Exploring Glypican-3 Targeted CAR-NK Treatment and Potential Therapy Resistance in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer and ranks as the second leading cause of cancer-related mortality globally. Despite advances in current HCC treatment, it remains a malignancy with poor prognosis. Therefore, developing novel treatment options for patients with HCC is urgently needed. Chimeric antigen receptor (CAR)-modified natural killer (NK) cells have shown potent anti-tumor effects, making them as a promising immunotherapy strategy for cancer treatment. Glypican-3 (GPC3), a cell surface oncofetal glycoprotein, is highly expressed in most HCC tissues, but not in normal tissues, and functions as a key driver of carcinogenesis. Given its high expression level on the cell surface, GPC3 is considered as an attractive immunotherapy target for HCC. In this study, two GPC3-specific CAR-NK cells, NK92MI/NH3 and NK92MI/HS20, were established using NK92MI cells, a modified IL-2-independent NK cell line. These cell lines were engineered with third generation GPC3-specific CAR, and their activities were subsequently evaluated in the treatment of HCC. We found that NK92MI/NH3 cells, rather than NK92MI/HS20 cells, exhibited a significant cytotoxicity effect against GPC3⁺ HepG2 cells in vitro and efficiently suppressed tumor growth in a xenograft model using NSG mice. In addition, irradiated NK92MI/NH3 cells displayed similar anti-tumor efficacy to unirradiated NK92MI/NH3 cells. Furthermore, we observed that NK92MI/NH3 cells showed higher killing activity against the GPC3 isoform 2 overexpression cell line (SK-Hep1-v2) than those with GPC3 isoform 1 overexpression cell line (SK-Hep1-v1) both in vitro and in vivo. This suggest that the presence of different GPC3 isoforms in HCC may impact the cytotoxicity activity of NK92MI/NH3 cells and potentially influence therapeutic outcomes. These findings highlight the effective anti-HCC effects of NK92MI/NH3 cells, as well as the underlying therapy resistance, suggesting their potential as a promising therapy for HCC.
Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer and ranks as the second leading cause of cancer-related mortality globally. Despite advances in current HCC treatment, it remains a malignancy with poor prognosis. Therefore, developing novel treatment options for patients with HCC is urgently needed. Chimeric antigen receptor (CAR)-modified natural killer (NK) cells have shown potent anti-tumor effects, making them as a promising immunotherapy strategy for cancer treatment. Glypican-3 (GPC3), a cell surface oncofetal glycoprotein, is highly expressed in most HCC tissues, but not in normal tissues, and functions as a key driver of carcinogenesis. Given its high expression level on the cell surface, GPC3 is considered as an attractive immunotherapy target for HCC. In this study, two GPC3-specific CAR-NK cells, NK92MI/NH3 and NK92MI/HS20, were established using NK92MI cells, a modified IL-2-independent NK cell line. These cell lines were engineered with third generation GPC3-specific CAR, and their activities were subsequently evaluated in the treatment of HCC. We found that NK92MI/NH3 cells, rather than NK92MI/HS20 cells, exhibited a significant cytotoxicity effect against GPC3+ HepG2 cells in vitro and efficiently suppressed tumor growth in a xenograft model using NSG mice. In addition, irradiated NK92MI/NH3 cells displayed similar anti-tumor efficacy to unirradiated NK92MI/NH3 cells. Furthermore, we observed that NK92MI/NH3 cells showed higher killing activity against the GPC3 isoform 2 overexpression cell line (SK-Hep1-v2) than those with GPC3 isoform 1 overexpression cell line (SK-Hep1-v1) both in vitro and in vivo. This suggest that the presence of different GPC3 isoforms in HCC may impact the cytotoxicity activity of NK92MI/NH3 cells and potentially influence therapeutic outcomes. These findings highlight the effective anti-HCC effects of NK92MI/NH3 cells, as well as the underlying therapy resistance, suggesting their potential as a promising therapy for HCC.

Key Words
Glypican-3, CAR-NK, immunotherapy, Isoform, Hepatocellular Carcinoma

1. Introduction
Liver cancer ranks as the sixth most commonly diagnosed cancer and the fourth leading cause of cancer-related death worldwide, with continuously increasing incidence and mortality rates, in recent years (1). By 2030, the burden of liver cancer is projected to exceed 1 million cases (2). Hepatocellular carcinoma (HCC), accounting for 75%-85% of all diagnosed cases, is the most common type of primary liver cancer (1). Despite extensive exploration of therapeutic options, HCC remains poor prognosis due to postoperative recurrence and metastasis. Therefore, developing novel strategies and long-life therapies for the patients with HCC are still urgently needed.

