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Targeted Therapy of CEA-CAR-NK Cells Against Colorectal Cancer Cells

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Abstract: *Objective:* Investigate the cytotoxic effect of CAR-NK cells targeting CEA on colorectal cancer cells with positive CEA expression. *Methods:* The mRNA and protein levels of CEA in different CRC cell lines were detected by qRT-PCR and Western blot analysis. Lentiviral transduction was used to construct CAR-NK cells and empty vector CON-NK cells targeting CEA. Fluorescence microscopy and WB were used to determine whether the cells successfully constructed and expressed CAR structures. The effector NK cells were co-cultured with target cells, and the levels of LDH, IFN-γ, and GM-CSF were detected. The killing rate of effector cells was calculated, and the release of cytokines during the killing of target cells by different effector cells was compared. *Results:* The expression level of CEA in colorectal cancer patients was significantly higher than that in normal samples and other tumor samples, and the prognosis survival time of patients with high CEA expression was lower than that of CRC patients with low or no CEA expression ($P < 0.05$). The CEA expression of the HT29 cell line was significantly higher than that of the SW1116 cell line at both the mRNA and protein levels. CEA-CAR-NK92 cells and CON-NK92 cells expressed green fluorescence under a microscope, and WB results showed that CEA-CAR-NK92 cells successfully expressed the CAR structure. Compared with CON-NK92 cells and NK92 cells, CEA-CAR-NK92 cells effectively killed HT29 cells ($P < 0.05$). CEA-CAR-NK92 cells secreted a large amount of IFN-γ and GM-CSF during the killing of HT29 cells, while the cytokine secretion of CON-NK92 cells and NK92 cells was not significant ($P < 0.05$). *Conclusion:* CAR-NK92 cells targeting CEA can effectively kill CEA-positive colorectal cancer cells.

Keywords: Colorectal cancer; Chimeric antigen receptor; Natural killer cells; Carcinoembryonic antigen; Immunotherapy

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer globally, with approximately 150,000 new cases diagnosed each year [1]. It is the second leading cause of cancer-related deaths, following lung cancer. Therefore, finding new treatment methods has become one of the key issues in the field of colorectal cancer treatment.

Natural killer (NK) cells are a type of lymphocyte with innate cytotoxic activity, capable of recognizing and killing tumor cells, and are an important part of the immune system [2]. As an "off-the-shelf" cell, NK cells can be expanded in vitro and used for treatment. During the killing process, the cytokines and growth factors

released by NK cells do not cause severe adverse reactions, making them a safer option [3]. CAR-NK cells not only rely on antigen targets but also exert their effects through classical cytotoxicity generated by antibody-dependent cellular cytotoxicity (ADCC) and cytokine pathways, thus reducing the risk of relapse due to antigen loss. Based on the various advantages of NK cells, CAR-NK cell therapy is expected to become a new research direction following chimeric antigen receptor (CAR) T-cell therapy.

Carcinoembryonic antigen (CEA) is an antigenic glycoprotein that is present in low levels in the blood circulation of healthy adults but is overexpressed in about 80% of colorectal cancers. Therefore, CEA could serve as a therapeutic target for NK cells, enhancing their cytotoxic effects against colorectal cancer cells and providing a new therapeutic strategy. This study investigates the cytotoxic effects of CAR-NK cells *in vitro*, laying the groundwork for future research on the *in vivo* killing mechanisms of CAR-NK cells and evaluating their potential application in clinical treatment.

2. Materials and methods

2.1. Materials

2.1.1. Cell lines

Colorectal cancer cell lines HT29 and SW1116 were purchased from Zhongqiao Xinzhou, NK92 cells were obtained from the ATCC cell bank in the USA, and 293T cells were preserved by the Marshall Experimental Center.

2.1.2. Major reagents and consumables

(1) The construction and identification of the lentiviral vector were completed by Henan Jiurui Biotechnology Co., Ltd.

