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Targeting CD276: a promising strategy for CAR-NK cell immunotherapy in human oral tongue squamous cell carcinoma

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Abstract

Human oral tongue squamous cell carcinoma (OTSCC) is a prevalent form of head and neck squamous cell carcinoma (HNSCC), often presenting at an advanced stage with a grim prognosis. Traditional therapeutic approaches such as surgery, adjuvant radiotherapy, and chemoradiotherapy have shown limited efficacy in treating advanced OTSCC, underscoring the urgent need for innovative treatment strategies. Our bioinformatics analysis identified CD276 as a significant biomarker in OTSCC, with high protein expression levels correlating to a markedly reduced survival rate in late-stage patients. This discovery has led us to develop chimeric antigen receptor-natural killer (CAR-NK) cells derived from umbilical cord blood cells (UCBCs), specifically targeting CD276. Our aim is to investigate this novel therapeutic approach for its potential to combat OTSCC under pre-clinical conditions. Our in vitro and ex vivo studies have demonstrated that CD276-targeted CAR-NK cells exhibit remarkable efficacy in lysing OTSCC cell lines and primary cells, as well as in eliminating OTSCC organoids. These promising results underscore the pivotal role of CD276 in OTSCC pathogenesis and highlight the potential of CAR-NK cell therapy as a groundbreaking treatment option for advanced-stage OTSCC, offering new hope for translational medicine in the field of stomatology.

keyword Oral squamous cell carcinoma, CD276, CAR-NK

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Introduction

Due to the lack of mature early-stage detection strategy in clinic and obvious characteristics on the lesions, the human oral tongue squamous cell carcinoma (OTSCC) was often diagnosed at an advanced-stage with a poor prognosis of the patient [1].

As a malignant tumor occurring in the tongue and one of the most common head and neck squamous cell carcinoma (HNSCC), OTSCC has features including high rate of relapses, lymphatic metastasis, invasion of deep lingual muscle and adjacent artery or nerve [1, 2]. In recent years, the incidence of OTSCC continues to rise, and the age of onset shows an earlier trend [3, 4]. However, the traditional methods such as surgery, adjuvant radiotherapy and chemoradiotherapy presented limited effects on improving the quality of patients' life, that the drug resistance occurred and 5-year survival ratio was 50% [5–7]. Finding new approaches for the OTSCC treatment is still an urgency in clinic.

A novel method such as chimeric antigen receptor (CAR)-T cell therapy, provides a more precise and safer approach against cancer [8]. However, this method demonstrated remarkable clinical success in hematologic cancers but less progress in solid tumors. It may be partly due to the unique microenvironment of the solid tumor, such as limited cell trafficking and infiltration in tumors [9]. Recently, CAR modified Natural Killer (CAR-NK) cell therapy was presented as an alternative to CAR-T method for solid tumors. Unlike T cells, NK cells do not need human leukocyte antigen (HLA) for the compatibility when attacking cancer cells, which means that CAR-NK cells could have a faster immune response in patient body and be applicable with the allogeneic sources [9]. Moreover, a previous study of CD19 targeting CAR-NK therapy had reported the 73% of positive results in patients without any serious adverse event (AE), such as cytokine release syndrome, graft-versus-host disease and neurotoxicity [10].

Selecting an efficient and available target is the key for the application of CAR-NK therapy. Thus, we screened the database corresponding to OTSCC and chose CD276 (a.k.a. B7-H3) as the targeted protein to the CAR. CD276 is a glycoprotein, which being located on the cell membrane and having low/normal expression on squamous cells. However, rising researches proved that CD276 could exhibit abnormal overexpression on some squamous cell associated cancers including lung squamous cell carcinoma (LUSC), esophageal squamous cell carcinoma (ESCC) and HNSCC [9, 11, 12]. Our previous work also demonstrated that CD276 had strong overexpression in OTSCC tissues, which indicating that CAR-NK cell therapy on targeting CD276 should be an available strategy against OTSCC.

