Combination Therapy with EGFR Specific CAR_NK_92 Cells and Cabozantinib against Human Renal Cell Carcinoma

Youqing Huang[1],Yong Zhong[2],Chunhui Qin[2],Hao Wang[3*]

[1]The Second Affiliated Hospital of Kunming Medical University,Kunming,650101, China.

[2]Department of Anesthesiology,Shuguang Hospital ,Shanghai University of Traditional Chinese Medicine,Shanghai, 201203, China.

[3]Teaching Center of Experimental Medicine, Shanghai Medical College, Fudan University, 138 Yixueyuan Rd, Shanghai 200032, China.

*Corresponding Author: Hao Wang

Teaching Center of Experimental Medicine, Shanghai Medical College, Fudan University, 138 Yixueyuan Rd, Shanghai 200032, China. Electronic

Email: wh91351@126.com(China)

Abstract

Adaptive therapy using immune effector cells engineered by means of chimeric antigen receptors (CAR) has risen as a hopeful cancer management option. Despite their unprecedented success in haematological malignancies, CAR-modified T cells have shown limited efficacy in solid tumours, as the tumor's immune-suppressive microenvironment inhibits CAR-modified immune effector cells' functionality by different pathways, counting checkpoint receptor ligands expression like PD-L1 & recruitment tregs like suppressive immune cells. Receptor of epidermal growth factor (EGFR) could be the target of a II-generation Chimeric antigen receptor T cellshat was transduced to NK-92 cell. In our research, we examined the antitumor efficacy of EGFR specific NK-92 (CAR-NK-92) cells using a xenograft mice model & in conjunction with tyrosine kinase inhibitor cabozantinib. We discovered that EGFR positive renal carcinoma cells (RCC) 786-O and ACHN may specifically detect and activate CAR_NK_92 cells. They also displayed particular cytotoxicity against RCC in *in vitro* & *in vivo* models. Furthermore, we discovered that cabozantinib improves RCC-specific cytotoxicity by enhancing the expression of EGFR while reducing PD-L1 expression in RCC. Our research shows that CAR_NK_92 cells possess anti cancer therapeutic potential for EGFR-positive tumour cells, and that cabozantinib can boost CAR_NK_92 cell cytotoxicity when treated together.

Keywords: EGFR, NK-92, Adaptive therapy,

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Introduction

RCC - Renal cell carcinoma is responsible towards 90 to 95 percent of carcinoma in the kidney. With about 30% recurrence rate after radical resection, it is one of the most deadly urological neoplasms. RCC is also resistant to standard chemotherapy and radiotherapy[1]. Targeted therapies such as tyrosine kinase inhibitors [TKI], cytokine therapy, and immune checkpoint blockers are at the center of much attention for RCC[2,3]. TKI and immune checkpoint inhibitors have demonstrated only a 15% to 35% objective response rate[4]. These call for novel therapeutic strategies that will be more amicable and efficient for RCC management.

The immune effector cells utilization in cancer therapy has sparked a lot of interest, and there's a lot of evidence that it works. Ex-vivo-expanded autologous tumor-infiltrating T lymphocytes (TILs) and recombinant human interleukin-2 (rhIL-2) resulted in continuous complete remission of metastatic malignant melanoma in 25% to 50% of patients treated in the 1980s and 1990s[5,6]. TILs infusion enriched for patient-specific neoantigens has also shown promising benefits in metastatic colorectal or breast cancer patiens [7,8]. However, challenges in producing an appropriate number of bioactive TILs from patients with non-melanoma malignancies obstruct the implementation of these techniques. TCRs for tumor-reactive T cells (TCRs) were cloned from TILs responsive patients and expressed in T cells expanded from another tumor patient's blood, allowing researchers to manufacture an almost infinite number of cells for therapeutic use. Cloned TCR and chains, on the other hand, link with endogenous receptors, resulting in low transgenic TCR levels.[9]. TILs found in melanoma tumours can detect self-antigens produced at low levels in healthy tissues, resulting in considerable on-target and off-target damage.[10,11] as well as crossreactivity[12–15].

Immune effector cells with chimeric antigen receptors (CAR) have emerged as a promising new class of cancer therapies. MHC has no effect on CAR-modified T cells, hence they can be used on patients of any HLA type. They also prevent MHC downregulation-induced resistance. Although certain first-generation CHIMERIC ANTIGEN RECEPTOR T CELLS cells have demonstrated long-term persistence[16–18], their use is limited due to their limited growth and inability to elicit clinically significant anti-tumor effects[19–22]. Second-generation CARs with a co-stimulatory endodomain upstream of CD3z, such as CD28 or 4-1BB, demonstrate significantly increased expansion and anti-tumor activity[23,24]. In high-grade B cell lymphoma[25–29] and B cell acute lymphoblastic leukemia[23,30–33], CD19 specific II-generation chimeric antigen receptor T cells have showed robust and long-lasting responses. The cells also effectual in ALL patients[34], & Patients with multiple myeloma benefit from chimeric antigen receptor T cells that target the B cell maturation antigen (BCMA)[35–38]. Treatment with chimeric antigen receptor T cells showed severe adverse drug ractions, E.g. cytokine release syndrome (CRS)[27,39,40] & immunological effector cell-associated neurotoxicity syndrome (ICANS)[41].

Natural killer cell (NK) - immune effector cell. These have generated much interest for beneficial platform for tumor. Key benefits from NK cells are that they are cytotoxic and do not have an endogenous TCR, thus they do not cause graft-versus-host reactions when given to MHC-incompatible patients.[42]. NK cells also have the potential to extravasate and migrate to tumor tissue[43]. However, NK cells utilization is limited because of difficulty in obtaining a sufficient number of therapeutically active NK cells in blood, it make up 10% of lymphocytes and are generally inactive. Moreover, allogeneic NK cell preparation needs depletion of T cells to prevent GVSD. These issues were solved by creating the immortal cell line NK-92, which exhibits all of the features of activated NK cells44, from a patient with clonal NK-cell lymphoma. In mice, parental NK-cells showed strong anti-tumor efficacy against melanoma, leukaemia, and

myeloma. NK-92 cells have been customized to CARs expression in order to improve their anti cancer potency. CAR-modified NK-92 cell have demonstrated remarkable efficacy in eliminating AML[45], lymphoma[46], myeloma[47] prostate cancer[48], breast cancer[49], neuroblastoma[50], and glioblastoma[51]. Engineered NK-92 cells have also been utilised in conjunction with other therapeutic platforms, with CAR_NK_92 cells exhibiting improved anti cancer effectiveness. A combination of EFGR specific CAR-altered NK-92 cells and oncolytic herpes simplex virus 1 demonstrated encouraging results in a mice model of breast tumour metastases, for example.[52]. In human colorectal cancer models, combining EpCAM detailed CAR_NK_92 cells & Regorafenib enhanced tumour suppression efficacy. [53].