(2) WB antibodies, including rabbit CEA antibody, mouse GAPDH antibody, sheep anti-mouse secondary antibody, and goat anti-mouse secondary antibody, were all purchased from Wuhan Sanying Biotechnology Co., Ltd.

(3) The reverse transcription kit was purchased from Suzhou JinAn Protein Technology Co., Ltd.

(4) The lactate dehydrogenase detection kit was purchased from Beyotime Biotechnology Co., Ltd.

(5) Human GM-CSF and IFN-γ ELISA detection kits were purchased from Hangzhou Lianke Biotechnology Co., Ltd.

2.2. Cell line culture

HT29, SW1116, NK92, and 293T cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.3. Construction of CEA-targeted CAR-NK92 cells

293T cells were seeded in a 6-well plate and cultured until the cell density reached approximately 70%. Transfection plasmids and packaging plasmids (psPAX2 and pMS2.G) were added to jetPRIME buffer and then introduced into the 6-well plate containing the cells. After 48 hours of culture, fluorescence was observed, and the cell supernatant was collected. The culture medium was replenished, and the cells were cultured for an additional 72 hours. The viral supernatant was then collected. NK92 cells were transduced with the viral supernatant and polybrene. After culturing, the cells were centrifuged, the supernatant was discarded, and a complete medium with 5 μg/mL puromycin was added to select and screen the cells. Finally, stable CEA-CAR-

NK92 and CON-NK92 cells were obtained.

2.4. Western blot

Proteins were extracted from HT29 and SW1116 cells. The marker and protein samples were loaded into the gel wells and run at a constant voltage of 120 V for 1.5 hours. After electrophoresis, the proteins were transferred to an NC membrane using a wet transfer method at a constant current of 400 mA for 45 minutes. The membrane was then blocked, incubated with primary and secondary antibodies, and placed in an exposure instrument to observe the development results.

2.5. Quantitative fluorescent PCR

RNA was extracted from colorectal cancer cells HT29 and SW1116 using the TRIzol method. Genomic DNA was removed and cDNA was obtained using ChamQ Universal SYBR qPCR Master Mix (gDNA Purge). The reaction system was prepared as follows: Mix 5 μL, Primer1 (10 μM) 0.5 μL, Primer2 (10 μM) 0.5 μL, cDNA 4 μL. The reaction was then performed on a qPCR instrument.

2.6. Lactate dehydrogenase (LDH) release assay

HT29 and SW1116 cells were seeded into 96-well cell culture plates. Effector-to-target ratios of 10:1, 5:1, and 1:1 were set up, and the cells were co-cultured for 24 h. After 23 h of culture, LDH release reagent was added to the "maximum enzyme activity control well" and incubated for 1 hour. The supernatant was collected by centrifugation, LDH detection working solution was added, and absorbance was measured at 490 nm using a microplate reader.

2.7. Enzyme-linked immunosorbent assay

HT29 and SW1116 cells were seeded in 6-well plates. CEA-CAR-NK92, CON-NK92, and NK92 cells were added to each well and co-cultured for 24 hours. The supernatant was collected by centrifugation. Enzyme-linked immunosorbent assay (ELISA) kits for human GM-CSF and human interferon-gamma (IFN-γ) were used for the assay, and OD values were measured at a wavelength of 450 nm using a microplate reader.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 26.0 software, and graphs were generated using Adobe Photoshop and GraphPad Prism 9.0 software. Measurement data are expressed as mean ± 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. Differences in CEA expression among different colorectal cancer cell lines

HT29 and SW1116 cell lines were selected for this study. Quantitative analysis of CEA at the mRNA level was performed using qRT-PCR. The results showed that the expression level of CEA in the HT29 cell line was significantly higher than that in the SW1116 cell line, with a statistically significant difference ($P < 0.001$, **Figure 1A**). To further verify this result, Western Blot analysis was conducted to detect CEA at the protein level. The results confirmed that the HT29 cell line expresses CEA protein, whereas the SW1116 cell line does not express CEA protein (**Figure 1B**).

