T cells expressing a HER2-specific chimeric antigen receptor as treatment for breast cancer

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Abstract

HER2 is a member of the growth factor receptor family. It is very weakly expressed in the few epithelial cells in normal tissue. The HER2 gene and protein are overexpressed in many solid tumors. Thus, there are many advantages of targeting HER2 in tumor therapy.

Tumor relapse can be prevented by chimeric antigen receptor (CAR) T-cell therapy. The efficacy of CAR-T-cell therapy against tumors can be improved by adding a hinge region to the CAR structure. Here, we constructed a second-generation CAR with a high-affinity scFv derived from a humanized anti-Her2 antibody and a CD8 hinge region. The CAR was transduced into T cells by lentiviral transfection. The modified CAR-T cells specifically targeted Her2+ tumor cells in vitro and in clinically relevant syngeneic and xenogeneic mouse models of Her2+ breast cancer. The modified CAR-T cells specifically recognized Her2+ breast cancer cells. The effect of the CAR-T cells against Her2+ breast cancer cells in vitro was in line with their efficacy in xenogeneic mouse models. Thus, these modified CAR-T cells may be a therapy for Her2+ breast cancer.

Introduction

Cancer is an important disease that threatens human survival and development. Cancer therapies in the clinic mainly include surgical removal, radiotherapy, and chemotherapy. These common therapies often have low cure rates and high recurrence rates, and satisfactory results are often not achieved, making the identification of new therapies an urgent issue.

With in-depth research on the mechanisms of tumorigenesis and development and systematic analysis continuously improving the understanding of human immunity, tumor immunotherapy has gradually gained attention (June et al. 2018). In particular, adoptive immune cell therapy with CARs has many advantages: strong targeting, good stability, low immunogenicity, high safety, good cell/blood vessel penetration, and so on (Schubert et al. 2018; Eshhar et al. 1996). In addition, CAR immunotherapy given to tumor patients once tumor cells return can significantly prolong the survival time of tumors (Eshhar et al. 1990).

CARs contain a CD3 zeta region, including scFv, transmembrane, and 41bb or CD28 domains and downstream immune receptor tyrosine-based activation motifs (Till et al. 2012). Various mouse xenograft models have been used to verify the effectiveness of CAR-T cells. Two CAR-T cells targeting cd-19 B-cell lymphoma have been approved by the FDA (Gross et al. 1989).

Human endothelial growth factor receptor-2 (HER2) is a leucine kinase receptor that is closely related to cell growth and differentiation. It is very weakly expressed in a few epithelial cells in normal tissue (Cox et al. 2001). Abnormal expression of HER2 usually leads to sustained activation of downstream signaling pathways, enabling epithelial cell growth, proliferation, and differentiation; this disturbs normal physiological processes and causes tumor formation. Overexpression of HER2 is related to the occurrence and development of breast cancer, gastric cancer, prostate cancer and other cancers (Slamon
et al. 1987; Bang et al. 2010). HER2 has become a well-established immunotherapy target for breast cancer. There are monoclonal antibodies against HER2 and a number of CAR-T-cell clinical and preclinical trials. These trials have confirmed the safety and effectiveness of therapies targeting HER2 (Shu et al. 2020; Hurvitz et al. 2018; Bellon et al. 2019; Takegawa et al. 2017).

In our previous study, we humanized Herceptin, a monoclonal antibody against HER2, and showed that the modified antibody also had biological activity in vivo and in vitro (Johnson et al. 2015; Loo et al. 2012). Therefore, we used an scFv derived from a modified antibody for CAR targeting (Savoldo et al. 2011). Although fab, nanoantibody and natural ligands could also have been selected, the modifications to the spacer region can affect the specificity of the CAR; for example, CD28, cd137.ox40 and other costimulatory factor modifications can enhance the amplification efficiency and tenacity of CAR-T cells, but the ITAM region is usually derived from CD3 zeta (Kowolik et al. 2006). Therefore, we chose to construct a second-generation CAR targeting HER2. The constructed CARher2-T-cell line could kill tumor cells in vitro. Experiments in an animal model showed that CARher2-T cells had an inhibitory effect on tumors in tumor-bearing mice.

**Materials and methods**

**Compliance with ethical standards**

The experiments involving animals were implemented after approval of the Animal Ethics Committee of Jinan University. Efforts were made to avoid all unnecessary distress to the animals.