NK-92 (CAR-modified immune effector cells), doesn't showed compelling confirmation of action to solid tumours despite their extraordinary effectiveness in several hematologic malignancies. Inefficient trafficking and an immune-suppressive microenvironment within solid tumours hampered CHIMERIC ANTIGEN RECEPTOR T CELLS cell functionality by different pathways, via Checkpoint receptor ligand expression (e.g., PD-L1) & suppressive immune cells that interfere with CHIMERIC ANTIGEN RECEPTOR T CELLS cell activity in a variety of ways (e.g., Tregs, MDSCs)[54,55].

Recent research has shown that certain tyrosine kinase inhibitors (TKIs) alter the tumour microenvironment and enhance anti-tumor immunity in addition to direct anti-tumor action. Doxorubicin[56], sunitinib[57], sorafenib[58,59], and gemcitabine[60,61] have been reported to reduce immune suppression and enhance anti-tumor immune response. These observations suggest that concurrent application of immunotherapy and TKI can enhance the efficacy of immunotherapy[60,62–65].

Cabozantinib comes under TKI that is FDA approved in renal cell tumor, hepatocellular tumor and medullary thyroid tumor in patients who have previously received sorafenib. It inhibits the enzymes MET and VEGFR2[66]. They enhance number of CD8+ &CD4+ T cells in spleen & reduce immune suppressive cells migration like MDSCs and Tregs to tumors[67]. Cabozantinib also eliminates MDSC in the tumour microenvironment in a mouse model, demonstrating significant synergistic effects when coupled with immune checkpoint inhibition & tumor vaccine treatment.[67,68]. As a consequence, we believe cabozantinib might be used in combination with EGFR- detailed CAR_NK_92 cells increases immune-based solid tumour therapies efficacy. Mouse model of human RCC, we developed a 2nd generation EGFR specific CAR in opposition to EGFR+ tumours and investigated how cabozantinib influenced the therapeutic efficacy of CAR- customized NK-92 cells.

Materials and Methods

Human Renal Cancer Cell Lines (2.1) The cell lines 786-O & ACHN, as well as the human colorectal cancer cell lines SW620 and HT29, were provided by the American Type Culture Collection. 786-O & HT29 cells grown in RPMI-1640 media added with 10% FBS (Thermo Fisher Scientific, USA) & 1% penicillin/streptomycin (Thermo Fisher Scientific, USA) (Thermo Fisher Scientific, USA). The ACHN and SW620 cell lines grown in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, USA) supplemented with 10% FBS & 1% penicillin/streptomycin. NK-92 cells & transduced NK-92 cells cultured in an alpha modification of Eagle's minimum essential medium (Thermo Fisher Scientific, USA) added with 2 mM L-glutamine, 0.2 mM myo-inositol, 0.02 mM folic acid, 0.1 mM 2-mercaptoethanol, 400 IU/ml IL-2 (Peprotech, America), 12.5 percent FBS.

2.2 The second stage is Flow Cytometric Analysis. To evaluate lentivirus transduction rate in NK-92 cells, flow cytometry have been used

for analyse GFA expression in Ctrl-NK-92 & CAR_NK_92 cells. Flow cytometry was used to assess the surface expression of EGFR and PD-L1 in tumor cells that had previously been treated with cabozantinib. Cancer cells were washed and resuspended in PBS supplemented with 2% BAS at 1x106 cells/ml. 100l of cell suspensions were treated with PE-labeled mouse anti-human EGFR antibody (BioLegend, USA) or PE/Cy7 labelled mouse anti-human PD-L1 antibody (BioLegend, USA) for 30 minutes at room temperature in the dark (BioLegend, USA). The cells were then washed and resuspended in 0.5ml PBS before being analysed by a CytoFlex flow cytometer (Beckman Coulter, USA). Using methods mentioned above, a second batch of cells was stained with Isotype Control Antibodies (BioLegend, USA).

2.3. Analysis of Western Blots To produce cell lysates, phosphatase & protease inhibitors being given to the NP-40 solution (Thermo Fisher, USA). After determining the protein content, Each sample had 25 g of total protein dispersed in an equal amount of sample buffer and ran on a 10% SDS-PAGE gel. Protein bands being transmitted across nitrocellulose membranes. (Amersham, Sweden) following separation and 60 minutes at ambient temperature blocking with 5% BSA. Afterwards, the membranes incubated with a rabbit anti-human anti-CD3 antibody. Incubate rabbit anti-human GAPDH antibody (1:1000, Invitrogen, USA) or rabbit anti-human GAPDH antibody (1:1000, Invitrogen, USA) at 4°C overnight (1:1000, Invitrogen, USA). Sections were incubated for 60 minutes at ambient temperature with an HRP conjugated anti-rabbit IgG (1:5000, Invitrogen, USA) in blocking buffer after 3 washing with TBST. The ECL system (Millipore) was used to identify the target proteins, and the ChemiDoc XRS system was used to display them (Bio-Rad).

2.4. Analyze with ELISA kit. ELISA kits (Abcam, UK) were used to measure human IFN-, perforin, and granzyme B in cell-free supernatants according to the manufacturer's procedure. 1x104 target cells co-cultured among ctrl-NK-92 / CAR_NK_92 as effector cells in a 96-well plate for 24 hours in effector to target ratios of 0.5:1, 1:1, & 2:1, respectively. 100µl of cell-free supernatants or standard were applied to antibody-coated wells of the provided well strips. Each well received 50µl of biotinylated tagged detector antibody, which was incubated at ambient temperature for 120 minutes. After three washes with washing buffer, each well received a 100µl streptavidin-HRP solution incubated for additional 30 minutes. After cleaning the wells again, 100l of chromogen TMB solution added & incubated for 15 minutes at ambient temperature. Finally, 100l stop reagent was added to all well, & the absorbance measured at 450nm wavelength by a microplate reader.

2.5. A cytotoxicity test is performed. As per procedure, an LDH cytotoxicity test kit (Abcam, UK) was used to determine cytotoxicity. 1104 target cells cocultured with CAR_NK_92 / Ctrl-NK-92 cells at E/T ratios of 1:1, 3:1, 10:1, or 30:1 for 4 hours in RPMI-1640 with 15mM HEPES & 5% FBS. In an optically transparent 96 well microplate, 100µl of cell-free supernatant was added to the appropriate wells. Each well received 100µl of newly produced reaction mixture, It was kept at ambient temperature for 30 minutes while being protected from light. The product's recommendations were followed for setting the background control, low control, and high control. A microplate reader worn to measure absorbance at 490nm. 610nm was chosen as the standard wavelength. The technique 100x (absorbance of test sample – absorbance of low control)/ (absorbance of high control – absorbance of low control) was used to compute the percent cytotoxicity.

2.6. Kit for Counting Cells No. 8 (CCK-8) Assay. The cytotoxicity of Cabozantinib was evaluated using the Cell Counting Kit-8 (Sigma-Aldrich). 5x103 cells were treated with DMSO or 2.5g cabozantinib for 0, 24, 48, 72 & 96 hours in 96 well microplates. After that, each well was

supplied with 10 l of CCK-8 solution, and the cells were cultured for 1 hour at 37oC. A monochromator microplate reader was used to measure absorbance at 450nm wavelength, which was used to evaluate cell viability.