Natural killer (NK) cells mediate potent cytotoxicity against tumor cells, making them attractive candidates for effective immunotherapies in the treatment of HCC (3, 4). NK cells possess the unique ability to recognize target cells via a major histocompatibility complex (MHC)-independent mechanism, allowing them to directly eliminate tumor cells, without being sensitized. Like T cells, NK cells can be genetically modified with chimeric antigen receptors (CARs) that can recognize antigens expressed by tumors. These CAR-engineered NK cells are also equipped with signaling components that enhance NK cell activity, thereby increasing their cytotoxicity against tumor cells. In comparison to CAR-T cell-based immunotherapy, which is known to have toxic side effects, the use of CAR-NK cells offers a promising approach to enhance efficacy while mitigating adverse effects such as acute cytokine release syndrome (CRS), neurotoxicity and graft-versus-host disease (GvHD) (5, 6). Several studies have demonstrated that CAR engineering of NK cells significantly enhances their cytotoxicity against various types of cancers (7-11). For instance, NK92 cells engineered with CARs targeting CD19 displayed an increased cytotoxicity activity against B-cell malignancies (12). Similarly, CAR-NK cells designed to recognize CD20 and Flt3 have demonstrated effective anti-tumor effects against B-cell tumors (13). Thus, CAR-NK cell therapy has emerged as a promising immunotherapy strategy for the treatment of cancers.

Glypican-3 (GPC3), a member of the heparan sulfate proteoglycan family, functions as an oncofetal glycoprotein that is attached to the cell membrane via glycosylphosphatidylinositol (GPI) (14). Several studies have consistently shown that both mRNA and protein levels of GPC3 are significantly elevated in HCC tissue, but
not in healthy adult liver tissues (15). This overexpression of GPC3 has been found to be strongly correlated with a poorer prognosis in individuals diagnosed with HCC (16, 17). Survival analysis of HCC patients with high GPC3 expression has revealed significantly reduced overall survival compared to those with low GPC3 expression. Moreover, the risk of recurrence after liver resection was found to be increased up to three-fold in HCC patients with high GPC3 expression, as compared to those with low GPC3 expression (18). Given the oncogene functions and high expression level of GPC3 on the cell surface in HCC, it is considered an attractive target for immunotherapy approaches such as vaccines and CAR-NK cell therapy.

The human GPC3 gene is transcribed and alternatively spliced into four distinct mRNA isoforms (19), with isoform 2 being the most commonly expressed (20). All GPC3 isoforms share the same C-terminal subunit, while the N-terminal subunits exhibit slight differences. Indeed, the alternative splicing of variants leading to the generation of different isoforms has been implicated in resistance to immunotherapy, as observed in B-cell acute lymphoblastic leukemia (B-ALL) patients with different CD19 isoforms contributing to CAR-T cell escape and resistance to CAR-T immunotherapy (21). Isoforms arise from the combination of different exons through RNA alternative splicing, resulting in proteins with diverse biological properties (22). The differential isoform expression can alter cellular activities, including cell proliferation, drug responsiveness and therapy outcomes (23, 24). The existence of various GPC3 isoforms and their potential impact on CAR-NK cytotoxicity may raise questions about the correlation between alternative mechanisms of GPC3 isoforms and their effects on CAR-NK immunotherapy. Further investigation of the expression and functional properties of GPC3 isoforms in the context of CAR-NK immunotherapy may provide valuable insights into their role in determining the outcomes of immunotherapeutic approaches targeting GPC3 and other antigens with alternative splicing isoforms, and potentially guide the development of more effective CAR-NK therapies for cancer treatment.

In the study, we established two kinds of CAR-NK cells, NK92MI/HN3 and NK92MI/HS20, by genetically engineering NK92MI cells, a highly cytotoxic NK cell lines, and evaluated the function and effectiveness of CAR-NK92MI cells against different HCC cells to identify a viable cell-based therapeutic strategy and potential mechanisms of therapy resistance mechanism in HCC treatment.

2. Material and Methods

2.1 Cell culture

Human HCC cell lines, including HepG2, Huh7, Huh7.5 and Sk-Hep1, as well as the 293T cell line, were purchased from ATCC (Manassas, VA). HCO2 and LH86 hepatoma cell lines were established in our laboratory (25). 293T cells and HCC cell line HCO2, HepG2, Huh7, Huh7.5, Sk-Hep1, LH86, Sk-Hep1-v1, and Sk-Hep1-v2 cells were maintained in Dulbecco’s minimal essential medium (DME) supplemented with 10% (v/v) FBS and antibiotics (100 IU/ml of penicillin and streptomycin) at 37°C in a 5% CO2 air-humidified incubator. Cells were routinely passaged with 0.25% trypsin. Human NK92MI, NK92MI/HN3 and NK92MI/HS20 cells were cultured in α-minimum essential medium supplemented with 0.2 mM inositol, 0.1mM 2-mercaptopethanol, 0.02 mM folic acid, 12.5% horse serum and 12.5% FBS.

2.2 Construction of retroviral vector and transduction of NK92MI cells

The CAR construct used in this study contains the HN3 or HS20 single chain antibody fragment, human IgG1 CH2CH3 hinge region, and CD28 transmembrane region, followed by the intracellular domains of co-stimulatory CD28, 4-1BB, and CD3ζ, as previously described (26). Briefly, 293T cells were transfected with Retro-HN3 or Retro-HS20 for 72 h to package CAR retroviral particles. The supernatant containing retroviral particles was collected and used to transduce NK92MI cells in a flat bottomed 48-well plate. To analyze the surface expression of CARs, the transduced NK92MI/HN3 and NK92MI/HS20 cells were harvested after 4-5 days and stained with anti-CD56 and CAR F(ab)2 domain [IgG (H+L)] for flow cytometry.