**Cells and cell culture**

BT474, SKBR3, MDA-MB468, K562 and K562-HER2 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% antibodies. MCF-7 and 293T cells were maintained in DMEM with 10% FBS and 1% antibodies. All of the above cells were purchased from ATCC. Monocytes were isolated from the peripheral blood of healthy donors, and the cells were cultured in X-VIVO 10 medium with 5% human serum. For experiments, 100 U/mL IL-2 and anti-human CD3/CD28 beads (Colombo et al. 2002) were used. All cells were incubated at 37 °C in 5% CO_2_.

**Cell ELISAs**

Her2 protein (0.25 μg) and 1×10^4 BT474, SKBR3 and MCF-7 cells were coated in 96-well plates overnight. BT474, SKBR3 and MCF-7 cells were fixed with 2.5% glutaraldehyde the next day, and the plates with Her2 protein, BT474, SKBR3 and MCF-7 cells were blocked in 5% BSA. The plates were washed with PBST. Anti-Her2 mAbs were diluted, added to the plates and incubated for 1 h. The plates were washed with PBST five times. Then, anti-human IgG Fc conjugated with HRP mAbs were added to the anti-Her2 mAbs. One hour later, the plate was washed with PBST five times. Finally, the OD450 was measured with a microplate reader.
Lentiviral packaging and generation of CAR-expressing cells

293T cells were seeded in 100 cm² dishes overnight. The medium was replaced with 5 mL Opti-MEM when the transient transfection was carried out. The core plasmid carrying the CAR and two packaging plasmids, pSPAX2 and PMD2G, were mixed at 1 μg/mL at a ratio of 1:5, and the compounds were added to 293T cells. The normal growth media were substituted 12 h later. The lentiviral supernatant was collected at 48 h and 72 h after transfection and then concentrated. The virus was harvested at 48 h and 72 h after transfection and concentrated. T cells (1×10⁶) were infected with the concentrated lentiviral supernatant and 8 μg/mL polybrene, and the medium was then replaced with fresh medium after 24 h. The cells were screened with 10 μg/mL puromycin, and they are hereafter referred to as CARHER2 T cells.

Analysis of CAR expression

For FACS, 1×10⁶ cells were collected and resuspended in PBS. Then, the cells were incubated with anti-Her2-Cy5 antibody at 4 °C overnight. The cells were washed the next day after incubation and were analyzed using a BD FACSCalibur. For immunoblotting, cells were collected and lysed. Lysates were separated by SDS–PAGE and detected by immunoblotting using anti-CD3ζ and GAPDH mAbs.

Cytotoxicity

For the LDH assay, 1×10⁴ target cells, BT474 cells, SKBR3 cells, and MCF-7 cells were seeded into 96-well plates overnight. Then, the effector cells were added to the 96-well plate with the target cells, at three different E/T ratios (E:T=1:1, 5:1, 10:1), and the mixtures were cocultured for 6 h. The LDH assay kit was used according to the manufacturer’s instructions to detect the cytotoxicity of the effector cells. For apoptosis analysis, CFSE dye was first used to mark effector cells. The marked effector cells and target cells were cocultured at the same three different E/T ratios used in the LDH assay. Next, the mixtures were collected and washed with PBS. An apoptosis detection kit and FACS were used for the analysis.

Cytokine measurement

Cell coculture supernatants and serum from the mouse model were extracted and collected for measurement. Cytokine detection kits, including kits for detecting human IL-2 and IFN-γ and mouse IL-2, IFN-γ and IL12, were purchased from MultiScience (Mulé et al. 1984). The analysis was performed according to the manufacturer’s instructions. The OD450 value was measured with a microplate reader.

In vivo antitumor activity in NSG mice

Six- to eight-week-old female NSG mice were injected with 1×10⁶ BT474 cells mixed with Matrigel at an E/T ratio of 1:1. When the tumor volume reached 60 mm³, the mice were treated with 5×10⁶ or 1×10⁷ T cells or CAR-T cells administered via tail vein injection (Ishikawa et al. 2005; Campana et al. 2014). Tumor progression and mouse weight were monitored every day. Peripheral blood was obtained and tumor volume was calculated at the end of the experiment. The tumor tissues were digested into single cells, and the cells were incubated with an anti-CD2 flow cytometry antibody (ab256295, Abcam) for 1 h. After
washing in PBS three times, the cells were analyzed by FACS, and the proportion of CD2+ lymphocytes was analyzed using FlowJo 7.6 software.