2.7. In vivo studies on efficacy. The in vivo efficacy of EGFR specific CAR_NK_92 cells was tested using a tumour xenograft model in NOD SID mice. In summary, 5x106 786-O & ACHN cells injected into right side in acclimatised 5-week-old female NOD SID mouse suspended in 100l FBS and antibiotic-free RPMI 1640 or DMEM medium. After then, the mice were split into five groups: untreated, cabozantinib, Ctrl-NK-92, CAR-NK-92, & cabozantinib+CAR-NK-92. Cabozantinib & cabozantinib+CAR_NK_92 groups started receiving cabozantinib (10mg/kg) via gavage 5 times a week for 6 weeks 5 days after inoculation with cancer cells (day 5). The Ctrl-NK-92 group got 3x106 Ctrl-NK-92 cells once a week for a total of six times starting on day 6. CAR_NK_92 & cabozantinib+CAR_NK_92 groups received 3x106 CAR_NK_92 cells once per weak, a total of 6 times, starting on day 6. Every other day, all NK-92 cell groups given with 2000 IU intraperitoneally administered recombinant human IL-2 (rhIL-2). The tumor's length and breadth were measured using a digital calliper, and the quantity deliberated by (tumour volume = length width2/2). Bioluminescent imaging was used to quantify tumour size at the end (BLI). Animals then euthanized, and tumors parts excised for histological examination.

2.8. Immunohistochemistry. Tumor tissues were paraffin embedded & made as 3–5m slices after being fixed in 10% formalin. By labelling tumour tissue sections with rabbit anti-human CD3 antibody, NK-92 cells in tumour tissue sections were identified (1:200, Abcam). Under 200 magnifications, The marked samples were processed in 10 intratumoral regions of the each slide by randomly.

2.9. Analytical Statistics All data was analysed with GraphPad Prism 5 software & given as mean ± SEM.

Results

3.1. CAR_NK_92 Cells with EGFR Specificity

As illustrated in Figure 1, an EGFR specific scFv was connected in tandem to the hinge & transmembrane domains of CD8, the intracellular signalling domains of CD28, & CD3 to create a CAR (a). CAR was included into a lentiviral vector system that also contained puromycin & green fluorescent protein sequences (GFP).

EGFR-CAR or empty vectors were transduced into the NK-92 cell line, yielding EGFR-CAR_NK_92 & Ctrl-NK-92 cells, respectively. Puromycin selection of EGFR-CAR_NK_92 and ctr-NK-92 cells resulted over 60% GFPpositive cells (Figures 1(b) and 1(c) (c)). Western blot examination with rabbit antihuman CD3 monoclonal antibody (Figure 1(d)) confirmed the expression of EGFR-CAR in EGFR-NK-92 cells but not in ctr-NK-92 cells.

3.2. EGFR-CAR_NK_92 Cells are cytotoxic to EGFR+ Renal Cancer Cells in vitro.

EGFR expression in EGFR+ cell lines 786-O assessed by flow cytometry & ACHN, EGFR- cell lines HT-29 and SW620. Figure 2 shows that EGFR was expressed substantially in 786-O and ACHN cells but not in HT-29 or SW620 cells.

To determine whether EGFR+ cell lines 786-O & ACHN activate CAR_NK_92 cells, we used a cytokine release assay. Figure 3 shows that when CAR_NK_92 cells were cocultured with 786-O or ACHN, the release of IFN-, perforin, and granzyme B was significantly and dose-dependently increased compared to Ctrl-NK-92 cells. When CAR_NK_92 & Ctrl-NK-92 cells co-cultured with EGFR- cell lines SW620 and HT29, inconsequential difference in cytokine production by CAR_NK_92 & Ctrl-NK-92 cells. The findings revealed that CAR_NK_92 cells could recognise and activate EGFR-positive cells.

Lactate dehydrogenase (LDH) release tests

were used to see if CAR_NK_92 cells could kill EGFR+ 786-O and ACHN cells. At effector to target ratios of 3:1, 10:1, & 30:1, CAR_NK_92 cells outperformed Ctrl-NK-92 cells in terms of dose-dependent cytotoxicity against 786-O and ACHN cells. CAR_NK_92 & Ctrl-NK-92 had comparable cytotoxicity against EGFR- SW620 and HT29 cells in same E/T ratio. CAR_NK_92 cells are solely cytotoxic to EGFR+ cells, according to these data.

3.3. Cabozantinib's In Vitro Effect on EGFR-Specific CAR_NK_92 Cell Functions

We estimated the potency of cabozantinib in 786-O & ACHN cell proliferation using cell counting kit-8 assay. When given at 2.5g/ml, cabozantinib inhibited the development of both 786-O & ACHN cells for 24, 48, 72, & 96 hours (Figure 5). In the presence of 2.5g/ml cabozantinib, cell proliferation persisted for another 96 hours. In all future in vitro studies, we utilised this cabozantinib concentration.

Cancer cells are known to express cell surface markers that help them escape therapies[69]. We looked at EGFR & PD-L1 expression in 786-O & ACHN cells after 24 hours of cabozantinib therapy to determine if it affected the phenotype of the cells and therefore modified their susceptibility to EGFR-CAR_NK_92 cell-mediate cytotoxicity. Cabozantinib treatment increased EGFR expression significantly in both 786-O & ACHN cells, as seen in figure 6. PD-L1 expression, on the other hand, was significantly decreased in both 786-O & ACHN cells. Cabozantinib may help in the identification and activation of EGFR-CAR-KN-92 cells by increasing EGFR expression, according to the findings. Furthermore, cabozantinib's decrease of PD-L1 expression may contribute to the enhanced cytotoxicity of EGFR-specific CAR_NK_92 cells against tumour cells.

Then, over the next 24 hours, we cocultured CAR_NK_92 or CTR-NK-92 cells with 786-O & ACHN cells that had previously undergone cabozantinib therapy. Cell lysis was determined using LDH release assays. Cabozantinib treatment made 786-O & ACHN cells more susceptible to EGFR- detailed CAR_NK_92 cell-mediated cytotoxicity, as seen in figure 7.

These findings show that cabozantinib changes the phenotypes of 786-O and ACHN cells, making it highly vulnerable to CAR_NK_92 cell-mediated cytotoxicity.3.4. Combination with Cabozantinib Enhances In Vivo Cytotoxicity of EGFR-Specific CAR-NK92 Cells.

We created a subcutaneous xenograft in NOD-SCID mice utilising 786-O or ACHN cells expressing firefly luciferase (786-O-Luc and ACHN-Luc, respectively) to examine the effect of cabozantinib on CAR_NK_92 cells' in vivo antitumor efficacy. (Figure 8(a))

Cabozantinib, ctrl-NK-92, CAR-NK-92, and the amalgamation of CAR_NK_92 & cabozantinib drastically slowed progression in both 789-O-Luc and ACHN-Luc tumours, as shown in figure 8(b)-8 (d). In addition, when compared to Ctrl-NK-92, CAR-NK-92, and cabozantinib groups, CAR_NK_92 plus cabozantinib treatment significantly reduced the progression of both tumours.

