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CD70 as a target for chimeric antigen receptor T cells in head and neck squamous cell carcinoma

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Abstract

Objectives—In accordance with the Precision Medicine Initiative, new treatment strategies for head and neck squamous cell carcinoma (HNSCC) are needed to yield better therapeutic outcomes. The purpose of this study was to establish and validate chimeric antigen receptor (CAR)-T cells targets in HNSCC.

Methods—Putative CAR-T antigens were identified in The Cancer Genome Atlas database. To validate antigen suitability, quantitative RT-PCR, flow cytometry, and immunofluorescent staining were performed. A retroviral human CD70 CAR construct, using truncated CD27 conjugated with 4-1BB and CD3-zeta costimulatory molecules, was used to transduce activated human T cells to generate CD70 CAR-T cells. Cell-based cytotoxicity and cytokine ELISAs were used to measure efficacy of killing.

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Results—Nine potential CAR-T targets (CD276, EGFR, MICA, MICB, MAGE-A4, FAP, EPCAM, CD70, B4GALNT1) were identified based on their high expression in tumors compared to flanking control tissues. CD70 was selected for further proof-of-principle analysis based on its differential expression in several tumor subtypes, and showed substantial heterogeneity in individual tumors analyzed. Cell surface CD70 protein and CD70 mRNA were detected from low to high levels in established HNSCC cancer cell lines. CD70 was highly expressed in 4 of 21 tumor biopsies (19%), and 3 of 4 specimens showed strong CD70 expression on the tumor cell surface. CD70-specific CAR-T cells were generated and further demonstrated to recognize and kill CD70-positive HNSCC cells efficiently, but not CD70-negative cancer cells.

Conclusion—CD70-specific CAR-T cells specifically recognized and efficiently eliminated CD70-positive HNSCC cells. This study provides the basis for further investigation into CD70 and other CAR-T targets.

Graphical abstract

Keywords

CD70; chimeric antigen receptor; head and neck squamous cell carcinoma; IFN- γ

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world, with an estimated 63,030 new diagnosed cases and 13,360 deaths in 2016 [1]. Moreover, recurrent and metastatic cases increase annually and treatment is mostly limited to chemotherapy and radiation therapy. Targeted therapies using epidermal growth factor receptor (EGFR)-blocking monoclonal antibody, either alone or in combination with chemotherapy and/or radiotherapy, were recently introduced. However, treatments have been limited due to unfavorable toxicity and new treatment options are still urgently needed to improve patient outcomes [2]. Recently, chimeric antigen receptor (CAR)-T cell technology has revolutionized cancer immunotherapy. This strategy uses synthetic CARs to redirect T cells to recognize specific antigens expressed on the surface of tumor cells. Representative studies using CD19-specific CAR-T cells have shown great success in patients with B cell malignancies, eradicating tumors permanently and achieving durable remission of leukemia [3–5]. Adoptive transfer of these CD19 CAR-T cells successfully treated circulating tumors, but there are still many obstacles to establishing efficacy in therapy for solid tumors. One limitation in developing CAR-T therapy is the selection of target molecules overexpressed in

solid tumors. To date, about 30 CAR-T antigens have been reported and a wide range of potential targets are under clinical investigation [6, 7]. However, there remains a need to identify and validate tumor-associated antigens in the most challenging malignancies, such as head and neck cancers. In this study, we identified nine candidate CAR-T antigens from previously reported CAR-T antigens, based on analysis of The Cancer Genome Atlas (TCGA) characterization of HNSCC [8]. One antigen, CD70, was further investigated and used to demonstrate CAR-T cell function in specific recognition and killing of HNSCC *in vitro*.

Materials and Methods

Analysis of CAR-T target gene expression in TCGA database

The TCGA mRNA expression data (RNASeq V2) of HNSCC and adjacent controls were downloaded from <https://portal.gdc.cancer.gov>. CAR-T target antigen expression was analyzed using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA).