In this research, we constructed CAR-NK cells targeting the biomarker CD276 to evaluate the feasibility of CAR-NK cell therapy on OTSCC. The resource of NK cells was umbilical cord blood cells (UCBCs), which had been considering as a potential candidate for future clinical application [13]. We measured the effectiveness of CD276-CAR-NK cells in multiple pre-clinical models and find its high efficiency on lysing TSCCa cell line, eradicating patient-specific primary cancer cells (PSPCs) and attacking patient-specific cancer organoids (PSOs). These results suggested that CD276-CAR-NK could be a promising immunotherapeutic option for OTSCC patients.

Materials & methods

Bioinformatics data sources

RNA-seq data and clinical information of TCGA-HNSC were analyzed based on the source of the TCGA database (https://portal.gdc.cancer.gov/), containing 32 normal tissue samples and 332 OSCC samples. 214 samples with complete survival information and clinical information were obtain in this study. GSE74530, GSE138206 and GSE160042 were sourced from the GEO database (https://www.ncbi.nlm.nih.gov/), contain four normal cells and OSCC.

Identification of differentially expressed genes (DEGs)

The data form GSE74530, GSE138206, GSE160042 and TCGA-HNSC were selected for identification of the DEGs datas through Limma package in R programming software. The$|Log2FC| > 0.5$ and multiple testing adjusted p value < 0.05 were used as threshold. The DEGs were displayed in the volcano map by ggplot2 package. The common DEGs between GSE74530, GSE138206, GSE160042 and TCGA-HNSC were selected by Venn tool.

Functional enrichment analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) enrichment analyses were applied to the differentially expressed genes (DEGs), utilizing a bioinformatics approach inspired by the Metascape algorithm. The GO terms and KEGG pathways at the significant level (p value < 0.05, adjusted by Benjamini and Hochberg method) were employed.

Cox regression analysis

Univariate Cox proportional hazards regression analysis was conducted on the gene expression profiles of patients with oral squamous cell carcinoma (OSCC), focusing on the expression levels of the shared differentially expressed genes (DEGs). A stringent p-value threshold of less than 0.01 was employed to ensure the robustness of the gene selection criteria, thereby enhancing the reliability of the prognostic gene candidates identified.

OTSCC cell lines

The OTSCC cell line TSCCa (ml097685, Mlbio, Shanghai, China) was cultured in RPMI-1640 (CM-0235, Procell, Wuhan, China) supplemented with 10% fetal bovine serum (FBS; 164210-50, Procell, Wuhan, China), 1% Penicillin and Streptomycin (P/S; PB180120, Procell, Wuhan, China). The cells were regularly cultured in a 37 °C incubator with 5% CO_2 and were routinely monitored for mycoplasma contamination.

Generation of PSOs and PSPCs

The tumor tissues were collected from the excised cancer samples in the surgery with full acknowledgments of the patients before treatment and the informed consent was approved by every participant. The tumor tissues were washed 3 times with washing buffer (provided by Guangdong Procapzoom Biosciences, Inc.) and then being cut into pieces in a diameter of proximately 200 μm in the medium. Followed, the pieces were digested with trypsin-EDTA (Gibco, USA) for about 15 min, being suspended in the medium and poured into a filter with the diameter of 100 μm. Subsequently, the tissues with size between 100 and 200 μm were selected out for generating patient specific organoid (PSO) of OTSCC. These PSOs were embedded in the GelMA gel (Engineering for Life, Suzhou, China) and seeded into 96-well plates for growth. The single cells passed through the filter were also collected, resuspended with medium and transferred into flasks, which had coated with gelatin (#48722, Sigma-Aldrich, Shanghai, China), for generating patient specific primary cell (PSPC) of OTSCC. Both PSOs and PSPCs were cultured in the 37 °C incubator with 5% CO2, and prepared for the examination of different NK cells killing efficiency.