3.5. Cabozantinib's Effect on CAR_NK_92 Cell Migration.

Tumor samples from 786-O tumours were stained with monoclonal antihuman CD3 primary antibody to appraise infiltration rate of CAR_NK_92 cells. A previous study [13] found that CD3 is expressed by wild type NK-92 cells. As a result, staining with antihuman CD3 primary antibody should show both Ctr-NK-92 and CAR_NK_92 cells infiltrating the tumour. NK-92 cells were found in tumour samples from all NK-92 treated animal groups, but not from untreated or cabozantinib treated animals, as depicted in figure 10. When compared to the Ctrl-NK-92 group, infiltration was substantially greater in CAR-NK-92+ cabozantinib treated mice. Surprisingly, there was no significant difference in infiltration rates between the

CAR_NK_92 & CAR-NK-92+ cabozantinib category.

4. Discussion

NK cells are immune cells that play an important role in the surveillance against cancer and virus-infected cells. There are several advantages in using NK cells as a therapeutic platform. NK cells can extravagate and migrate to solid tumor tissue. They are not restricted by MHC, so they can be used in all patients irrespective of HLA types. Moreover, NK cells do not express TCR; thereby do not cause graft-versus-host reactions. However, generating a sufficient amount of NK cells from peripheral blood for clinical use is a big challenge. This limitation of NK cell therapy has been circumvented by using the NK-92 cell line, which demonstrates all the characteristics of activated NK cells. Furthermore, the anticancer activity of primary NK cells is significantly suppressed by the immune suppressive mechanisms of cancer cells. Previous studies have demonstrated that modification of NK cells to express CAR against specific cancer-associated-antigen significantly enhances the anticancer efficacy of NK cells. However, in developing a CAR-modified NK cell, selection of the target antigen is of paramount importance. Such an antigen has to be expressed by the cancer cell in abundance, while expression in normal cells should be at a minimum. EGFR is overexpressed or undergo mutation in most cancers, including RCC, breast cancer, glioblastoma, and lung cancer[71–73]. In our study, we developed second-generation wild type EGFR specific CAR-NK-92 cells. Our results convincingly demonstrated the anticancer efficacy of CAR-NK-92 cells against RCC in the mice xenograft model.

Cabozantinib is a tyrosine kinase inhibitor that has been reported to modulate the immune suppressive microenvironment of solid tumors and alter the phenotype of cancer cells. Cabozantinib has also been shown to have a commensal response in immune therapy in cancer. Considering the above observations, we hypothesized that concurrent application of cabozantinib might enhance the anticancer efficacy of CAR-NK-92 cell therapy. In our experiments, we observed that cabozantinib enhanced CAR-NK-92-mediated cytotoxicity against RCC cells as well as increased the expression of EGFR in RCC cells. It has been reported that the efficacy of immune therapy in solid tumors is significantly influenced by the expression level of the target antigen in the cancer cells. We, therefore, attributed the higher anticancer efficacy of CAR-NK-92 cells to enhanced expression of EGFR in RCC cells caused by concurrent cabozantinib treatment. Furthermore, we observed that cabozantinib suppresses the expression of PD-L1 in the target cells. Expression of PD-L1 on tumor cells is one of the mechanisms of immune evasion since this inhibits the functional activity of cytotoxic lymphocytes, including NK cells. Suppression of PD-L1 expression on target cells by cabozantinib, therefore, enhances the susceptibility of EGFR positive cells to CAR-NK cell cytotoxicity. Cabozantinib is also known to suppress the functions of MDSCs and Tregs, two most potential immune suppressive cells, present in cancer tissues. Further studies are needed to elucidate the influence of MDSCs and Tregs in cabozantinib and CAR-NK-92 cell combination therapy in RCC.

Cabozantinib is ill tolerated by the renal cell carcinoma patients at its clinical dose 60mg/day. In our experiments in mice, 10mg/kg/day of cabozantinib significantly enhanced the cytotoxicity of EGFR specific CAR-NK-92 cells against EGFR positive RCC cells. According to the FDA guidance for the conversion of animal dose to human dose, 10mg/kg in mice is equivalent to (10 x .081) 0.81mg/kg. Considering the average weight of human being 60kg, that would be 48.6mg/day, a significantly lower dose than present clinical dose. This dose may be better tolerated by the patient.

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Figure Legends

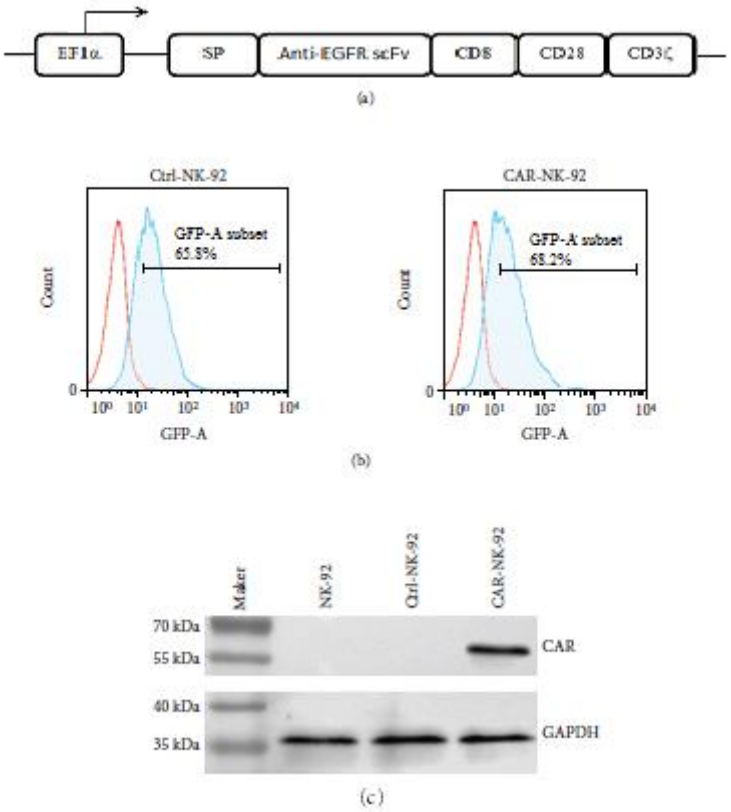

Figure 1: Generation and characterization of EGFR-specific CAR-NK-92 cells. (a) Structure of EGFR-specific CAR. CAR was constructed with wild type EGFR specific scFv linked to the hinge and transmembrane domains of CD8, and the intracellular signaling domains of CD28 and CD3ζ. (b) Transduction efficiency of lentivirus in NK-92 cells. NK-92 cells were transduced either with lentivirus vector (Ctrl-NK-92) or lentivirus containing the EGFR-specific CAR encoding sequence (CAR-NK-92) and enriched by repeated selection with puromycin. The abundance of the cells expressing GFP was determined by flow cytometric analysis. (c) CAR expression in NK-92 cells was confirmed by Western blot with a monoclonal anti-human CD3ζ antibody. GAPDH was detected as an internal control.