Patient specimens, cell culture, and reagents

Human oral tissues were collected at the H. Lee Moffitt Cancer Center (Tampa, FL; Protocol MCC-15730) and approved by the Institutional Review Board of the University of South Florida (106444) as previously reported [9–11]. Seven head and neck cancer cell lines (BHY, CAL27, FaDu, HN, OQ01, RPMI 2650, SCC-25) were cultured as described [9]. K562 cells (chronic myelogenous leukemia) were purchased from ATCC. RS4 (acute lymphoblastic leukemia) and HOS (osteosarcoma) cell lines were obtained from the stock in Dr. Lung-Ji Chang laboratory. K562 and RS4 were maintained in RPMI 1640 medium containing 10% FBS and HOS cells were cultured in DMEM medium containing 10% FBS. To design bioluminescence cell killing assays, CAL27 and OQ01 were transduced by lentivirus to express firefly luciferase (Luc) and a puromycin resistance gene. Stable cell lines (CAL27.Luc and OQ01.Luc) were subjected to puromycin selection and maintained in DMEM medium containing 10% FBS. Monoclonal antibodies (mAbs), including PE-labeled and unconjugated anti-human CD70 (clone Ki-24), were purchased from BD Pharmingen (San Diego, CA, USA). Alexa Fluor 568-conjugated secondary antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Quantitative real-time PCR

RNA isolation, cDNA synthesis, and TaqMan real-time PCR were performed as described [9]. CD70 mRNA expression was measured using TaqMan human CD70 gene expression assay (Assay ID Hs00174297_m1, Thermo Fisher Scientific). 18S rRNA was used as an internal control to normalize relative expression of CD70. Fold change values were calculated using the 2^{-C_t} method.

Flow cytometry

Adherent cells were washed with PBS and incubated in Cell Dissociation Buffer (Thermo Fisher Scientific) for 20 min in a 37°C incubator to detach cells. After centrifugation, the cells were collected and counted. Cells were incubated either with or without PE-labeled anti-CD70 antibody for 1 h on ice and then cells were washed three times in PBS. Cells

were analyzed with an LSRFortessa flow cytometer (BD, Heidelberg, Germany) and FlowJo software (FlowJo, Ashland, OR, USA).

Indirect immunofluorescence of head and neck tumor specimens

Formalin-fixed paraffin-embedded (FFPE) tissue sections of 5 μm thickness were used for this study. Heat-induced antigen retrieval was performed with Trilogy (Cell Marque, Rocklin, CA, USA) for 20 min at 95°C. The specimens were stained with 1 μg of unconjugated anti-CD70 antibody at 4°C overnight. Then, slides were washed three times in PBST (0.1% Tween-20 in PBS) and incubated with Alexa Fluor 568-conjugated secondary antibody for 30 min in the dark, followed by washing with PBST. Specimens were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and images were captured at 200 $\times$ magnification using a Zeiss Axiovert 200M microscope equipped with a Zeiss AxioCam MRm camera.

Production of retrovirus and expansion of T cells

The pMSGV8-trCD27-41BB-CD3 zeta CAR plasmid was kindly provided by Dr. Steven Rosenberg (National Cancer Institute, Rockville, MD) [12]. To produce retrovirus, GP2-293 cells were co-transfected with 2 μg pMSGV8-trCD27-41BB-CD3 zeta plasmid and 2 μg pMD2.G plasmid (Addgene, Cambridge, MA, USA) using Lipofectamine 2000 (Thermo Fisher Scientific). Some cells were also co-transfected with EGFP expression plasmid together with pMD2.G plasmid to estimate transduction efficiency. After two days, the culture supernatants containing virus were harvested and used to transduce activated human T cells. Healthy peripheral blood mononuclear cells (PBMCs, LifeSouth, Gainesville, FL, USA) were activated by beads coated with anti-CD3 and anti-CD28 antibody (Dynabeads Human T-Activator CD3/CD28, Thermo Fisher Scientific) in the presence of recombinant human IL-2 (rhIL-2, 300 IU/ml) for 3 days before transduction. The stimulated T cells were added to 24-well plates initially pre-coated with recombinant human fibronectin (RetroNectin, Takara Bio, Mountain View, CA, USA) and then coated with retroviral supernatants by spinoculation (3200 rpm, 32°C, 2h). The plates were then centrifuged at 1700 rpm for 10 min, and incubated overnight at 37°C in a 5% CO₂ incubator. Four days later, transduction efficiency was estimated by EGFP expression of T cells. Typically, 20-50% transduction efficiency was obtained. At least 20% of transduced CAR-T cells were effective at killing tumor cells. Transduced cells were maintained in AIM V Medium CTS (Thermo Fisher Scientific) containing rhIL-2 (50-100 IU/ml) with 5% human AB serum, and then used for the bioluminescence assay.

