

Investigation of the Cytotoxicity of CAR-NK-92 Cells Targeting CEA Against Gastric Cancer Cells

Xinyu Zheng, Xiaoxiao Zhou, Xingzhou Xia*, Xiaomeng Chen

Department of Gastroenterology, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

*Corresponding author: Xingzhou Xia, Zhouxia@163.com

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Abstract: *Objective:* To construct CAR-NK-92 cells targeting carcinoembryonic antigen (CEA) and study their killing effect on gastric cancer cells. *Methods:* CAR-NK-92 cells targeting CEA were constructed. After co-culturing CAR-NK-92 cells with MKN-45 gastric cancer cells, the killing effect of CAR-NK-92 cells was detected by a lactate dehydrogenase release assay. The secretion levels of gamma interferon and granulocyte-macrophage colony-stimulating factor were measured using an ELISA assay. *Results:* The lactate dehydrogenase release assay showed that CAR-NK-92 cells had a significant killing effect on MKN-45 cells compared to CON-NK-92 cells, and the difference was statistically significant ($P < 0.001$). ELISA results indicated that the levels of gamma interferon and granulocyte-macrophage colony-stimulating factor secreted by CAR-NK-92 cells and MKN-45 target cells were significantly increased after co-culture ($P < 0.001$). *Conclusion:* CAR-NK-92 cells targeting CEA exhibit a significant killing effect on CEA-positive gastric cancer cells.

Keywords: Gastric cancer; Natural killer cell; Carcinoembryonic antigen; Immunotherapy

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1. Introduction

Gastric cancer (GC) is a severe global disease, ranking fifth in incidence among cancers worldwide. Although there have been significant advances in traditional gastric cancer treatments, the median overall survival for many patients with advanced gastric cancer still does not exceed one year due to the continuous progression, metastasis, and recurrence of the disease^[1]. Therefore, there is an urgent need to find more effective treatments.

Over the past decade, tumor immunotherapy has become an important method for treating advanced malignant tumors. Natural killer (NK) cells are immune cells capable of mediating cytotoxicity^[2], which can rapidly eliminate multiple abnormal cells. Chimeric antigen receptors (CARs) are membrane-bound fusion proteins that enhance immune cells' ability to recognize antigens and activate themselves, thus eliminating tumors^[3]. Carcinoembryonic antigen (CEA) belongs to the carcinoembryonic antigen cell adhesion molecule (CEACAM) family and is highly expressed in gastric cancer tissues. Based on this, the present study focuses on the killing effect of CAR-NK-92 cells targeting CEA on gastric cancer cells. The aim is to evaluate the preclinical application value of CAR-NK-92 cells targeting CEA in the treatment of gastric cancer. The results

of this study will provide important experimental evidence for understanding the mechanism of CAR-NK-92 cells in killing gastric cancer cells and their clinical application, while also offering a relatively safe treatment method for developing gastric cancer immunotherapy.

2. Materials and methods

2.1. Cells and reagents

Human gastric cancer MKN-45 and AGS cells (Shanghai Cell Bank, Chinese Academy of Sciences), NK-92 cells (ATCC, USA), recombinant lentiviral vector (pCDH-C2-45-CD137-CD3 ζ /CAR-GFP-Puro) (constructed by the Cell Biology Laboratory of the Fifth Affiliated Hospital of Zhengzhou University). ECL chemiluminescence kit (Beijing Kangwei Century Biotech Co., Ltd., China), rabbit CEA antibody (Wuhan Sanying Biotechnology Co., Ltd., China), rabbit CD3 ζ antibody (Abcam, USA), real-time fluorescent quantitative PCR detection kit (Vazyme Biotech Co., Ltd., Nanjing, China), lactate dehydrogenase detection kit (Beyotime Biotechnology, China), human IFN- γ enzyme-linked immunosorbent assay (ELISA) detection kit (Lianke Biotech Co., Ltd., Hangzhou, China).