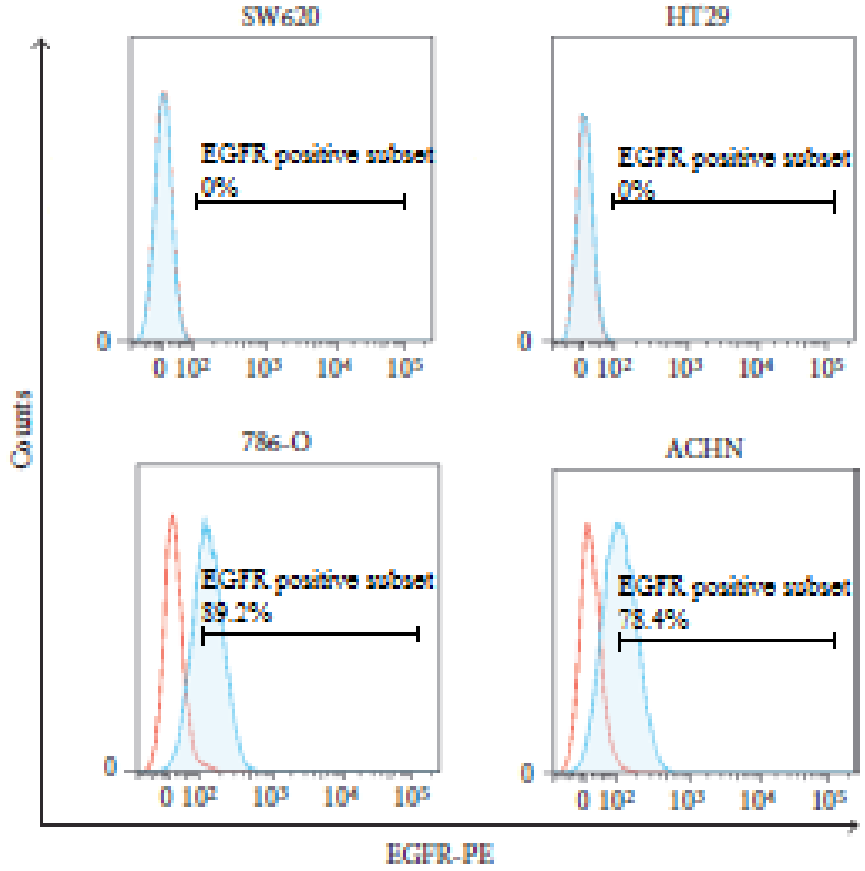

Figure 2: Surface expression of EGFR in human cancer cell lines. Expression of EGFR in human colon cancer cell lines SW620, HT 29, and renal cancer cell lines 786-O, ACHN were determined with flow cytometry.

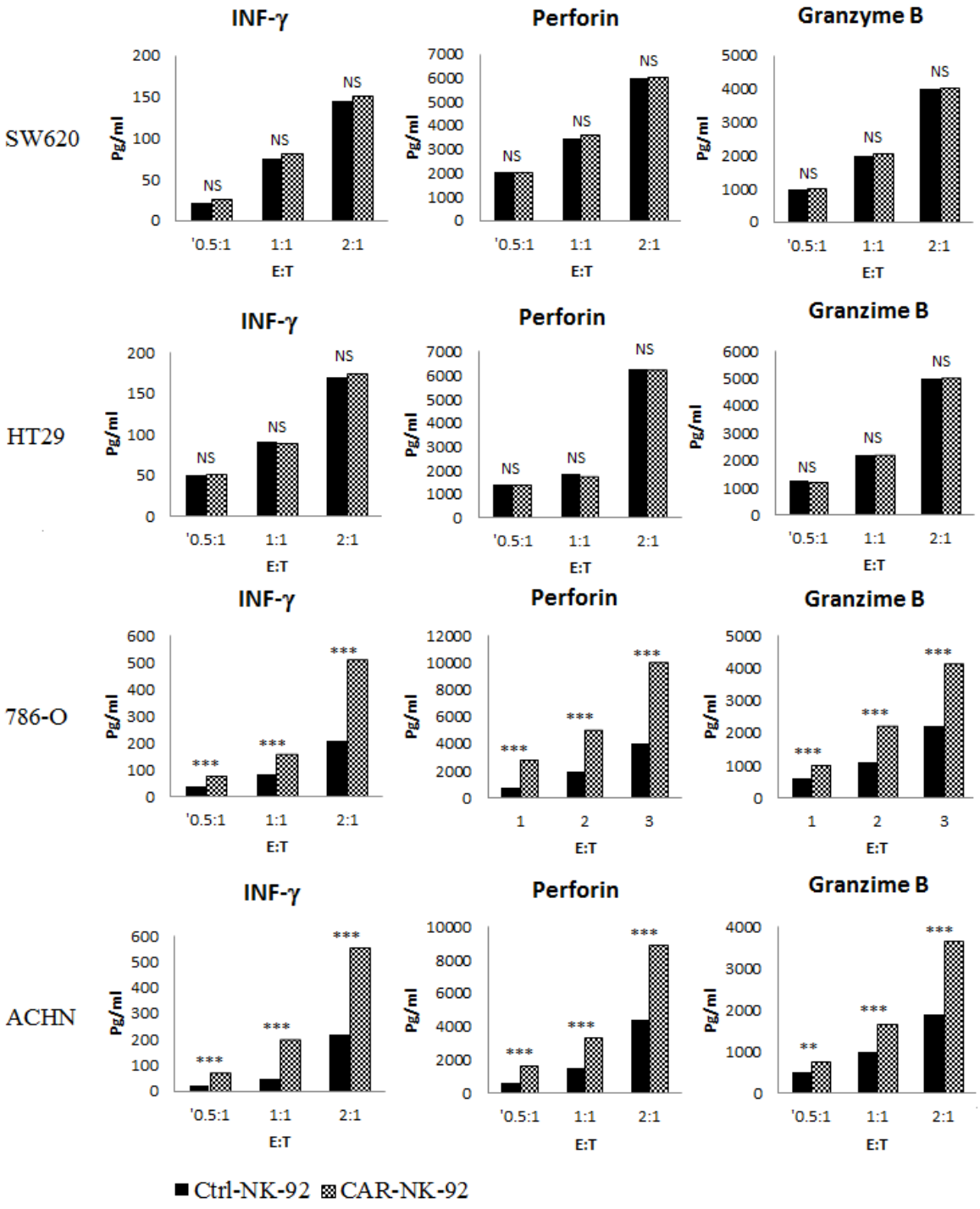

Figure 3: Specific cytokine release of EGFR-specific CAR-NK-92 cells against EGFR$^+$ cells. Ctrl-NK-92 and CAR-NK-92 cells were cocultured with either EGFR$^-$ or EGFR$^+$ target cells for 24h at an E/T ratio of 0.5 : 1, 1 : 1, and 2 : 1 and the release of cytokines were measured by ELISA. $^{**}p < 0\ 01$; $^{***}p < 0\ 001$. ns: not significant.