2.3 Analysis of NK92MI and CAR-NK92MI cell phenotype

The NK92MI, NK92MI/HN3 and NK92MI/HS20 cells were collected and stained with the following antibodies for flow cytometry: anti-F(ab)2 domain [IgG (H+L)] (Jackson ImmunoResearch Laboratories INC, PA),
PE-conjugated anti-NKG2C Ab (clone S19005E), AF647-conjugated anti-CD56 Ab (clone HCD56), PECy7-conjugated anti-NKG2A (clone S19004C), FITC-conjugated NKp46 (clone 9E2), PE-conjugated CD94 Ab (clone DX22), APC-conjugated CD69 Ab (clone H1.2F3), APC-Cy7 conjugated NKp44 Ab (clone P44-8), FITC-conjugated CD25 Ab (clone BC96), PE-conjugated NKG2D Ab (clone 1D11), APC-conjugated NKp30 Ab (clone P30-15) and APC-Cy7 conjugated PD-1 Ab (29F.1A12).

2.4 Polymerase chain reaction (PCR)

The cultured cells were collected for RNA extraction and reverse transcription. cDNA was synthesized from the extracted RNA, and this cDNA was then used as a template for PCR amplification of different GPC3 using the following primers: GPC3F1: CCGTATAGGGCTAGACTTACAG; GPC3R1: CAGCTCATGGA-GATTGAACTGG; GPC3F2: TAGAAACTCCGTGCCAG; GPC3R2: GCCGTAGAGACACATCTG- GTG. Primers were synthesized by Yale Keck oligo synthesis. The PCR cycling conditions were as follows: denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds, and primer extension at 72 °C for 1 minute, for a total of 30 cycles. A final extension step of 10 minutes at 72 °C was performed. PCR products were analyzed on a 2% agarose gel stained with SYBR safe and visualized under UV light.

2.5 GPC3 isoform plasmid construction

The GPC3 isoform 1 and 2 fragments were amplified by PCR with primers as follows: F1: TATATTAT-TATATTATGCTAGCGCCACCATGGGCGGCCGACCGTGCCAGGCG; R1: CTGTAAGTCTAGC-GCT; F2: CTTCCTGTGTATAGGGCTAGACTTACAG; R2: ACTAGCGCATATGGATCCTCAGTGCA-CAGGAAAGAAAGCA and with primers F: TATATTATATTATATATGCTAGCGCCACCATGGGCGGCCGACCGTGCCAGGCG; and with primers R: ACTAGCGCATATGGATCCTCAGTGCA-CAGGAAAGAAAGCA, respectively. HepG2 cDNA was used as the template for PCR amplification. The obtained fragments were then cloned into the pLenti-III-HA plasmid, which contains multiple modified cloning sites. The recombinant pLenti-III-HA-hGPC3-v1 and pLenti-III-HA-hGPC3-v2 plasmids were constructed by utilizing NheI and BamHI restriction enzyme sites. These plasmids were subsequently used for generating recombinant lentivirus particles.

2.6 Lentivirus production and transduction of Sk-Hep1 cells

The 3rd generation packaging system from abm company (Applied Biological Materials Inc, Canada) was utilized for generating recombinant lentivirus. 293T cells were transfected with the vector plasmid pLenti-III-HA-hGPC3-v1 or pLenti-III-HA-hGPC3-v2, along with a helper plasmid mixture, using TransIT Lent reagent. The transfected cells were maintained at 37 °C in a 5% CO2 for 48 h. The recombinant lentiviruses were then collected from the supernatant, concentrated, and stored at -80 °C for future use. Subsequently, Sk-Hep1 cells were incubated with medium containing GPC3-v1 or GPC3-v2 lentivirus to establish the Sk-Hep-1 GPC3 isoform 1 (Sk-Hep1-v1) or Sk-Hep-1 GPC3 isoform 2 (Sk-Hep1-v2) cell models, respectively. After incubation, puromycin (4 μg/ml) was added for selections of Sk-Hep1-v1 and Sk-Hep1-v2 cells.

2.7 RNA isolation and qRT-PCR analysis

Total RNA isolation was performed on cultured cells using RNeasy Mini Kit (QIAGEN, MD), followed by reverse transcription with a cDNA synthesis kit (Thermo Scientific, IL). Quantitative real-time polymerase chain reaction (qRT-PCR) was then performed using a two-step SYBR green qPCR assay and the target genes were amplified using following primers. Human GPC3, forward: CATTGGAGGCTCTGGATGGA; reverse: TTGTCTCTTGAGGTGCTGCT; Human GAPDH, forward: GTTCCTCTGACTTCAACAGCG; reverse: ACCACCTGGTCTGGTACCCAA. The qRT-PCR data were acquired using the Step One real-time PCR system (Applied Biosystem, CA), with the following cycling procedure: one cycle at 95 °C for 30 seconds, followed by 40 cycles at 95 °C for 5 seconds and 64 °C for 31 seconds. Each assay plate included negative control with no template. The mRNA levels of the gene of interest were normalized to the mRNA levels of GAPDH and analyzed using the 2-ΔΔCt method.