Tissue source: The human gastric cancer tissue samples used in this study were obtained from the Fifth Affiliated Hospital of Zhengzhou University. The study was conducted with the approval of the Ethics Review Committee, and informed consent was obtained from the patients.

2.2. Methods

2.2.1. Cell line culture

- (1) Preparation of NK-92 cell culture medium: Add 62.5 mL of fetal bovine serum (FBS), 62.5 mL of horse serum, 0.5 mL of folic acid, 0.5 mL of inositol, 1 mL of β -mercaptoethanol, 2 mL of IL-2, and 5 mL of 1 \times penicillin-streptomycin to 375 mL of RPMI-1640 medium.
- (2) Preparation of AGS, GES-1, and MKN-45 cell culture medium: Add 50 mL of fetal bovine serum (FBS) and 5 mL of 1 \times penicillin-streptomycin to 445 mL of RPMI-1640 medium. All media were incubated in a constant temperature incubator at 37°C with 5% CO₂.

2.2.2. Construction of CAR-NK-92 Cells Targeting CEA

NK-92 cells were seeded in a 24-well plate. The experimental group was transduced with a recombinant lentiviral vector carrying the *CEA-CAR* gene, while the negative control group was transduced with an empty vector without the *CEA-CAR* gene (CON). After 72 h of culture, the expression of green fluorescent protein (GFP) in NK-92 cells was observed to obtain chimeric antigen receptor-modified NK-92 cells targeting carcinoembryonic antigen (CEA), referred to as CAR-NK92 cells, and NK-92 cells transfected with the empty vector, referred to as CON-NK92 cells.

2.2.3. Real-time quantitative PCR (qPCR)

RNA was extracted from GES-1, MKN-45, and AGS cells. RNA was reverse transcribed using a PCR machine to obtain cDNA. The qPCR reaction system was prepared using a qPCR kit, and the reaction mixture was added to the cDNA. Detection was performed using a real-time quantitative PCR instrument.

2.2.4. Western blot

Proteins were extracted from MKN-45 and AGS cells. A 10 μ L protein sample was loaded and subjected to electrophoresis at 120 V for 1 hour 30 minutes, followed by transfer to a PVDF membrane at 400 mA for

45 minutes. The membrane was blocked with skim milk for 2 hours, incubated with primary antibody CEA overnight, and secondary antibody incubation for 1 hour. Finally, the membrane was exposed and developed.

2.2.5. Lactate dehydrogenase (LDH) release assay

Target cells (MKN-45, AGS, and GES-1) were seeded into a 96-well plate. After cell adhesion, effector cells (CAR-NK92, CON-NK92, and NK92) were added to the 96-well plate at an effector-to-target ratio of 5:1 for co-culture. After co-culture, the supernatant was collected and transferred to a new 96-well plate as the enzyme-labeled plate, with a portion reserved. LDH detection solution was added to the enzyme-labeled plate and incubated at room temperature in the dark. Stop Solution was then added to each well, and absorbance was measured at 490 nm using a microplate reader.

2.2.6. Enzyme-linked immunosorbent assay (ELISA)

The supernatant collected from the previous step was used. 100 μ L of standards and diluted samples were added to the enzyme-labeled plate, followed by shaking incubation for 2 hours. Human IFN- γ ELISA and Human GM-CSF ELISA detection kits were used for the assay. Within 10 minutes, the optical density (OD) values of the standards and samples were measured at a maximum absorption wavelength of 450 nm using a microplate reader.

