expression, and transduction of human T cells. (a) PTK7-CAR was generated by fusing PTK7-specific scFv to the co-stimulatory signaling domain of the 4-1BB (BB-ICD) and
activating signaling domain of CD3ζ (CD3ζ-ICD), a T2A ribosomal skipping sequence, and tEGFR was included for the detection of CAR modified T cells. (b) 293T cells transfected with control or PTK7-CAR constructs express both CAR and the marker gene tEGFR. (c) PTK7-CAR expression on transduced human CD4 and CD8 T cells was generally greater than 60%, as determined by staining tEGFR.

Figure 2

PTK7 is overexpressed on several tumor cell lines. CHO and PTK7-CHO cells served as negative and positive controls, respectively. PTK7 overexpression was observed on NSCLC (H520, H1975, H1299), SCLC (H446, H69), MDA-DB-468 breast cancer (BC), BxPC3 pancreatic cancer (PC), OVCAR3 ovarian cancer (OC) cells. Black and red line denote the control (secondary antibody alone) and PTK7 staining respectively.
Figure 3

PTK7-CAR T cells release IFN-γ and IL-2 and proliferate in response to PTK7-positive target cells. (a) Control or PTK7-CAR T cells from healthy donors (n = 3) were cocultured with CHO and PTK7-CHO and various PTK7-expressing tumor cell lines for 24 hours before performing IFN-γ and IL-2 ELISA. Mean and SEM are shown. (b) T cells were labeled with CFSE and cocultured for 3 days with CHO, PTK7-CHO, H520,
H1975 or H1299 cells in the absence of exogenous IL-2, and CFSE dilution was analyzed by flow cytometry. A representative histogram from 3 independent assays is shown.

**Figure 4**

PTK7-CAR T cells kill PTK7-positive tumor cell lines. Percent tumor cell killing of different target cells cocultured with control or PTK7-CAR T cells at the indicated effector-to-target ratios for 18 hours, calculated against the numbers of viable tumor cells when cultured in the absence of effector cells. Shown are means ± SEM of % cell killing in triplicate wells.
Figure 5
PTK7-CAR2 T cells retain effector function upon recursive target exposure. (a) Schematics of the long-term cytotoxicity assay. (b) Counts of H520 target cells after each round of recursive co-culture (rounds 1–3, R1–R3) with control or PTK7-CAR T cells. (c) Counts of H446 target cells after each round of recursive co-culture with control or PTK7-CAR T cells. (d) Intracellular staining for granzyme B of control or PTK7-CAR T cells at the end of round 1 and 3 co-culture with H520 tumor cells. (e) PD-1 expression in control or PTK7-CAR T cells after rounds 1 and 3 of recursive co-culture with H520 tumor cells. (f) Percentage of PD-1+TIM-3+ cells in control or PTK7-CAR T cells after rounds 1 and 3 of recursive co-culture with H520 tumor cells. Data are shown as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, and ****P < 0.0001, determined by repeated-measures two-way ANOVA with Turkey’s post hoc test.
Figure 6

Systemic treatment with PTK7-CAR2 T cells leads to tumor growth control and increased survival of mice in both human tumor xenograft models. (a) NSG mice were s.c. implanted with H520 or H69 tumor cells, after 7 days, received two intravenous infusion of control or PTK7-CAR2 T cells week apart and tumor growth quantified by measuring tumor size. Data are shown as mean ± SEM (n = 5 mice per group). *P < 0.05, ***P < 0.001, and ****P < 0.0001, determined by repeated-measures two-way ANOVA with Turkey's post hoc test. (b) Kaplan–Meier survival curves summarizing 3 independent experiments (n = 11 mice per group). ***P < 0.001, and ****P < 0.0001 determined by log-rank test. (c) Frequency of human CD3+tEGFR+ CAR T cells in the peripheral blood collected 10 days after T cell infusion or at the end of experiment. Data are shown as mean ± SEM (n = 4 mice per group). **P < 0.01, and ***P < 0.001, determined by repeated-measures two-way ANOVA with Turkey’s post hoc test. (d) Representative IHC images and quantification of T-cell infiltration in tumor tissues (n = 3) from treated mice harvested at the end of experiment. Scale bars, 100 µm. Data are shown as mean ± SEM (n = 3 mice per group). ***P < 0.001, determined by repeated-measures two-way ANOVA with Turkey’s post hoc test.
Figure 7

PTK7-CAR2 T cells do not mediate detectable on-target off-tumor toxicity. Control or PTK7-CAR T cells were tested reactivity against a panel of primary human normal epithelial cells or HUVECs in the cytotoxicity assays at the indicated effector-to-target ratios. Shown are mean ± SEM of % cell killing in triplicate wells. **P < 0.01, determined by repeated-measures two-way ANOVA with Turkey’s post hoc test.

Supplementary Files

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