2.8 Western blot analysis
Samples from the cultured cells were lysed in ice-cold RIPA buffer (Sigma, MA) containing phosphatase/protease inhibitors (Thermo Scientific, IL). The lysates were then centrifuged at 11,000 g for 10 min at 4 °C, and the supernatant was collected and quantified using a BCA protein assay kit (Thermo Scientific, IL). After quantification, cell lysates were loaded and separated by electrophoresis with a 12 % sodium dodecyl sulfate-polyacrylamide gel. The protein was then transferred onto a polyvinylidene fluoride membranes (Millipore, MA), which were blocked for 1 h at room temperature with 1 % casein in PBS and probed with anti-human GPC3 and GAPDH monoclonal antibodies at 4 °C overnight. After washing with PBS containing 0.05 % Tween 20 three times at 10-min intervals, the membrane was incubated with goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies for 1 h at room temperature. After further wash steps, the membranes were treated with a substrate (Thermo Scientific, IL) to visualize the protein bands using the ChemiDoc Imaging System (BioRad, CA).

2.9 Cytotoxicity effects of CAR-NK92MI cells to HCC cells

HepG2 cells at a density of 2 x 10^5 were co-cultured with NK92MI, NK92MI/HN3 and NK92MI/HS20 at E:T ratios of 2.5:1, 5:1 and 10:1 for 24 h and 48 h in vitro. GFP fluorescence signals were observed under a microscope, which served as an indicator to evaluate the killing efficacy of CAR-NK92MI cells. Similarly, Sk-Hep1, Sk-Hep1-v1 and Sk-Hep1-v2 cells at a density of 2 x 10^5 were cultured in a 24-well plate overnight. NK92MI/HN3 cells were added and incubated with the target cells with an E:T ratio of 5:1 for 24 h and 48 h in vitro. Fluorescence signals were observed under a microscope.

2.10 Functional assay of CAR-NK92MI cells

CAR-NK92MI cells were co-cultured with target cells at an E:T ratio of 1:1 for 24h. After incubation, the supernatant from the co-culture system was collected and the production of IFN-γ was detected using an enzyme-linked immunosorbent assay kit (R&D Systems, USA) according to the manufacturer’s instructions. Similarly, the CAR-NK92MI cells were co-cultured with target cells at an E:T ratio of 1:1 for 24h in 96-well V bottom plates. The cells were collected and washed with FACS buffer, and then stained with anti-CD107 for flow cytometry analysis.

2.11 Xenogeneic tumor-grafted mouse models

Six to eight-week-old NOD-scid IL2Rγnull (NSG) mice were purchased from the Jackson Laboratory. All experimental manipulation of mice was undertaken following the National Institute of Health Guide for the Care and Use of Laboratory Animals, with approval from Yale Institutional Animal Care and Use Committee (IACUC). The NSG mice were randomly assigned to four groups. HepG2 cells (1 x 10^6) suspended in 50% Matrigel were subcutaneously injected into the right flank of each mouse. When the tumor size reached approximately 100mm^3, the mice were intravenously (i.v.) injected with NK92MI, NK92MI/HN3, NK92MI/HS20 cells or PBS twice at seven-day intervals. Tumor sizes were monitored and calculated every two days thereafter using the formula V= length x width^2/2. The mice were euthanized when the tumor size reached 1.5 cm in diameter, according to the Yale IACUC guideline.

Similarly, a total of 1 x 10^6 Sk-Hep1, Sk-Hep1-v1, or Sk-Hep1-v2 cells suspended in 50% Matrigel were subcutaneously injected into the right flank of each mouse. When tumor size reached approximately 100mm^3, the mice were intravenously (i.v.) injected with NK92MI/HN3 cells or PBS, at the indicated time points. Tumor sizes were then monitored and calculated every two days.

2.12 Effects of irradiation on cytotoxicity of NK92MI/HN3 cells in vitro and in vivo

NK92MI/HN3 were irradiated with 5 Gy using an X-ray irradiator. HepG2 cells were co-cultured with irradiated and unirradiated NK cells at various ratios of E:T at 1:1, 2.5:1, 5:1 and 10:1. Fluorescence signals were observed under the microscope to assess the cytotoxicity of CAR-NK92MI cells towards HepG2 cells. Six-week-old mice were randomly divided into three groups. A total of 1 x 10^5 HepG2 cells were suspended in 50% Matrigel and subcutaneously injected into the right flank of each mouse. When tumor size reached approximately 100mm^3, the mice were intravenously (i.v.) injected with irradiated or unirradiated
NK92MI/HN3 cells, or PBS, at the indicated time points. Tumor sizes were then monitored and calculated every two days.