Bioluminescence assay and analysis of IFN- γ secretion

For the cell-based bioluminescence assay, 1×10^4 OQ01.Luc or CAL27.Luc cells were co-cultured with either CD70 CAR-T cells or non-transduced cells at indicated effector-to-target ratios overnight. Bioluminescence was measured with the Bright-Glo Luciferase system (Promega, Madison, WI, USA) according to the manufacturer's instructions. CAR-T cell killing activity was calculated by comparing bioluminescence values between cultures of target cells alone (100%) and target cells co-cultured with CAR-T cells. 1×10^5 CD70 CAR-T cells were co-cultured with an equal number of target cells for 18h. Cell-free supernatants were assayed for IFN- γ secretion by DuoSet ELISA (R&D Systems,

Minneapolis, MN, USA) according to the manufacturer's instructions. Data depicted in figures show mean $\pm$ SEM values for triplicate wells from representative experiments. Each experiment was performed at least three times using different T cell donors.

Statistical analysis

Mann-Whitney *U* test, one-way ANOVA, and Student's *t*-test (unpaired) were used to compare groups in this study. GraphPad Prism 5.0 was used for all statistical calculations and differences in groups were considered statistically significant if $P < 0.05$.

Results

Identification of CAR-T targets in head and neck cancers

In order to screen for putative CAR-T targets appropriate for HNSCC, the expression levels of 18 reported CAR-T targets (Supplemental Table 1) were analyzed in the 303 HNSCC compared to the 37 adjacent control tissues in the TCGA database. With a focus on predicted cell surface protein targets, only nine candidates showed significantly increased mRNA expression in cancer compared to controls (Fig. 1A). CD70 was selected for further analysis because of the availability of a CD70 binding receptor (CD27)-engineered CAR-T construct and its recent success in a mouse model of another type of solid tumor in brain [13]. CD70 mRNA was overexpressed more than five-fold in HNSCC compared to controls (Fig. 1A). CD70 overexpression was limited to certain cancer subtypes, including larynx, oral cavity, and tongue (Fig. 1B). This data were clearly limited by sample size but CD70 overexpression was not detected in hard palate, hypopharynx, lip, and oropharynx tumors. In addition, substantial heterogeneity was observed in CD70 expression in 22 individual cancer cases compared to their respective adjacent normal tissues (Fig. 1C).

Heterogeneous expression of CD70 in head and neck cancers

CD70 was previously thought to be expressed exclusively in activated T and B lymphocytes and natural killer cells. However, overexpression of CD70 has been reported in several tumor types, such as kidney [12], brain [13, 14], lung [15], B cell lymphoma [16], and head and neck [17]. We analyzed seven available head and neck cancer cell lines for CD70 expression. Of these, OQ01 showed the highest mRNA expression, above RS4 and HOS cells that are known to highly express CD70 (Fig. 2A). CAL27 also showed significantly elevated CD70 expression compared to K562, known to be CD70-negative. In order to verify that CD70 is overexpressed on the surface of head and neck cancer cells, we stained these cancer cell lines with anti-CD70 mAb. Flow cytometry analysis clearly demonstrated surface CD70 expression in OQ01, CAL27, and RPMI 2650, but not in HN, BHY, FaDu, and SCC-25 (Fig. 2B). As expected, positive controls RS4 and HOS showed high expression of both CD70 mRNA and protein levels compared to the negative control cell, K562. CD70 mRNA levels generally correlated with cell surface expression, except in SCC-25. We measured comparable CD70 mRNA expression in SCC-25 and RPMI2650, yet SCC-25 showed little or no cell surface expression (Fig. 2A and 2B).

For preclinical validation of CD70 surface expression, we stained patient FFPE tumor specimens with anti-CD70 antibody. Representative immunofluorescence images are shown

in Fig. 3. Cytoplasmic, membranous, and dot-like staining were also observed as previously reported [17]. CD70 staining was determined by the reference pathologist (KMF) as positive or negative. EGFR antibody was used as a positive control (Fig. 3, lower right panel) and showed strong expression evenly distributed on the tumor cell surface; negative controls included staining without primary antibody (Fig. 3, lower middle panel) and using unrelated anti-giantin antibody (Fig. 3, lower left panel). Immunofluorescent staining of CD70 demonstrated that 4 of 21 tumor biopsies showed strong expression of CD70 and 3 of those 4 showed expression detectable on the tumor cell surface. A previous report also indicated that CD70 expression was not detectable in 52 normal tissue types from different organs [13]. Taken together, these data suggest that CD70 is a viable CAR-T target for a subset of head and neck cancers with little or no off-tumor toxicity.