2.2.7. Statistical analysis

All data in this study were statistically analyzed using SPSS 25.0 software, and graphs were generated using Adobe Photoshop and GraphPad Prism 9.0 software. Data are presented as mean \pm standard deviation (SD). Depending on the data type, *t*-tests or one-way analysis of variance (ANOVA) were used for comparisons. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Expression differences of CEA between human gastric mucosal epithelial cells and gastric cancer cell lines

The expression of CEA mRNA in human gastric mucosal epithelial cell line GES-1 and gastric cancer cell lines AGS and MKN-45 was detected using qRT-PCR technology. The results showed that the expression level of CEA mRNA was significantly higher in the gastric cancer cell line MKN-45, while it was not expressed in the human gastric mucosal epithelial cell line GES-1 and the gastric cancer cell line AGS, with a statistically significant difference ($P < 0.0001$, **Figure 1A**). To further verify this result, Western Blot was used to detect protein levels, and the results also indicated positive CEA protein expression in the MKN-45 cell line, while other cell lines were negative (**Figure 1B**).

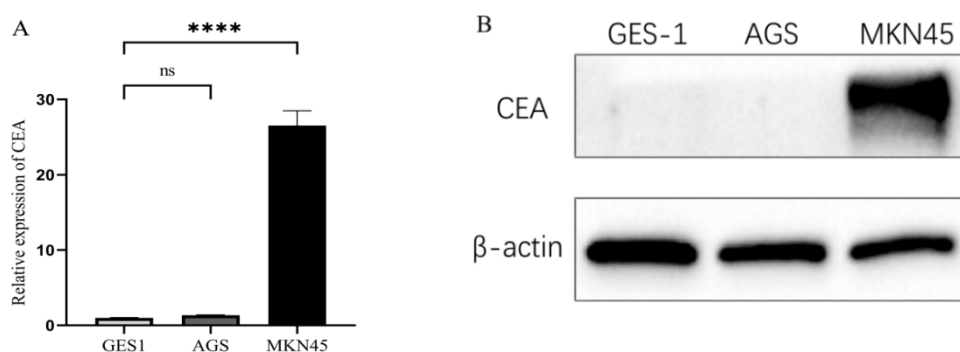


Figure 1. Expression of CEA in three cell lines

3.2. Observation of lentiviral transfection results under fluorescence microscope

After 72 hours of lentiviral transfection of NK-92 cells, the cells were observed under a microscope. Fluorescence signals expressing GFP were detected in the cells, preliminarily indicating that the lentivirus successfully transfected the NK-92 cells (**Figure 2**).

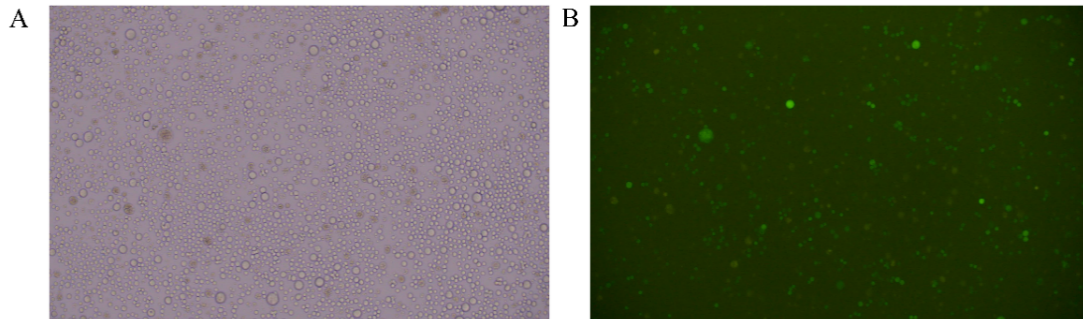


Figure 2. Results of lentiviral transfection of NK-92 cells. **(A)** Observation of CEA-CAR-NK92 cells using an inverted phase-contrast microscope (100×); **(B)** Observation of CEA-CAR-NK92 cells under a fluorescence microscope (100×)

3.3. Detection of CEA expression in NK-92 cells by Western blot

The results showed high expression of CD3 ζ protein in CEA-CAR-NK92 cells, while CON-NK-92 cells and NK-92 cells, which did not carry the target gene, did not express this protein. Western blot results indicated that CEA-CAR-NK-92 cells could express CEA-CAR protein (**Figure 3**), confirming the successful construction of CEA-CAR-NK-92 cells.