Statistical analysis

The experiment was performed three times independently. The curve was fitted by Origin 9.1 software. The statistical significance of the differences of in vitro and in vivo results was determined with GraphPad Prism7, and the p values are reported (*= p % 0.05, **= p % 0.01, *** = p % 0.001).

Result

Construction of second-generation CAR molecules

As previously described, the tumor-targeting antigen domain is often derived from monoclonal antibodies in the form of the scFv. To investigate whether the anti-Her2 mAb could recognize the Her2 antigen on the cell surface, we tested the expression level of the Her2 antigen on different cells and measured the affinity of the anti-Her2 mAb in a series of cells. The EC50 of the anti-Her2 mAb and Herceptin bound to Her2 is reported relative to the expression level of Her2 antigen because lower expression level of the Her2 antigen leads to inferior binding (Fig. 1A & B). The EC50 of the anti-Her2 mAb in BT474 cells was nearly 0.25 μg Her2 protein, 0.162 nM, and the EC50 of the Herceptin Standard product in BT474 cells was nearly 0.25 μg Her2 protein, 0.187 nM. Because of its high affinity, we obtained an scFv from the anti-Her2 mAb and used it as the extracellular domain to target the Her2 antigen. We constructed a second-generation CAR with a costimulation factory, 41BB, and IMTA and CD3ζ in the intracellular domain (Fig. 1C) (Long et al. 2015; Kakarla et al. 2013).

Generation of T cells carrying the Her2-specific CAR

To obtain T cells with stable expression of the CAR, we utilized a lentiviral system for infection. We produced the lentivirus in packaging 293T cells and then infected the T cells with the lentivirus over a period of 6 h (Fig. 2A). To ensure that the CAR was expressed in the infected cells after a week of screening with 10 μg/mL puromycin, we analyzed the CAR expression of CARHER2 T cells at the gene and protein levels. The FACS results showed that the CARHer2 gene expression of CARHER2 T cells was far higher than that of the mock group (Fig. 2B). Thus, we successfully constructed T cells carrying the HER2-specific CAR (Grossman et al. 2004; Till et al. 2012).

Cytotoxicity of CARHER2 T cells

In this study, we constructed a T-cell line expressing a HER2-specific chimeric antigen receptor. The CARHER2 T cells were activated and cultured with CD3/CD28 beads (Kowolik et al. 2006; Lee et al. 1989). We measured the expression of relevant molecular markers (Fig. 3A). The stimulation of amplification
induced by different target cells was assessed (Fig. 3B). Then, we tested the cytotoxicity of CARHER2 T cells against target cells and the release of the Th1-type cytokines IL-2 and IFN-γ (Fig. 3C&D) (Salman et al. 2019). The CARHER2 T cells were cocultured with the target cells (BT474, SKBR3 and MDA-MB468) at E/T ratios of 1:1, 5:1 and 10:1. The CARHER2 T cells and T cells had no significant effect against MDA-MB468 target cells (approximately 40% specific lysis at E/T ratios of 5:1 and 10:1) because there was little Her2 expression on the MDA-MB468 cell surface. Compared with that of T cells, the killing rate of CARHER2 T cells against BT474 and SKBR3 cells was approximately 40% higher at an E/T of 10:1. This result indicated that the CARHER2 T cells could selectively kill cells with high Her2 expression (Mulé et al. 1984).

**Analysis of the in vivo tumor-suppressive activity of PBMC-activated/CARHer2 T cells**

To verify the tumor-suppressive activity of PBMC-activated/CARHer2 T cells in vivo, we constructed a BT474 cell NSG mouse tumor model (Table 1). When the tumor volume of PBS control mice reached 2300 mm$^3$ on average, the mice were euthanized, and this was treated as the end point of the cell therapy. The tumor size and mouse body weight were determined, and the extracted tumors were photographed. The results showed that PBMC-activated/CARHer2 cells had stronger in vivo tumor suppressive activity than PBMC-activated cells, and administration of PBMC-activated/CARHer2 cells significantly improved the survival of tumor-bearing mice (Fig. 4A&B&C). The levels of the cytokines IL2, IFN-γ and IL12 in the serum of each group of mice were measured in blood samples (Lieschke et al. 1997). The results showed that PBMC-activated/CARHer2 cells induced the production of more Th1 cytokines in tumor-bearing NSG mice than PBMC-activated cells, which is likely a reason why the PBMC-activated/CARHer2 cells had a better in vivo tumor treatment effect than the PBMC-activated cells (Fig. 4D).