CD70 CAR-T cells effectively eliminate head and neck tumor cells *in vitro*

It is noteworthy that human CD70 CAR-T cells successfully eradicated CD70-positive renal cell carcinoma after adoptive transfer into a NOD/SCID xenograft model [12]. Another recent study demonstrated that human and mouse CD70 CAR-T cells were capable of crossing the blood-brain barrier in both xenograft and syngeneic mouse glioma models, leading to complete regression with little or no toxicity observed [13]. Thus, we decided to explore whether CD70 CAR-T cells were effective in killing HNSCC *in vitro*. We generated human CD70 CAR-T cells with the same CD70 CAR construct [13] using healthy PBMCs from three different donors, in order to verify their effector functions. A bioluminescence assay showed that co-culture of CD70 CAR-T cells with OQ01.Luc or CAL27.Luc led to significant killing of the Luc-expressing cells compared to co-culture with non-transduced T cells (Fig. 4A). CD70 CAR-T cells derived from different donors showed similar trends in killing target cells, but also showed some variations. Donor #1 CAR-T cells killed targets in an effector-to-target ratio-dependent manner, and levels of secreted cytokine IFN- γ gradually increased up to 4 days later (Fig. 4B). Donor #2 CAR-T cells had similar capacity to kill target cells as donor #1 with similar secretion of IFN- γ . However, donor #2 CAR-T cells also showed potential non-specific killing that might be mediated by non-transduced cells. Donor #3 CD70 CAR-T cells were capable of killing target cells at low effector-to-target ratios. IFN- γ was greatly increased on Day 1 compared to other donors; however, it slowly declined on Day 4.

Discussion

Given the substantial clinical success of CD19 CAR-T cell therapy, the U.S. Food and Drug Administration recently approved it for the treatment of B-cell acute lymphoblastic leukemia and non-Hodgkin's lymphoma. For hematological malignancies, uniquely expressed cell surface molecules on lymphocytes, like B cell maturation antigen [18], CD22 [19], and CD19 [3, 5, 20], were investigated as specific CAR-T targets. Ideally, mutated or uniquely expressed antigens, as well as overexpressed tumor cell surface molecules, are suitable candidate targets for CAR-T therapy. However, identifying candidate targets in solid tumors has been more challenging than in blood cancers. Considering the increasing incidence of recurrent and metastatic head and neck cancers annually, and the lack of new treatments

beyond chemotherapy and radiation, we realized that identification of novel targets for CAR-T therapy would be critical to improving outcomes for head and neck cancer patients.

Here, we identified nine candidate CAR-T target antigens for head and neck cancers using data from the TCGA database. Among these candidates, CD70 was of particular interest, given the successful work already done in experimental mouse models of glioma [13]. CD70 antigen was found to be highly overexpressed in larynx, oral cavity, and oral tongue subsets and showed heterogeneous expression in individual patients. For example, individual cancer cases 2, 9, 17, and 22 showed greater than 100-fold higher expression in cancers compared to controls (Fig. 1C). Yet, other cases (e.g. 5, 6, 7, 14, 19, and 20) showed essentially no difference in CD70 expression between tumor and control tissue. These data show that CD70 is clearly not an appropriate CAR-T target for all HNSCC. However, for the subset of 10-20% HNSCC with high CD70 expression, CAR-T therapy may be a suitable and attractive option.

Our data showed some variability in the capacity of CD70 CAR-T cells derived from three different PBMC donors to kill CD70-positive cancer cells. There are a few possible reasons for this variability and further technical optimization may be needed to overcome this issue. First, the transduction efficiency of activated T lymphocytes by the viral CAR construct might vary between donors. Based on our experience, as low as 20% transduction efficiency may be enough to generate sufficient CD70 CAR-T cell activity and death of target cells. Thus, ensuring high transduction efficiency could improve effective tumor target killing. A second factor might be variability in the CD4⁺/CD8⁺ T cell ratio in different PBMC donors. However, it was previously shown that the majority of CD70 CAR transduced T cells were CD8⁺ T cells and that the CD4⁺/CD8⁺ T cell ratio had negligible effect on this issue [13]. Third, some donor PBMCs appeared more responsive to IL-2 than others during activation of T cells [21]. PBMCs from donor #2 had an increased response to IL-2 stimulation compared to other donors. Thus, activated T cells from this donor might require a longer resting time prior to assaying for tumor cell killing. This is supported by our data showing that non-transduced T cells in donor #2 were highly active and non-specifically kill tumor cells (Fig. 4A). These variables are clearly important to address and further work is needed to optimize the assay protocol.