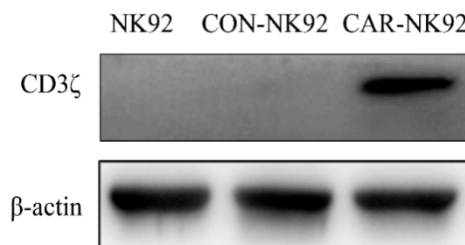


Figure 3. Expression of CD3 ζ protein in three cell lines

3.4. Detection of targeted killing of CEA-positive gastric cancer cells by CEA-CAR-NK92 cells using LDH release assay

The LDH release assay was used to evaluate the killing ability of the three types of effector cells on target cells at an effector-to-target ratio of 5:1 (4 hours). The results showed that the killing rate of CEA-CAR-NK92 cells on the CEA-positive gastric cancer cell line MKN-45 was significantly higher than that of the unmodified NK-92 cells (CON-NK92 and NK-92 cells), with a statistically significant difference ($P < 0.001$). Compared to CON-NK92 and NK-92 cells, the killing rate of CEA-CAR-NK92 cells on the CEA-negative gastric cancer cell line AGS was not statistically significant ($P > 0.05$). Human gastric mucosal epithelial cells GES-1 were used as a blank control, and no significant cytotoxicity was observed (**Figure 4**).

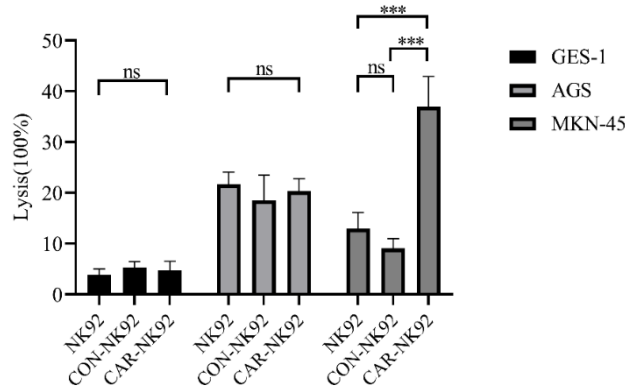


Figure 4. Killing effect of CEA-CAR-NK92 cells on CEA-positive gastric cancer cells detected by LDH release assay

3.5. Detection of cytokine secretion in the supernatant after CEA-CAR-NK92 cells target gastric cancer cells using ELISA

The production of IFN- γ and GM-CSF is a crucial indicator when CAR-NK92 cells exert their killing effect. To measure the levels of these cytokines, ELISA was used to detect the concentrations of IFN- γ and GM-CSF in the supernatant during the co-culture of effector cells and target cells. The results showed that the levels of GM-CSF and IFN- γ released by CEA-CAR-NK92 cells were significantly higher compared to the unmodified CON-NK92 and NK-92 cells ($P < 0.001$, **Figure 5**).