2.13 Cell Proliferation Assay

Briefly, 2 x 103 cells/well were seeded into 96-well plates. At the indicated time points, cell counting kit-8 assay (DOJINDO, MD) was performed for the determination of cell proliferation. An absorbance at 450 nm was measured using a microplate reader (BioTek, VT).

2.14 Cell Migration Assay

Migration assay was conducted using chambers (Corning, AZ). Briefly, 100 μL cell suspension (5 x 10⁵ cells/mL) was seeded into the upper chamber, and 600 μL DMEM containing 10% fetal bovine serum was added to the bottom chamber. The migrating cells were fixed with 4 % paraformaldehyde for 30 min and stained with 1 % crystal violet solution for 30 min after 24 h of cell culture at 37 °C with 5 % CO2. The cells in 3 random fields were photographed under an inverted microscope (magnification: 100×) and counted using Image J software.

2.15 RNA sequencing data analysis

The Sk-Hep1, Sk-Hep1-v1, and Sk-Hep1-v2 cells were submitted to the Yale Stem Cell center for stranded library preparation and sequencing. Data analysis was performed using Partek software.

2.16 Statistics analysis

Data are shown as mean ± SD. All calculations and statistical analyses were performed using Graphpad PRISM 5.0 (GraphPad Software, San Diego, CA) for Mac. Comparisons between groups were conducted using analyses of unpaired t-tests, and P<0.05 was considered as statistically significant.

3. Results

3.1 Generation of NK92MI/HN3 and NK92MI/HS20 cells

Given its oncogenic function, GPC3 has already been suggested as a potential target for cancer immunotherapy in the treatment of HCC, including CAR-T and CAR-NK strategies (27). These immunotherapeutic approaches aim to specifically target and eliminate GPC3-expressing tumor cells, while minimizing off-tumor effects. Considering the splendid advantages of easy expansion, cultivation and activation, NK92MI cell line with indefinite expansion capacity have been used in clinical practice (28). To develop an NK cell-based immunotherapy to HCC patients, we genetically engineered NK92MI cells with the third-generation CAR molecules, HN3 or HS20, which specifically target GPC3, a highly expressed antigen in HCC. As shown in Figure 1A, we cloned the scFv domain of HN3 and HS20 into an SFG retroviral vector. The CAR construct contains human IgG1 hinge CH2-CH3 domain, CD28 transmembrane (TM) domain and intracellular domain, 4-1BB ligand intracellular domain, and CD3ζeta intracellular domain (Figure 1A ). After construction, the 293T cells were transfected with a combination of plasmids containing HN3-CAR or HS20-CAR in the SFG backbone, RDF, and PegPam3, as previously described (29). The SFG retrovirus particles in the supernatant were utilized to transduce NK92MI cells with the GPC3-specific CAR construct to generate NK92MI/HN3 or NK92MI/HS20 cells (Figure 1B ). After 4-5 days, the cells were collected and labeled with CD56 and human IgG (H+L) for sorting to enrich the CAR-NK92MI cell population. Flow cytometry analysis demonstrated that more than 96% of CD56+CAR+ NK92MI cells were observed (Figure 1C ).

3.2 Characteristics of CAR-NK92MI cells

To assess the immunophenotyping of NK92MI, NK92MI/HN3 and NK92MI/HS20 cells, flow cytometry was performed to characterize the expression of several important activating and inhibitory receptors and markers on the surface of the cells, including CD56, CD25, CD69, PD-1, NKP30, NKP44, NKP46, NKG2A, NKG2C, NKG2D and CD94 (Figure 2 ). The results revealed that the expression profile of these receptors and markers did not show significant differences between NK92MI, NK92MI/HN3 and NK92MI/HS20 cells.
3.3 Cytotoxicity activity of NK92MI/HN3 and NK92MI/HS20 cells against HepG2 cells in vitro and in vivo

To determine the recognition of CAR-NK cells to the GPC3+ HCC cells, we first evaluated the expression of GPC3 in several HCC cell lines, including HCO2, HepG2, Huh7, Huh7.5, Sk-Hep1, LH86 and Hep3B cell lines. As shown in Figure 3A, we observed higher mRNA levels of GPC3 in HepG2, Huh7, Huh7.5 and Hep3B cells, and lower levels in HCO2 and LH86 cells. On the other hand, the protein expression of GPC3 was significantly higher in HepG2 cells (Figure 3B), while Huh7, Huh7.5, and Hep3B cells showed relatively lower GPC3 expression levels. Consistent with previous data from other groups, Sk-Hep1 cells did not exhibit detectable GPC3 protein expression (30). Thus, HepG2 cells were chosen as the cell model for subsequent studies.