Conclusion

Together with the recent report that intravenous administration of CD70-specific CAR-T cells was effective in the treatment of gliomas in mice [13], our current data provide a promising new target for the treatment of a subset of HNSCC patients. While we acknowledge that CD70 CAR-T may only be effective for the 10-20% of HNSCC patients with high CD70 expression, CD70 CAR-T therapy could be a useful component in a repertoire of therapies in the coming age of personalized medicine. Investigations of the efficacy of other candidate antigens, as well as the killing capacity of CD70 CAR-T cells in mouse models of HNSCC, are ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HNSCC	head and neck squamous cell carcinoma
CAR	chimeric antigen receptor
Luc	luciferase
mAb	monoclonal antibody
NT	non-transduced T cells
PBMCs	peripheral blood mononuclear cells
TCGA	The Cancer Genome Atlas

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Highlights

- Identified nine candidate CAR-T targets for head and neck cancers from The Cancer Genome Atlas based on expression data in tumors versus flanking control tissues.
- Since CD70 has been reported as a successful CAR-T target in other cancers, the expression of cell surface CD70 was experimentally validated in selected head and neck cancer cell lines and tumor biopsies.
- CD70 specific CAR-T cells were generated and demonstrated effective in killing of CD70 positive oral tumor cell lines *in vitro* and thus holding promise for future *in vivo* validation.

Fig. 1.

Analysis of candidate CAR-T targets, especially CD70, in head and neck cancers. (A) Analysis of The Cancer Genome Atlas database identified nine cell surface protein candidate CAR-T targets with significantly elevated expression in 303 tumors (Ca) compared to 37 adjacent tissue controls (Ctrl). (B) The CD70 mRNA expression data were further analyzed by tumor types and CD70 was significantly elevated in several tumor subtypes (T) compared to controls (N). Note that the normal controls for base of tongue and floor of mouth tumors only included two samples and hence not showing statistical differences to corresponding tumors. (C) CD70 mRNA was heterogeneously overexpressed in 22 individual comparisons of head and neck tumors (black bars, Ca) vs. adjacent normal tissues (white bars, Ctrl). All results are presented as mean $\pm$ standard error. Statistical significance was determined by the Mann-Whitney *U* test (** $P < 0.05$, *** $P < 0.0005$).

Fig. 2.

Heterogeneity of CD70 mRNA and protein overexpression in several representative head and neck cancer cell lines. (A) qRT-PCR results are expressed as mean $\pm$ SEM from total six experiments. Statistical significance was determined by one-way ANOVA multiple comparison test comparing to K562 (***, $P < 0.0005$, **, $P < 0.005$, *, $P < 0.05$; ns, not significant). (B) Cell surface CD70 protein expression in seven head and neck cancer cell lines was determined by flow cytometry. Gray-filled histograms represent signal without antibody, while red-line histograms show staining with PE-conjugated anti-CD70 mAb. Data were collected from at least two independent experiments. RS4 and HOS cells were used as high CD70 expression controls and K562 as a negative control.

Fig. 3.

CD70 expression in a representative patient tumor tissue positive for CD70. A HNSCC tumor section was stained with H&E (upper left panel) and adjacent serial section co-stained with anti-CD70 (red, upper middle panel; shown with DAPI counterstain, upper right panel), and an unrelated anti-giantin (green, lower left panel; arrows show Golgi staining). Arrows in enlarged inset show staining consistent with cell surface expression. Other sections were also stained without anti-CD70 (negative control, lower middle panel) or with anti-EGFR (red, positive control, lower right panel). All panels are shown at 200× original magnification. Scale bar: 20 µm.

Fig. 4.

Demonstration of *in vitro* killing of two head and neck cancer cell lines by CD70 CAR-T cells. (A) CD70-positive luciferase-expressing OQ01 (OQ01.Luc) and CAL27 (CAL27.Luc) cells were co-cultured with either CD70 CAR-T cells or non-transduced T cells (NT) at indicated effector-to-target (E:T) ratios for 24 hours. Specific killing activity of CD70 CAR-T cells was measured by bioluminescence assay. Cultures were set up in duplicate and mean $\pm$ error are shown. Similar results were obtained in independent experiments from three healthy T cell donors. (B) IFN- γ secretion of CD70 CAR-T cells in co-culture supernatants after 1 and 4 days was measured by ELISA. Cultures were set up in triplicate and mean $\pm$ SEM are shown. Statistical significance was determined by Student's unpaired *t* test (**P*<0.05; ****P*<0.005).