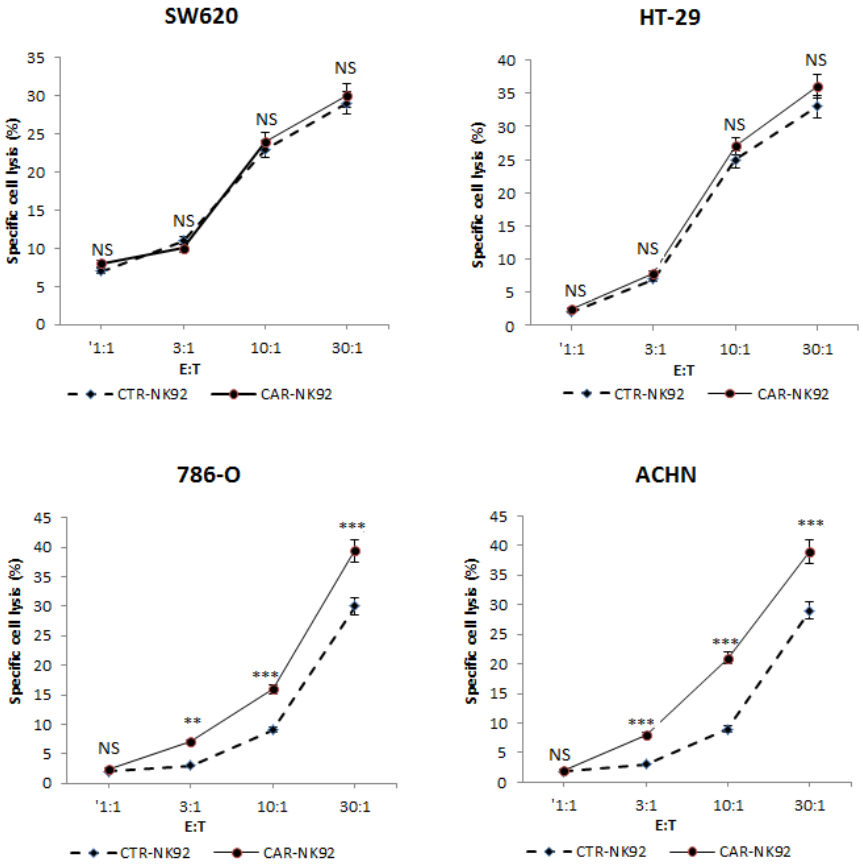

Figure 4: EGFR⁺ cell-specific cytotoxicity of EGFR-specific CAR-NK-92 cells. CAR-NK-92 and Ctrl-NK-92 cells were cocultured either with EGFR– or EGFR+ cancer cells for 4h at indicated E/T ratio and the cytotoxic activity of CAR-NK-92 and Ctrl-NK-92 cells was determined using lactate dehydrogenase (LDH) release assay. $*p < 0\ 05$; $**p < 0\ 01$; $***p < 0\ 001$. ns: not significant.

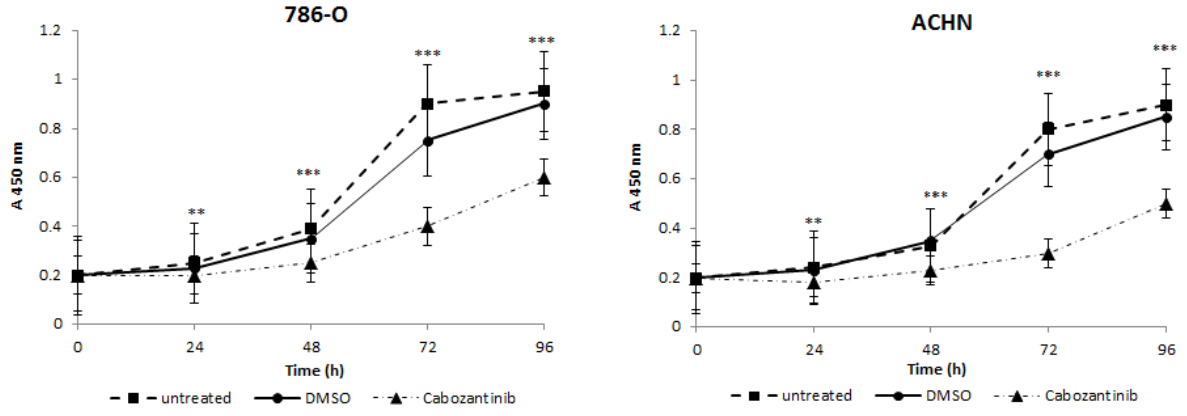

Figure 5: Suppression of proliferation of renal cancer cell 786-O and ACHN by cabozantinib. 786-O and ACHN cells were incubated with 2.5 μg/ml cabozantinib or vehicle (DMSO) for 24, 48, 72 and 96h and assayed for cell viability using CCK-8 kit. $*p < 0\ 05$; $**p < 0\ 01$; $***p < 0\ 001$.

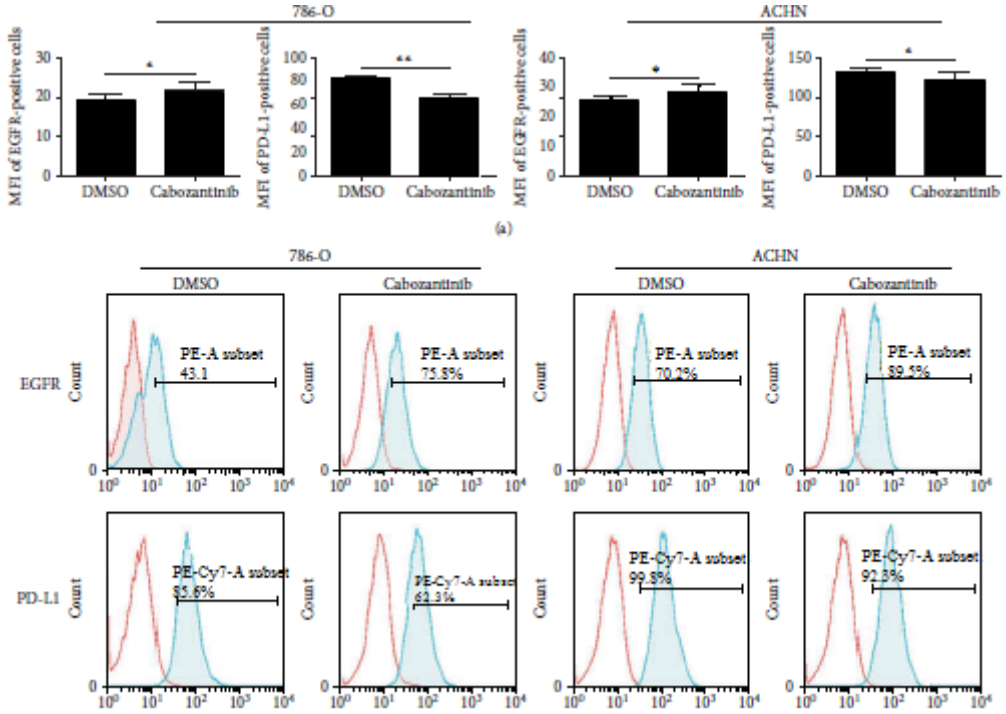

Figure 6: Cabozantinib alters the expression of EGFR and PD-L1 in RCC. 786-O and ACHN cells were treated with 2.5 μg/ml cabozantinib or vehicle for 24h and analyzed by flow cytometry for surface expression of EGFR and PD-L1.