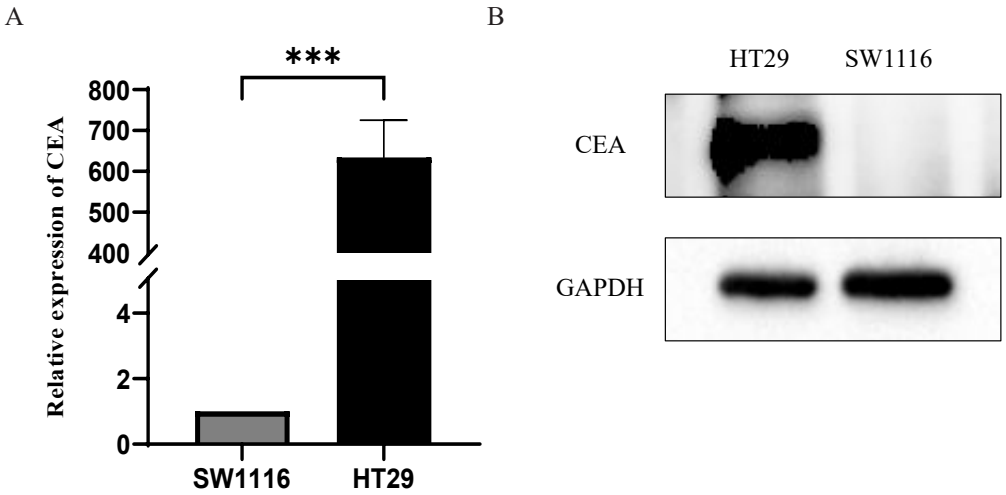

Figure 1. Expression of CEA in Two Cell Lines. **(A)** Relative expression levels of CEA in colorectal cancer cell lines HT29 and SW1116 as detected by qRT-PCR; **(B)** Expression of CEA protein in HT29 and SW1116 cell lines as detected by Western blot. *** $P < 0.001$, $n = 3$

3.2. Construction and identification of CEA-CAR-NK92 cells

3.2.1. Identification of CEA-CAR-NK92 cells

NK92 cells were transduced with either CEA-CAR lentivirus or GFP empty vector lentivirus, resulting in CEA-CAR-NK92 cells and CON-NK92 cells. After a period of selection, cells were observed under a confocal microscope through the GFP channel. The presence of green fluorescence indicated successful transfection (**Figure 2**).

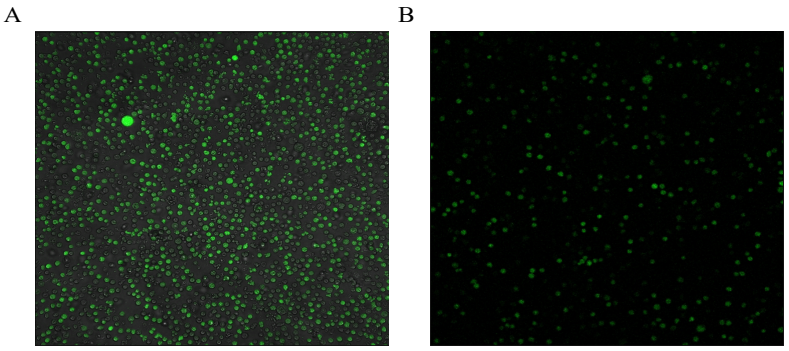

Figure 2. Results of lentiviral transduction of NK92 cells. **(A)** Observation of CEA-CAR-NK92 cells under a confocal microscope (10×); **(B)** Observation of CON-NK92 cells under a confocal microscope (10×)

3.2.2. Expression of CD3ζ in NK92 cells

Western blot analysis showed high expression of CD3ζ protein in CEA-CAR-NK92 cells, whereas CON-NK92 and NK92 cells did not express CD3ζ protein (**Figure 3**). This result confirms that CEA-CAR-NK92 cells were successfully transfected and expressed the CEA-CAR structural protein.