Generation and construction of CD276-CAR-NK from UCBCs

The purification and generation of NK cells from the UCBCs were performed following previously established protocols [14, 15]. briefly, UCBCs were collected from the umbilical cord blood using the Density Separation Solution Kit (CS006, Baso, China). After conducting the density gradient centrifugation method, the UCBCs were isolated and then cultured following the datasheet of the Frozen NK cell Culture Reagent Kit (T20214U4, Baso, China) with the FNK-Medium. The time points of medium refresh and regent change were referenced the protocols in the previous studies [14, 15]. Afterwards, the mature NK cells were collected in the serum free medium for lentivirus transduction, following the procedures from a previous study [14]. NK cells were suspended at a density of 5×10^5 cells/ml, 3 ml per well in 12-well plates with lentivirus (MOI = 10) containing the CD276 CAR sequence and polybrene (final concentration = 8 μg/ml). After the transduction, the NK cells were of static culture for an additional 24 h and isolated with a flow cytometer employing the antibody, which recognizing the scFv sequence of the CD276-CAR (customized from Cusabio, China). The resulting CD276-CAR-NK cells were then identified by the biomarkers including CD3, CD34, CD16, CD45 (ab16669, ab81289, ab183354 and ab40763, Abcam, Cambridge, UK) and CD56 (362508, BioLegend, USA) with the corresponding antibodies.

Immunohistochemistry

Before the generation of PSOs and PSPCs, A portion of tissue was isolated from the tumor, being fixed with 4% paraformaldehyde, for the preparation of immunohistochemistry (IHC). The fixed tissue underwent dehydration, paraffin embedding, slicing, rehydrated and 3% hydrogen peroxide treatment. Then the tissue sections were blocked with 10% BSA (SRE0096, Sigma, USA) for 1 h at the room temperature. Subsequently, primary antibody against CD276 (11188-T24, SinoBiological, China) was employed to incubation overnight at 4 °C, for detecting the protein expression. On the next day, anti-rabbit IgG (PV-9000, ZSGB-BIO, China) was used as a secondary antibody, and a DAB staining kit (D405772, Aladdin, China) was utilized for the color development.

Cell viability assay

PSPCs or TSCCa cells were cultured in a 96-well plate at a density of 1×10^4 cells per well in advance, and then being treated with different kinds of NK (Mock-CAR-NK or CD276-CAR-NK) cells for 24 h co-culturing. While PSPCs or TSCCa cells in blank control group were only treated with NK-culturing medium at the same volume. Afterwards, the NK cells and medium were washed out and the CCK-8 solution (C0039, Beyotime Biotechnology, Shanghai, China) was added into the plate for 10 μL in each well, following the procedures described in the datasheet. After the two hours of incubation at 37 °C, the absorbance of each well was detected with a Microplate Reader PT-3502B (Potenov, Beijing, China) at OD = 450 nm.

Monitoring CD276-CAR-NK cells Lysis efficiency by non-invasive cell analyzer

The real-time measurements of CD276-CAR-NK cells killing OTSCC cells were performed with the xCELLigence RTCA instrument (Agilent, Santa Clara, USA), and the normalized data was regarded as the CD276-NK cells lysis efficiency during the monitoring period. Before the measurement, TSCCa or PSPC cells were suspended at the concentration of 1×10^5 cells/mL and seeded in an E-Plate 16 (Agilent, Santa Clara, USA) for 100 μL in each well. Being static cultured overnight, the cells were divided as experimental group and control group.

CD276-CAR-NK or Mork-CAR-NK cells were added into the wells of experimental group at the E: T ratio of 3: 1, while the control group was added with NK-culturing medium at the same volume. Impedance was tracked every 15 min continuously for 24 h at least. The monitored cell index in each group was normalized to that in the control group over the entire timeline.

Statistical analysis

The software Prism GraphPad 8 (GraphPad Software Inc, San Diego, USA) was employed to analyze the quantified data with One-way ANOVA or two-way ANOVA method for comparing mean values of single variate or multiple variables among different groups, respectively. The "P-values < 0.05" were deemed as the significant difference statistically.