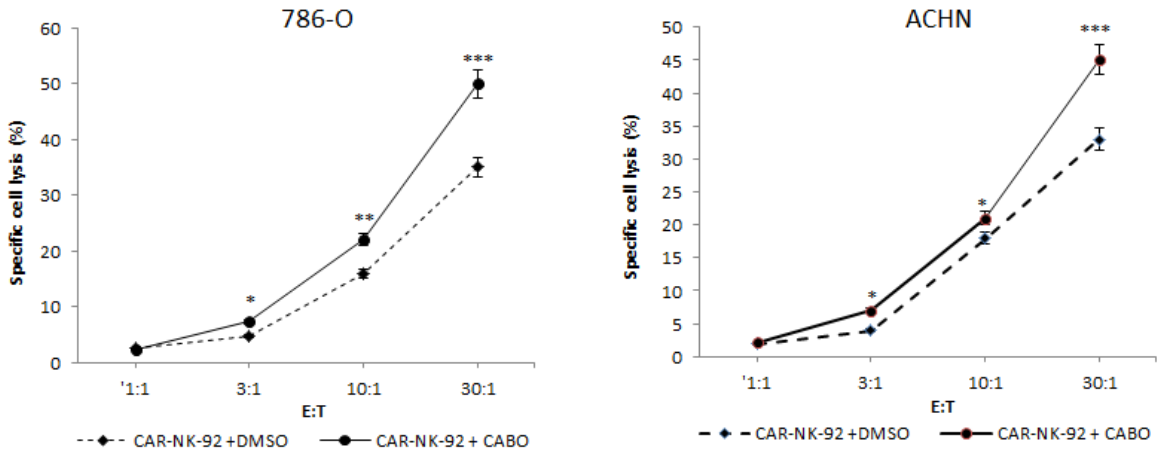

Figure 7: Treatment with cabozantinib increases the sensitivity of renal cancer cells to the CAR-NK-92 cell-mediated cytotoxicity. 786-O and ACHN cells were treated with 2.5 μg/ml cabozantinib or vehicle for 24h, then incubated with the CAR-NK-92 cells for 4h. Cytotoxicity of CAR-NK-92 cells was determined by LDH release assay. $*p < 0\ 05$; $**p < 0\ 01$; $***p < 0\ 001$. ns: not significant.

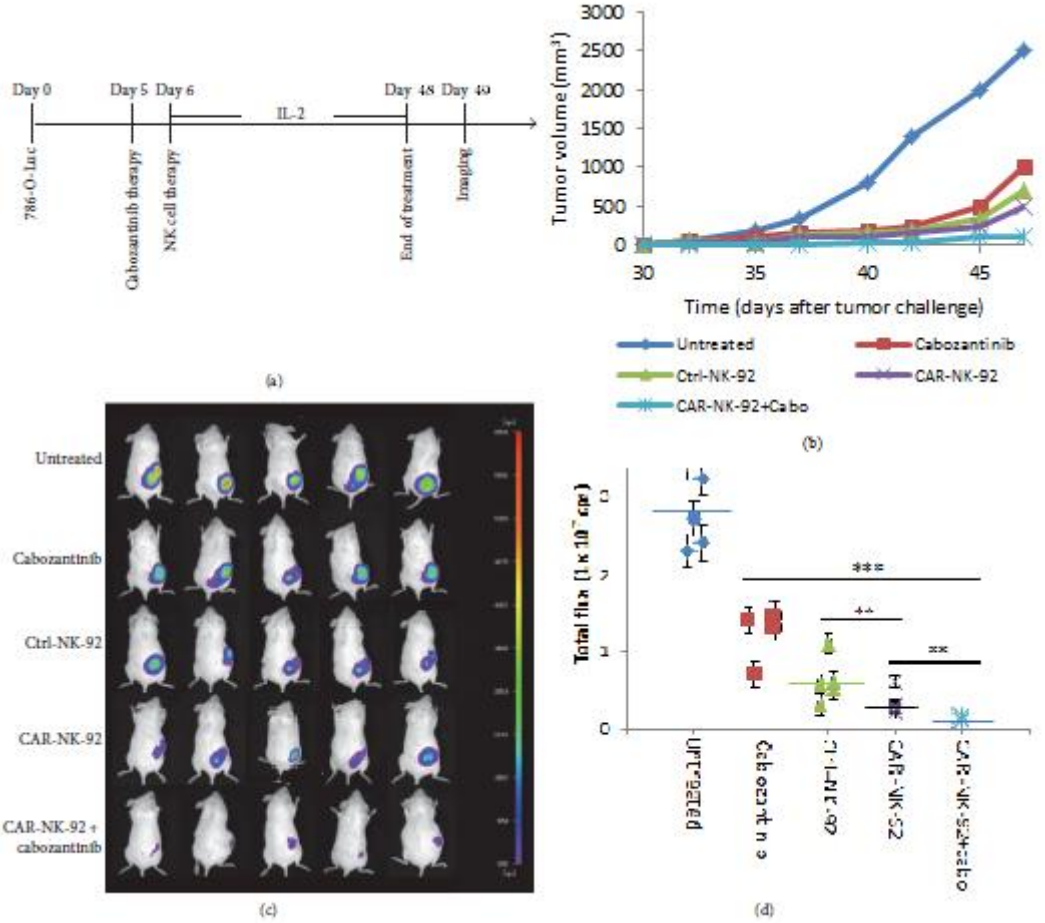

Figure 8: Therapeutic efficacy of EGFR-specific CAR-NK-92 cells combined with cabozantinib for human renal cancer xenograft established with 786-O cells. (a) Schematic diagram showing the treatment protocol of the mice. (b) The tumor growth curves during the experiment. (c) Luminescence images showing the tumor size at the end of the treatment. (d) Quantitative results of the tumor luminescence intensity shown in (c). $*p < 0\ 05$; $**p < 0\ 01$; $***p < 0\ 001$.