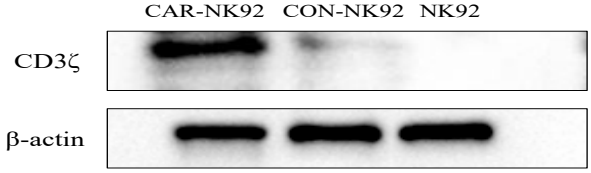

Figure 3. Western blot detection of CD3ζ expression in CEA-CAR-NK92, CON-NK92, and NK92 cell lines

3.3. Cytotoxic effect of CEA-CAR-NK92 cells on colorectal cancer cells

3.3.1. LDH assay for detecting the cytotoxic ability of CEA-CAR-NK92 cells

CEA-CAR-NK92, CON-NK92, and NK92 cells were co-cultured with target cells for 24 hours, and the LDH content in the supernatant was measured. The results showed that, compared to the two control groups (CON-NK92 and NK92), CEA-CAR-NK92 cells exhibited significant cytotoxicity against CEA-positive HT29 cells. Additionally, the cytotoxic effect of CEA-CAR-NK92 cells increased with a higher effector-to-target ratio, with statistically significant differences observed at effector-to-target ratios of 10:1 and 5:1 ($P < 0.0001$), and at a ratio of 1:1 ($P < 0.01$, **Figure 4A**). However, at different effector-to-target ratios, there was no significant difference in the cytotoxic effects of the three types of effector cells on CEA-negative SW1116 cells ($P > 0.05$, **Figure 4B**). This indicates that CEA-CAR-NK92 cells specifically target and kill colorectal cancer cells via the CEA target and that increasing the proportion of CEA-CAR-NK92 cells can enhance the cytotoxic effect.

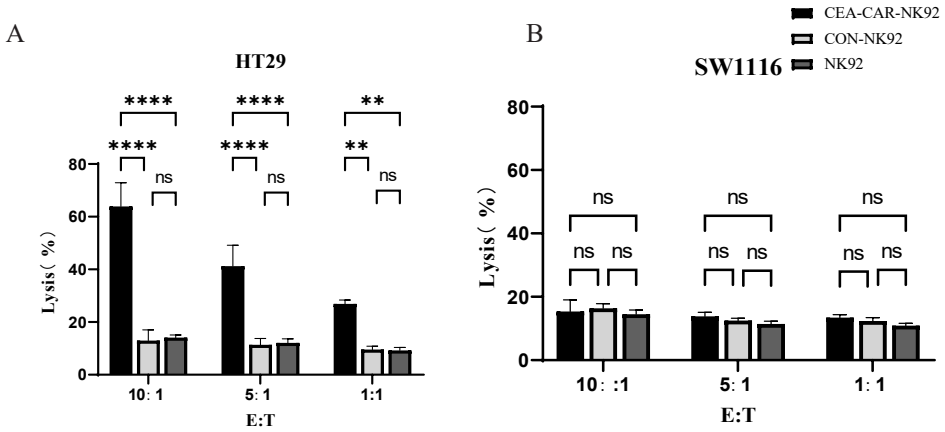

Figure 4. LDH assay for detecting the cytotoxic ability of CEA-CAR-NK92 cells. **(A)** Cytotoxic efficiency against CEA-positive HT29 cells; **(B)** Cytotoxic efficiency against CEA-negative SW1116 cells. ** $P < 0.01$, **** $P < 0.0001$, $n = 3$)

3.3.2. Cytokine release by CEA-CAR-NK92 cells detected by ELISA

Different effector cells were co-cultured with HT29 cells at an effector-to-target ratio of 5:1 for 24 hours. The cytokine secretion in the supernatant was measured using ELISA. As shown in **Figure 5**, the release of IFN-γ and GM-CSF by CEA-CAR-NK92 cells was significantly higher than that by CON-NK92 and NK92 cells, with a statistically significant difference ($P < 0.0001$). This indicates that CEA-CAR-NK92 cells may enhance their antitumor effect through cytokine release.