Results

Joint screening strategy on CD276 as a potential target against OTSCC

From GEO database, 1855, 630 and 1935 DEGs were screened in GSE74530, GSE138206 and GSE160042, respectively. Meanwhile, 219 common DEGs were selected from the database (Fig. 1A). Cox regression analysis from TCGA database shows that 64 differentially expressed genes were associated with prognosis. We used the R software package Survival to integrate data on survival time, survival status, and 64 features, and evaluated the prognostic significance of these features in 214 samples using the Cox method (Fig. 1B). Kaplan-Meier curve developed by bioinformatic analysis with the database institute database [16] showed a strong correlation between CD276 and poor prognosis in advanced OTSCC subclass. The overall prognosis difference is significant, as shown in log-test = 0.001314, sc-test = 0.000616, wald-test = 0.060122, with a C-index of 0.824088 (Fig. 1C). Enrichment analysis was performed on the 64 common DEGs. The significantly enriched GO terms and KEGG pathways were shown on Fig. 1D and E. The results demonstrated that the 64 common DEGs were significantly enriched in signal transduction and protein recognition. These genes are enriched in pathways related to metabolism and signal transduction (Fig. 1D and E).

Successful generation of PSPCs and CD276-CAR-NK cells

The detailed procedure of PSPCs derivation from human OTSCC tissues and CD276-CAR-NK cell construction were described in the method section. As shown in Fig. 2A, CD276 had positive expression in the OTSCC tissue but negative expression in the normal tissue (Fig. 2A and Supplementary Fig. 2). Moreover, CD276 presented the clear staining location on the membrane of the OTSCC cells (Fig. 2A), which supported the feasibility of CD276-CAR-NK cells recognizing OTSCC through CD276. Accordingly, the PSPCs derived from OTSCC tissue demonstrated the same positive expression of CD276 with flow cytometry detection (Fig. 2B). For the CD276-CAR-NK construction, the detailed information was also included in the method section. The transduction of CD276-CAR into the UCBCs derived NK (UCBC-NK) cells did not induce their morphology changes, being spherical and growing in cluster or not (Fig. 2C). The biomarkers of the mature CD276-CAR-NK cells presented as the negative expression of CD34 and CD3, while positive expression of CD45, CD16, CD56 and the CD276-CAR (Fig. 2D).

Efficacy of CD276-CAR-NK cells against human OTSCC cell line and PSPCs

We used both human OTSCC cell line, TSCCa, and the PSPCs from the clinical patients to evaluated the lysis efficiency of the CD276-CAR-NK cells. The co-culturing of CD276-CAR-NK and TSCCa cells in a dose-dependent manner had been performed, in which the ratios of effector cells versus targeting cells (E: T) were queued from 1: 10, to 30: 1 (Fig. 3A). Correspondingly, the residual cell viabilities of TSCCa cells after 6 h of co-culturing were listed from 93.7% in the 1: 10 group to 9.4% in the 30: 1 group, in gradient (Fig. 3A). The dose-dependent detection of CAR-NK and TSCCa cells co-culturing demonstrated a similar trend (Fig. 3B). Considering the PSPCs had more tolerance than the cell line, the cell viability detection had been postponed to 24 h. As the result, the CD276-CAR-NK cells still showed efficient lysis ability at the ratio of 3: 1, comparing with the NK constructed non-relevant CAR (Mock-CAR-NK) cells (Fig. 3B). The time-dependent curve of NK and TSCCa cells co-culturing was illustrated as Fig. 3C. At the E: T ratio of 3: 1, the CD276-CAR-NK cells could lyse 67.0% TSCCa cells within 12 h and restrict the growth of remained cancer cells, while the NK cells with mock or without CAR failed to suppress the TSCCa cell growth, according to the RTCA assay result (Fig. 3C). The CCK8 assay result at 24 h also exhibited the difference in capacity between CD276-CAR-NK and Mock-CAR-NK cells, being consist with the RTCA result (Fig. 3D). For the PSPCs time-dependent curve in 36 h monitoring, the CD276-CAR-NK cells at the E: T ratio of 10: 1 had successfully lysed 45.2% PSPCs within 12 h and continually lysed next 36.3% (81.5% in total), presenting a higher efficiency than the Mock-CAR-NK cells (Fig. 3E).