To assess the anti-tumor efficacy of different CAR-NK92MI cells, we co-cultured the HepG2 cells with NK92MI, NK92MI/HN3 and NK92MI/HS20 cells and observed the fluorescence signal under a fluorescence microscope. Compared to the NK92MI and NK92MI/HS20 cells, co-culturing with NK92MI/HN3 cells displayed less GFP fluorescence signals, indicating a high anti-tumor effect against HepG2 cells (Figure 3C). In addition, we found that CAR-mediated killing activity was associated with the elevated levels of IFN-γ production, as measured via ELISA, in the supernatant of HepG2 cells co-cultured with NK92MI/HN3 cells (Figure 3D). To further validate the cytotoxicity of NK92MI/HN3 cells, we co-cultured the NK92MI/HN3 cells with HepG2 cells by utilizing different effector-to-target (E:T) cell ratios ranging from 1:1 to 10:1. As shown in Figure 3E, the NK92MI/HN3 cells effectively eliminated the tumor cells at different ratios in vitro. To explore the in vivo anti-tumor activity of NK92MI/HN3 and NK92MI/HS20 cells, we established a xenograft model using immunodeficient (NOD/SCID) mice bearing subcutaneous HepG2 cells. Approximately 2 weeks after tumor cell inoculation, mice were grouped and treated with PBS, NK92MI, NK92MI/HN3 or NK92MI/HS20 cells. The results showed that administration of NK92MI/HN3 markedly inhibited the growth of the HepG2 xenografts (Figure 3F), indicating the potent anti-tumor activity of NK92MI/HN3 cells.

3.5 Irradiated NK-92MI/NH3 cells showed effective cytotoxicity against HepG2 cells in vitro and in vivo

To ensure the safety and minimize the risk of potential development of NK lymphoma in patients, NK92 cells are commonly irradiated prior to clinical application. Studies have shown that a radiation dose of 5Gy is suitable for maintaining the short lifespan and effectiveness of CAR-NK92 cells (31). Thus, we assessed the specific cytotoxicity of both irradiated and unirradiated CAR-NK against HepG2 cells in vitro and in vivo. It was observed that both irradiated and non-irradiated NK92MI/HN3 cells displayed cytotoxicity effects against HepG2 cells at different ratios in vitro (Figure 4A). Furthermore, both irradiated and non-irradiated NK92MI/HN3 cells significantly inhibited tumor growth in HepG2 xenograft models (Figure 4B). These results suggest that the irradiated NK92MI/HN3 cells displayed an anti-GPC3 malignancy capacity as unirradiated NK92MI/HN3, indicating the irradiation of NK92MI/HN3 does not compromise their anti-tumor efficacy. To optimize the treatment procedure, we further treated HepG2 xenografts with irradiated NK92MI/HN3 cells every 4 days for a total five-time injections. It was observed that this treatment regimen significantly inhibited the tumor growth (Figure 4C). This finding supports the use of irradiated NK92MI/HN3 as a viable option for CAR-NK immunotherapy to maintain safety and efficacy in the treatment of HCC.

3.4 Impact of GPC3 isoforms on the cytotoxicity activity of NK92MI/HN3 cells

3.4.1 Identification and analysis of GPC3 isoforms in HCC

It was reported that there exist four different kinds of GPC3 variants in HCC (Figure 5A), whereas no obvious evidence to show the impact of different GPC3 isoforms on cytotoxicity of CAR-NK cells. To explore the potential impact of GPC3 isoforms on anti-tumor efficacies, we first compared the sequence information of four GPC3 variants (NM_001164617.2, NM_004484.4, NM_001164618.2, and NM_001164619.2). Through the sequence comparison, it was found that the main difference among the GPC3 isoforms was the deletion...
of part or entire sequence of exon 2 and exon 4 (Figure 5B). This exon loss phenomena could potentially result in different biological functions of GPC3 in HCC. Based on sequence information, we designed two primer sets, GPC3F1/R1 and GPC3F2/R2, to examine the existence and distribution of GPC3 variants in HCC cell lines. The GPC3 variant 1 was detected with the GPC3F1 and GPC3R1 primer set at the size of 348 bp. The total GPC3 variants 1 and 2, variant 3, and variant 4 were detected at the size of 311bp, 286bp and 145bp, respectively, using the GPC3F2 and GPC3R2 primer set (Figure 5C). The results showed that GPC3 variants 1 and 2 were detected in HepG2, Huh7, Huh7.5, and Hep3B cells, only GPC3 variant 2, but not variant 1, was present in HC02 and LH86 cells (Figure 5D). In addition, the ratio of GPC3 variant 1 to variant 2 showed significant differences among these cell lines, with HepG2 cells expressing a high percentage of GPC3 variant 1 than Huh7, Huh7.5 and Hep3B cells. Interestingly, HepG2 cells with high levels of the GPC3 protein carry a high percentage of variant 1, but not in HC02 and LH86 cells carrying a high ratio of variant 2. These data indicate that the presence of GPC3 variant 1 may directly contribute to the increased expression level of GPC3 in HCC. There were no GPC3 variant expression detected in Sk-Hep1 cells. Additionally, we found that NK92MI/HN3 cell showed different killing activities to different HCC cells (Figure 4E), indicating that GPC3 isoform existence may have potential impact on anti-tumor efficiencies.