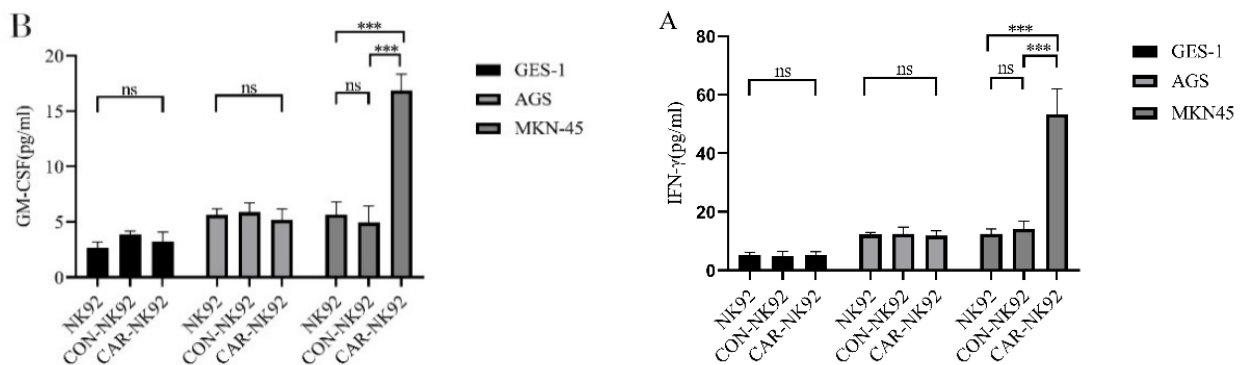


Figure 5. Detection of cytokine secretion by ELISA. (A) ELISA detection of IFN- γ in the supernatant; (B) ELISA detection of GM-CSF in the supernatant

4. Discussion

Gastric cancer (GC) is a common malignant tumor with high incidence and mortality rates. Therefore, exploring new treatment methods for gastric cancer is crucial. The advent of Chimeric Antigen Receptor (CAR)-modified T-cell therapy marks a milestone in the field of cancer immunotherapy. Although CAR-T cell therapy has made significant progress in hematological malignancies, it also has some drawbacks, such as cytokine release syndrome, neurotoxicity, antigen escape, and off-target effects [4], which limit its development. Due to the limitations of CAR-T cell therapy, NK cells, with their strong antitumor properties and relatively short lifespan, are considered a safer alternative and an “off-the-shelf” treatment option. NK cells are CD3⁻CD56⁺ lymphocytes that play a crucial role in defending against infections and tumors.

Unlike T cells, NK cells’ cytotoxic activity is not dependent on the activation of specific receptors, and they do not require prior sensitization [5]. Moreover, NK cells are insensitive to defects in the major

histocompatibility (MHC) class I molecule expression on target cells, enabling them to kill various types of tumor cells ^[6]. This unique biological characteristic suggests that tumor cells that escape T cell-mediated immune responses may still be susceptible to NK cell-mediated killing. Another important advantage of NK cells over T cells is that they do not cause graft-versus-host disease (GVHD), making NK cells suitable as “off-the-shelf” cells for transplant therapy ^[7]. Therefore, compared to CAR-T cell therapy, CAR-NK cell therapy may hold more promise in cancer immunotherapy.

With the deepening research on NK cells, the mechanisms by which they kill target cells are gradually being elucidated. When NK cells contact target cells, they interact by forming an immunological synapse, activating intracellular enzyme systems and signal transduction pathways, and releasing lytic granules, which increase the permeability of the target cell membrane and lead to lysis ^[8]. This process, known as degranulation, involves granzyme initiating apoptosis in target cells by cleaving substrates such as caspase-3, Bid, and DNA-PKc. Similar to CD8⁺ T lymphocytes, NK cells can perform sequential killing (the “serial killer” effect), eliminating up to six target cells simultaneously. In addition to direct cytotoxicity, activated NK cells secrete cytokines such as IFN- γ , TNF- α , GM-CSF, and chemokines, which play important immunoregulatory roles in NK cell activation, differentiation, and migration.

In summary, this study used CEA-targeted CAR-NK92 cells co-cultured with CEA-positive gastric cancer cell line MKN-45 *in vitro* to confirm the targeted killing effect of CAR-NK92 cells on MKN-45 gastric cancer cells. No impact was observed on the “normal” human gastric mucosal cell line GES-1, indicating that the constructed CEA-CAR-NK92 cells have good specificity for CEA-positive gastric cancer cells. Therefore, CAR-NK cells can avoid off-target effects in clinical treatment and provide a safer and more effective treatment option, laying an experimental foundation for further preclinical and clinical research on CAR-NK cells.

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Disclosure statement

The authors declare no conflict of interest.

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