CD276-CAR-NK cells rapidly killed PSO of OTSCC

To mimic the CD276-CAR-NK cells killing human OTSCC cells at the ex vivo condition, we made effort to generated PSOs using the three-dimensional culturing method with PSPCs. Then we assessed the efficacy of CD276-CAR-NK cells on killing the OTSCC tumor by

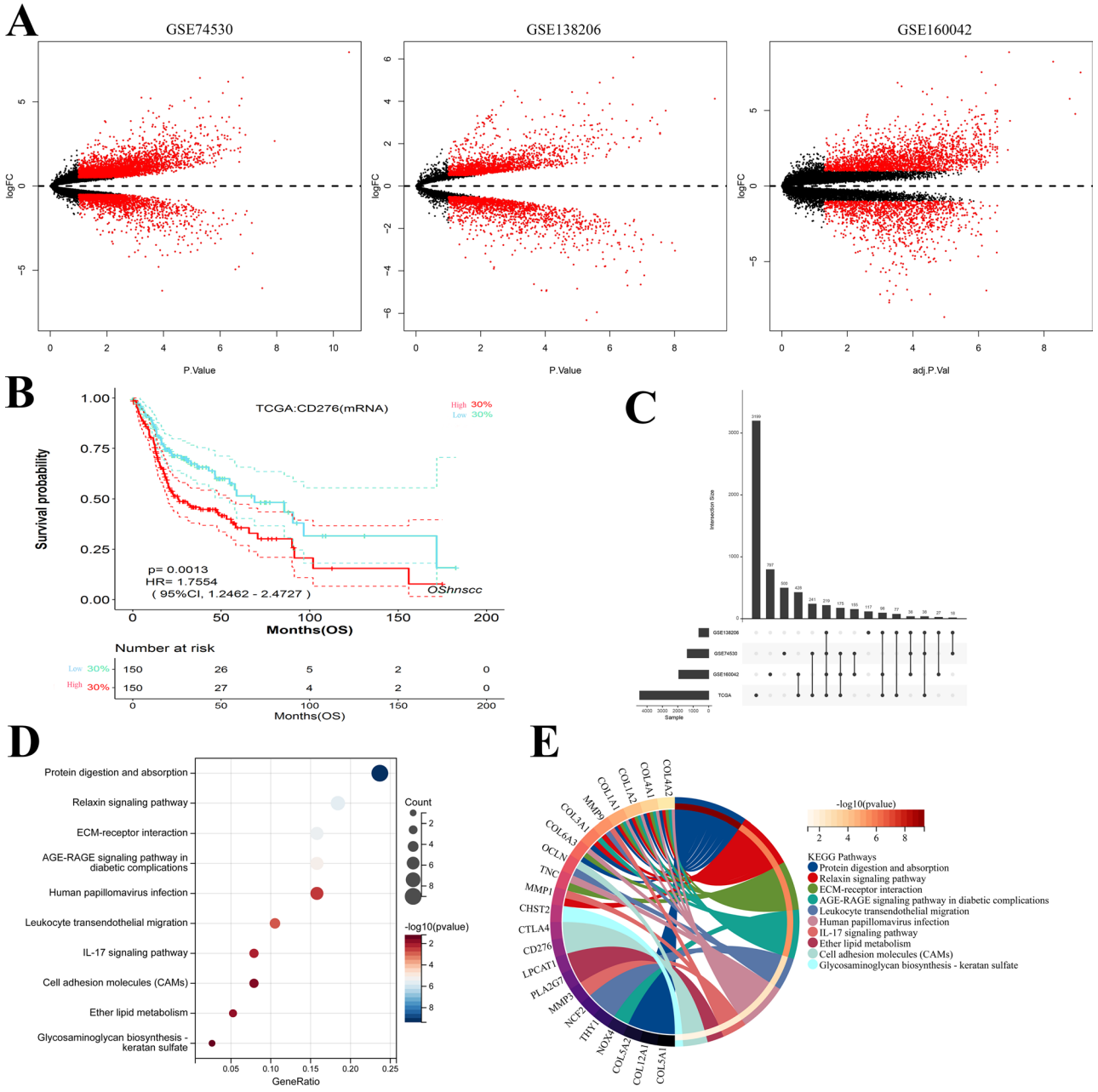

Fig. 1 Results of differential analysis and enrichment analysis. (**A**) The volcano plot of DEGs in different groups. (**B**) The Kaplan–Meier survival curve in TCGA-HNSC. The x-axis is time and y-axis is the survival probability. The red is the High-CD276 group and the blue is the Low-CD276 group. p value is calculated by the log-rank test. (**C**) The figure containing all DEGs. (**D**), (**E**) The significantly enriched KEGG pathways. The x-axis is the number of enriched genes and y-axis is the names of the KEGG pathways

regarding the PSOs as the small tumor tissues of OTSCC. The killing process was monitored by microscope with green (for living cells) and red (for dead cells) fluorescence. After 12 h, the PSO in CD276-CAR-NK group had been almost totally killed, shown as a red organoid comparing to the other three groups (Fig. 4A). The quantification data of green and red fluorescence was illustrated as Fig. 4B. We calculated the killing efficiency of NK cells by converting the fluorescence quantification result into lysis ratio, that CD276-CAR-NK cells lysed 77.2% cells in the OTSCC PSO while the NK cells without CAR and Mock-CAR-NK cells had lysed cells in PSOs were 0.0% and 12.5%, respectively (Fig. 4C).

Discussion

The cohort of patients with CD276 positive OTSCC should not be neglected due to their survival probability dramatically decrease at the late stage (Fig. 1B). Aiming