### 3.4.2 In Vitro and In Vivo Characterization of Sk-Hep1-v1 and Sk-Hep1-v2 cell lines

Because GPC3 cannot be detected in the Sk-Hep1 cells, it would be a good model to generate two stable cell line Sk-Hep1-v1 and Sk-Hep1-v2 that were overexpressing with GPC3 variants 1 and GPC3 variant 2, respectively, to elucidate different biological activities on regulating HCC tumorigenesis and potential anti-tumor efficacies of CAR-NK92MI cells. To assess the expression levels of GPC3, we collected both protein and mRNA from Sk-Hep1, Sk-Hep1-v1, and Sk-Hep1-v2 cells for western blot and qPCR, respectively. Notably, Sk-Hep1-v1 cells displayed significantly higher protein expression of GPC3, as shown in Figure 6A. To further investigate the cellular localization of GPC3 expression, its distribution on the cell membrane and in the cytosol was assessed. The results revealed that GPC3 variant 1 exhibited a higher level of expression on the cell membrane compared to GPC3 variant 2 (Figure 6B). These findings suggest that the existence of different GPC3 isoforms may impact the properties of the protein and cellular activities and could potentially contribute to therapy resistance.

To investigate the possible roles of different GPC3 variants in HCC, we initially performed the CCK8 and migration assays to detect the in vitro proliferation and migration of the parental and modified cell lines, respectively. As shown in Figure 6C, Sk-Hep1-v1 cells grow at a faster rate than Sk-Hep1 and Sk-Hep1-v2 cells in vitro. Also, Sk-Hep1-v1 cells displayed enhanced migration ability in vitro when compared to Sk-Hep1 and Sk-Hep1-v2 cells (Figure 6D). To further observe the effects of GPC3 variants on the tumorigenicity of HCC cells in vivo, Sk-Hep1, Sk-Hep1-v1 and Sk-Hep1-v2 cells were xenografted into the flank of the mice. The in vivo study on tumorigenicity showed that the growth rates of tumors derived from Sk-Hep1-v1 and Sk-Hep1-v2 were slightly increased compared with the tumors derived from Sk-Hep1 cells when these cell line were injected into NSG mice (Figure 6E, F). In contrast, tumors derived from Sk-Hep1-v1 grew aggressively than those derived from Sk-Hep1-v2 cells. To elucidate the underlying mechanisms of different GPC3 variants in HCC cells, we studied the expression of gene alternations associated with various biological activities in Sk-Hep1, Sk-Hep1-v1, and Sk-Hep1-v2 cells, using significance analysis of microarrays. Based on the gene expression profile, we found a profound change in the expression level of 150 gene candidates (Figure 6G, H, I). Among them, BMP-7 was significantly increased in Sk-Hep1-v1 cells, with at least 2-fold higher than that in Sk-Hep1-v2 cells (Figure 6J). These data imply that the expression of BMP7 is positively correlated with that of GPC3. The analysis with qPCR also confirmed this similar change in mRNA expression of BMP7, indicating a potential association between GPC3 and BMP7 (Figure 6K).

### 3.4.3 The cytotoxicity of NK92MI/HN3 cell to Sk-Hep1-v1 and Sk-Hep1-v2 cells

To evaluate the killing efficacy of NK92MI/HN3 cells in relation to different GPC3 isoforms, we co-cultured NK92MI/HN3 with Sk-Hep1, Sk-Hep1-v1 and Sk-Hep1-v2 cells in vitro at an E/T ratio of 5:1. Fluorescence signal of the HCC cells was observed under the microscope, which was served as an indicator to evaluate the killing efficacy of NK92MI/HN3 cells. It was observed that NK92MI/HN3 cells exhibit high cytotoxic
effects against Sk-Hep1-v2 than Sk-Hep1 and SkHep1-v1 cells (Figure 7A), followed by an increased IFN-γ production in the supernatant (Figure 7B), indicating that the presence of different isoforms may have an impact on therapy outcomes. To evaluate the in vivo anti-tumor activity of NK92MI/HN3 cells, we performed NK92MI/HN3 treatment in HCC xenograft NSG models that were subcutaneously implanted with either Sk-Hep1, Sk-Hep1-v1, or Sk-Hep1-v2 cells. The results revealed that tumors derived from Sk-Hep1-v1 and Sk-Hep1-v2 cells in mice treated with NK92MI/HN3 cells exhibited significantly slower growth compared to tumors derived from Sk-Hep1 cells, indicating that NK92MI/HN3 cells exerted stronger anti-tumor effects against Sk-Hep1-v1 and Sk-Hep1-v2 cells in the xenograft NSG model (Figure 7C). However, it was observed that the tumor volume derived from Sk-Hep1-v1 cells was larger than that from Sk-Hep1-v2 cells, suggesting that the presence of GPC3 isoform 1 may be associated with poor clinical outcomes after treatment with NK92MI/HN3 cells. To further validate the activity of NK92MI/HN3 cells, the expression of CD107a, a sensitive marker for NK cell functional activity, was analyzed by flow cytometry. Co-culturing NK92MI/HN3 cells with Sk-Hep1-v2 cells led to a significant upregulation of CD107a expression in NK92MI/HN3 cells (Figure 7D), indicating the enhanced NK cell cytotoxicity against Sk-Hep1-v2 cells, but not against Sk-Hep1-v1 cells. Thus, further analysis and investigation may be required to better understand the implications of GPC3 isoform 1 in the clinical outcome of HCC treatment with NK92MI/HN3 cells.