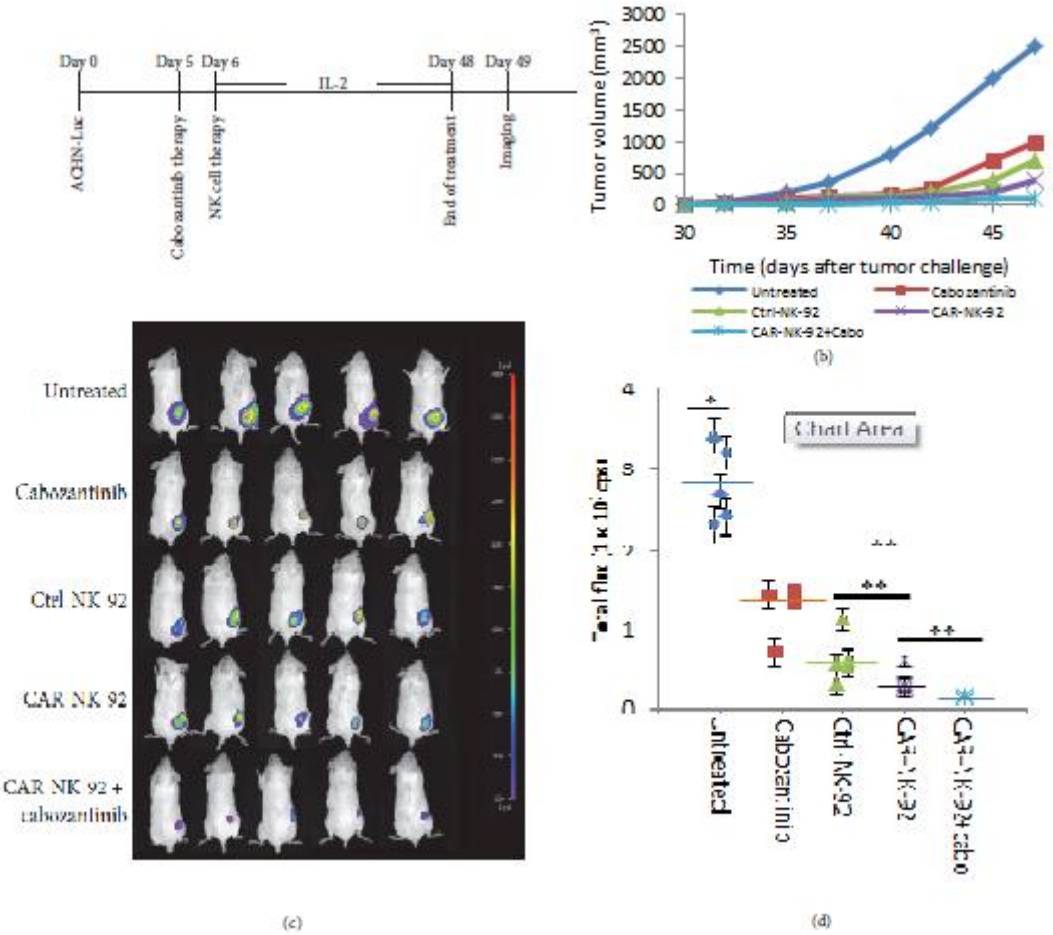

Figure 9: Therapeutic efficacy of EGFR-specific CAR-NK-92 cells combined with cabozantinib for human renal cancer xenograft established with ACHN cells. (a) Schematic diagram showing the treatment program of the mice. (b) The tumor growth curves during the experiment. (c) Luminescence images showing the tumor size at the end of the treatment. (d) Quantitative results of the tumor luminescence intensity shown in (c). ***$p < 0\ 001$.

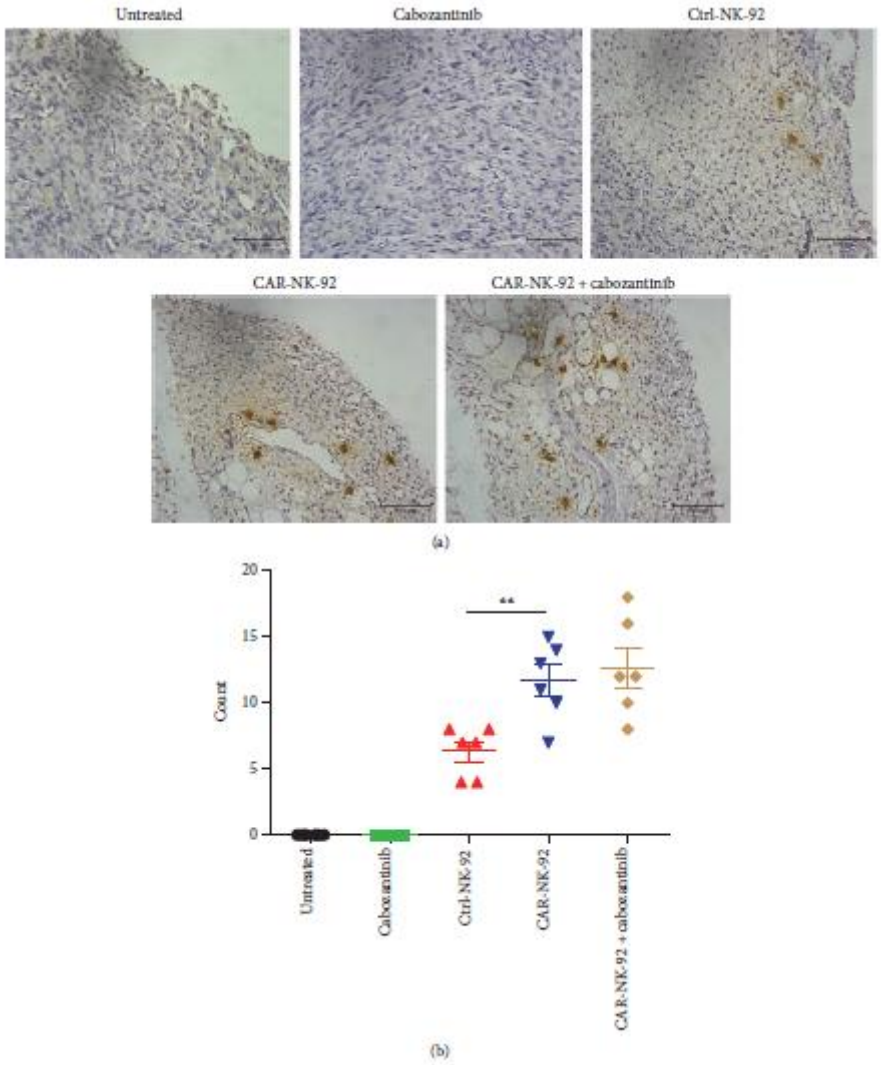

Figure 10: Tumor infiltration analysis of NK-92 cells in vivo. (a) Immunohistochemical analysis of human CD3+ NK-92 cells in established
s.c. xenografts. The images were obtained under ×200 magnification. (b) The corresponding quantitative analysis results of human CD3+ NK-92 cells shown in (a). **p < 0 01.