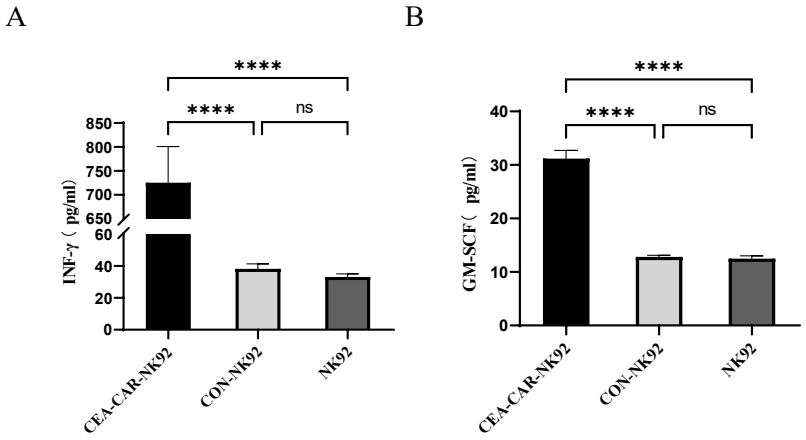

Figure 5. Cytokine release detected by ELISA. **(A)** Measurement of cytokine IFN-γ; **(B)** Measurement of cytokine GM-CSF. **** $P < 0.0001$, $n = 3$

4. Discussion

In recent years, the incidence and mortality rates of CRC have been increasing in China. In 2020, newly diagnosed CRC cases in China accounted for 28.8% of the global total, and CRC-related deaths accounted for 30.6% of the global total [4]. This places a significant burden on the healthcare system. Therefore, this study aimed to explore new treatment methods for CRC through immunotherapy, bringing hope to CRC patients.

The clinical activity of NK cells is currently limited by tumor immune evasion and low NK cell activity [5]. Thus, CAR gene reprogramming is considered a strategy to enhance the antitumor efficacy of NK cells. To date, most CAR-NK studies, including many clinical trials, have been conducted using the NK92 cell line. NK92 cells lack the ability to proliferate in vivo, and the infused cells are cleared after seven days. While multiple infusions are required to ensure therapeutic efficacy, this approach has a higher safety profile [6].

In this study, a second-generation chimeric antigen receptor (CAR) structure was used to successfully construct a CEA-CAR-NK92 cell line via lentiviral transduction, which could stably express the CAR structure. Compared to CON-NK92 cells (empty vector-transduced) and unmodified NK92 cells, CEA-CAR-NK92 cells effectively eliminated CRC cells *in vitro*. However, for CEA-negative SW1116 cells, the killing effect was not significant among the three types of effector cells, indicating that NK cells primarily exert their cytotoxic effects through specific targeting of the CEA antigen. Furthermore, co-culturing CEA-CAR-NK92 cells with HT29 cells resulted in increased levels of cytokines such as IFN-γ and GM-CSF in the supernatant. This finding aligns with other studies, suggesting that CEA-CAR-NK cells can indirectly enhance immune function by secreting cytokines in addition to directly killing target cells.

Other studies have used electroporation to construct NK cells that can transiently express CAR, significantly enhancing the tumor lysis activity of NK cells against CRC cell lines *in vitro* [7]. This study chose lentiviral vectors to integrate the CAR gene into the NK cell genome, enabling permanent CAR expression and reducing the need for multiple infusions due to short CAR expression duration.

This study successfully constructed second-generation CAR-NK cells and demonstrated their specific targeting and killing ability against colorectal cancer cells. Our results suggest that CEA could be a potential target for CAR-NK therapy in CRC. However, this study has limitations, such as the absence of animal model experiments, resulting in a lack of data on the systemic responses of CAR-NK cells *in vivo*. Future research should continue to address these limitations and lay the groundwork for the clinical application of CEA-CAR-NK cells in CRC patients.

Funding

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(2) Henan Province 2022 Science and Technology Development Plan "Study on Pyroglutamate Targeting DJ-1 to Trigger ROS-Induced Cell Death and Protective Autophagy in Pancreatic Cancer" (Grant No. 222102310725)

Disclosure statement

The authors declare no conflict of interest.

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