Discussion

HCC is a highly aggressive form of cancer, and curative treatment options are typically limited to patients with a limited tumor burden. Therefore, novel strategies for the patients with HCC are urgently needed. One strategy for implementing cell-based immunity involves the use of chimeric antigen receptor (CAR) technology. In the study, NK92MI/HN3 displayed potent and specific anti-tumor activities both in vivo and in vitro, suggesting its potential as a treatment option for HCC.

Chimeric antigen receptor (CAR) T cell therapy is recognized as a promising immunotherapeutic strategy for the treatment of cancers, especially in the treatment of relapsed and refractory B-cell malignancies (32). To date, two CD19-CAR-T-cell therapies have been approved for the treatment of lymphocytic leukemia (ALL) and diffuse B-cell lymphoma (DLBCL) (33). However, CAR-T cell therapy is still facing several challenges. One major obstacle is the requirement to collect and use autologous cells, which involves labor-intensive steps of isolation and in vitro expansion. Furthermore, the high costs, complex manufacturing processes, and potential severe toxicity, such as cytokine release syndrome, have hindered the widespread use of CAR-engineered T lymphocytes (34, 35). These concerns may limit the broader clinical applications of CAR-T-cell therapy and also increased the interest in exploring alternative CAR drivers for this promising approach (36). The safety and accessibility features of CAR-NK cells make them a potential replacement for CAR-T cells. The modification of NK cells with CARs is being investigated as an alternative to T cells in various areas. One advantage of NK cells over T cells is that they are not major histocompatibility complex (MHC)-restricted, which results in the activation or inhibition of target cells through germline-encoded activated or inhibitory receptors that interact with their specific ligands on target cells (37). Based on this, the NK cells can serve as an “off-the-shelf product” for patients in a cost-effective manner, as allogeneic sources of NK cells have shown reduced risk of alloreactivity. Allograft NK cells contribute have been shown to contribute to the graft-versus-tumor effect without causing GvHD, which has been confirmed in mouse model (38) and clinical studies (39). In addition, the limited life span of mature NK cells reduces the possibility of long-term adverse events while effectively killing target cells (31). To date, preclinical investigations of CAR-expressing NK cells have shown promising results. Thus, CAR-NK cells may serve as an alternative candidate for retargeting cancer because of their unique recognition mechanisms, cytotoxic effects especially on cancer cells in both CAR-dependent and CAR-independent manners and clinical safety.

Due to the molecular heterogeneity of the disease, CAR-NK therapy may have underlying limitations. In the study, we found that NK92MI/HN3 showed different killing efficacies against Sk-Hep1-v1 and Sk-Hep1-v2 cells, accompanied by the increased IFN-γ production and CD107a expression. These discrepancies may suggest that the existence of GPC3 alternative splicing variants could increase isoform diversity, potentially impacting therapy outcomes. Being that alternative splicing of mRNA precursors enables one single gene
to create multiple protein isoforms, the aberrant splicing activity may substantially modify crucial protein functions, and thus affect or disrupt typical cellular processes, such as antibody affinity or recognition. This can result in cancer cells escaping targeted therapy and increasing resistance to treatment. Studies have shown that alternative splicing variants are implicated in resistance to immunotherapy. For example, in breast cancer, a splicing variant that eliminates exon 16 in the extracellular domain of HER2 has been found to contribute to resistance to trastuzumab (40). Furthermore, melanoma cells have been found to develop resistance to vemurafenib through the dimerization of aberrantly spliced BRAF (V600E) (41). In patients with B-cell acute lymphoblastic leukemia (B-ALL), the presence of different CD19 isoforms has been shown to contribute to escape from CAR-T cell therapy, resulting in resistance to CAR-T immunotherapy (21). Therefore, addressing the clinical issue of resistance to CAR-NK therapy and understanding the preexistence of GPC3 isoforms as a possible mechanism of escape is crucial. Further investigation into the functional characteristics of GPC3 isoforms may provide insights into their role in mediating resistance to therapy in the context of CAR-NK immunotherapy and guide the development of strategies to overcome such resistance for more effective cancer treatment. To overcome the challenge of isoform existence, the biomarkers that can predict the preferential selection of preexisting GPC3 isoforms may become relevant in the early identification of CAR-NK therapy patients, as the molecular determinants of CD19 exon splicing dissection may be correlated to the therapeutic outcomes. This may suggest the necessary to consider any other clinical therapies to combine with, such as immune checkpoint therapy, which might improve NK cell antitumor activity and the outcome to achieve a long-term complete remission in these patients.

Reference


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