<table>
<thead>
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<th>GROUP</th>
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<tr>
<td>GROUP4</td>
<td>PBMC activated/CARHer2(T cells)</td>
<td>1x10^7</td>
<td>500μl</td>
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</tbody>
</table>

**Analysis of the growth of PBMC-activated/CARHer2 cells in mice**
CD2 is a differentiation antigen expressed by all kinds of cells except for red blood cells in human peripheral blood and can be used to monitor the number of and changes in effector cells. To observe the growth of PBMC-activated/CARHer2 cells in mice, we performed ocular blood sampling at Days 12 and 24 after effector cell infusion and at the end of treatment and then determined the survival of effector cells in the blood of each group of mice by flow cytometry analysis. At all three time points after cell infusion, PBMC-activated/CARHer2 cells grew significantly better than PBMC-activated cells (Fig. 5A&B). This result further explains why PBMC-activated/CARHer2 cells had significantly stronger effects in inhibiting tumor growth in NSG mice than PBMC-activated cells. The higher the number of activated effector cells that survived in NSG mice was, the better the effect of the tumor treatment.

**Discussion**

CAR immune cell therapy is very effective for blood tumors. However, for solid tumors, the therapeutic effects need to be further improved (Moon et al. 2014). This study of immune effector cells carrying a HER2-targeted CAR showed rapid and effective killing of malignant tumor cells and inhibition of tumor development in tumor-bearing mice. CARHer2 T cells effectively inhibited tumor progression in tumor-bearing NSG mice with HER2-positive tumors and may be a novel therapy for HER2-positive malignant solid tumors, but the therapeutic effect needs to be further improved. To verify the effectiveness of these second-generation CAR-T cells, CAR cellular therapy strategies targeting immune cells to overcome PD1/PDL1-related immunosuppressive signaling in the tumor microenvironment are needed (Kim et al. 2016). This study verified the activity of the constructed CAR immune cells in vitro and in vivo. However, studies in HER2-positive tumors and many malignant solid tumors, such as gastric cancer, ovarian cancer, lung cancer and glioblastoma, are needed so that the best indications for CAR-T-cell therapy can be explored in the future (Zhang et al. 2009; Verri et al. 2005; Brown et al. 2015).

**Declarations**

Funding Not applicable.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

The authors alone are responsible for the content and writing of the paper.

**References**


Figures
**Figure 1**

**Construction of second-generation CAR molecules.** (A) Relative affinity analysis of the anti-HER2 monoclonal antibody. (B) Detection of the expression of HER2 protein on the surface of target cells. (C) Structure of the CARHer2 gene and its lentiviral vector.

**Figure 2**

**Generation of T cells carrying the Her2-specific CAR.** (A) Analysis of the CARHer2 lentivirus titer. (B) Detection of CARHer2 protein expression on the surface of PBMC-activated/CARHer2 T cells.
Cytotoxicity of CARHER2 T cells. (A) Detection of the expression levels of CD3 protein, CD4 protein, and CD8a protein. (B) Activation and proliferation of PBMC-activated cells and PBMC-activated/CARHer2 cells during coculture with MDA-MB468, SKBR3 and BT474 cells. (C) Comparison of the killing activity of PBMC-activated cells and PBMC-activated/CARHer2 cells against target cells as assessed by LDH assay. The killing activities of PBMC-activated cells and PBMC-activated/CARHer2 cells against MDA-MB468,
SKBR3 and BT474 target cells were compared at effector to target ratios of 1:1, 5:1 and 10:1. (D) Detection of IL2 and IFN-γ during PBMC-activated cell and PBMC-activated/CARHer2 cell killing of MDA-MB468, SKBR3, and BT474 target cells.
Analysis of the in vivo tumor-suppressive activity of PBMC-activated/CARHer2 cells

(A) The changes in tumor volume in each group of mice were recorded. (B) The changes in body weight in each group of mice were recorded. (C) Tumor morphology at the end of treatment. (D) The levels of secreted IL2, IFN-γ, and IL12 in each group of mice at the end of treatment were determined. The data are presented as the mean±sd and were derived from six mice in each group.

Figure 5
Analysis of the growth of PBMC-activated CARHer2cells in mice. (A) The quantity of CD2-positive cells in each group of mice on the 12th day and, the 24th day after cell transfusion and at the end of treatment were detected by FCM. (B) The quantity of CD2-positive cells in each group of mice was recorded. The data are presented as the mean±sd and are derived from six mice in each group. The different letters indicate significant differences among the groups (P<0.05).