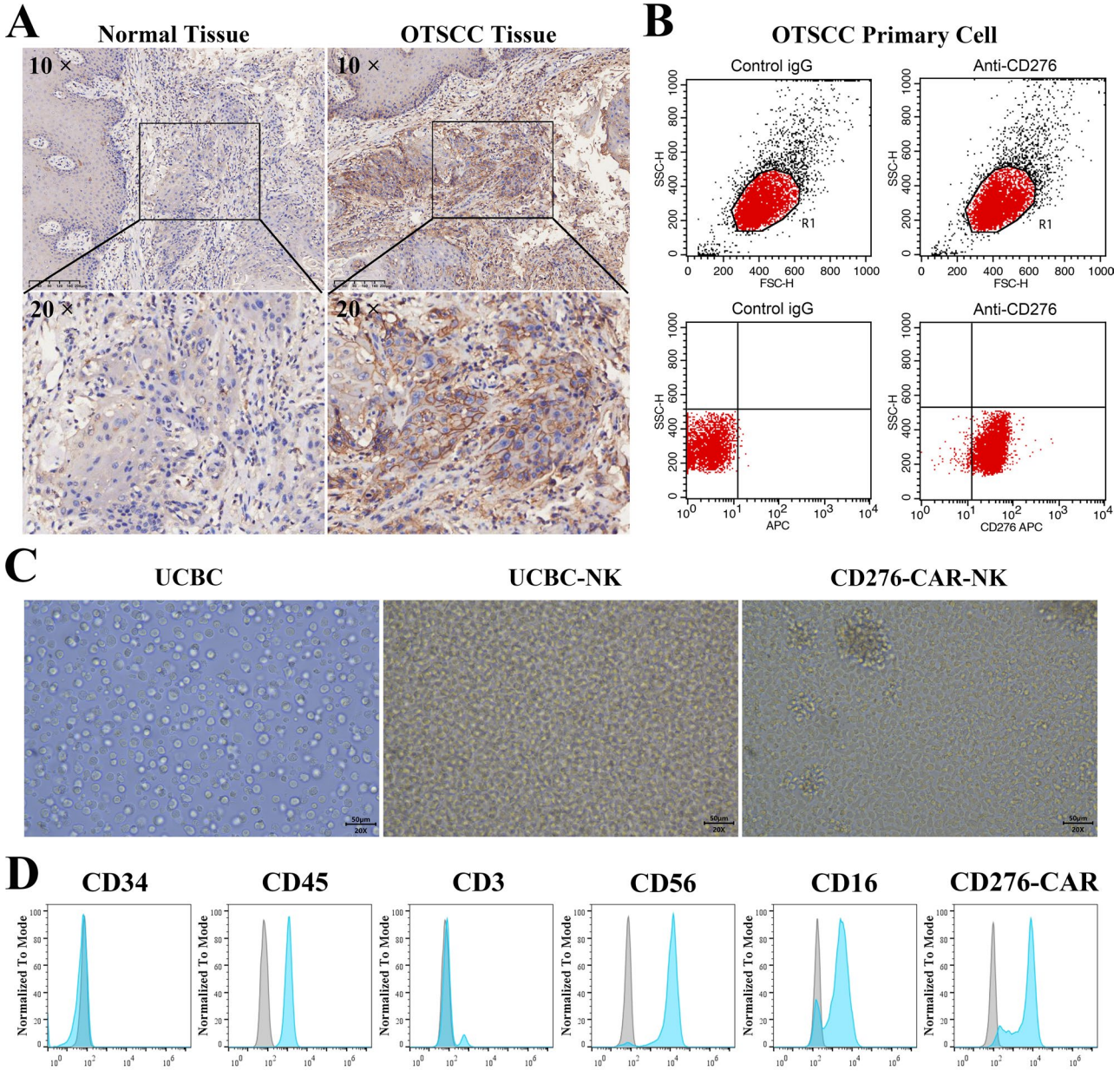

Fig. 2 Features of CD276 expression on OTSCC and identification of CD276-CAR-NK cells. (**A**) IHC of normal tissues and OTSCC tumor tissues. The strong positive of CD276 expression on cell membrane in the 10× region was enlarged as 20× image. (**B**) CD276 positively expressed in PSPCs of the OTSCC. (**C**) Morphology comparison of UCBCs (right panel), UCBC-NK cells (mid panel) and CD276-CAR-NK cells (left panel) under bright field, in which the radius being UCBCs > UCBC-NK cells = CD276-CAR-NK cells. (**D**) Biomarkers determination of CD276-CAR-NK cells by flow cytometry. For the biomarkers CD34, CD45, CD3, CD56 and CD16, negative controls were used irrelevant IgG replacing primary antibody for incubating, presented as gray peaks. For the CD276-CAR detection, negative control was used UCBC-NK without CAR incubating with anti-CAR antibody, presented as gray peak. Samples in detection were presented as blue peaks

at this group, the immuno-cell-therapy targeting CD276 revealed its potential on human OTSCC treatment, based on its efficiency and security. We successfully constructed the CD276-CAR-NK cells utilizing the UCBCs, which being credited with lower risk and scarce rejection to the host [13]. The previous study had demonstrated that CD276 was little expressed even unexpressed in the human major organs [17]. Moreover, the application of CD276-CAR-T cells in human clinically had reported the safety of the CD276-CAR [18]. Based on this, we have confidence to investigate the translational way of CD276-CAR-NK on oral clinic medicine continually.

Based on our investigation result, we found that CD276-CAR-NK cells exhibited the high efficiency on lysis OTSCC cell line and PSPCs, and it correlated to the dose-dependent manner (Fig. 3). An interesting finding

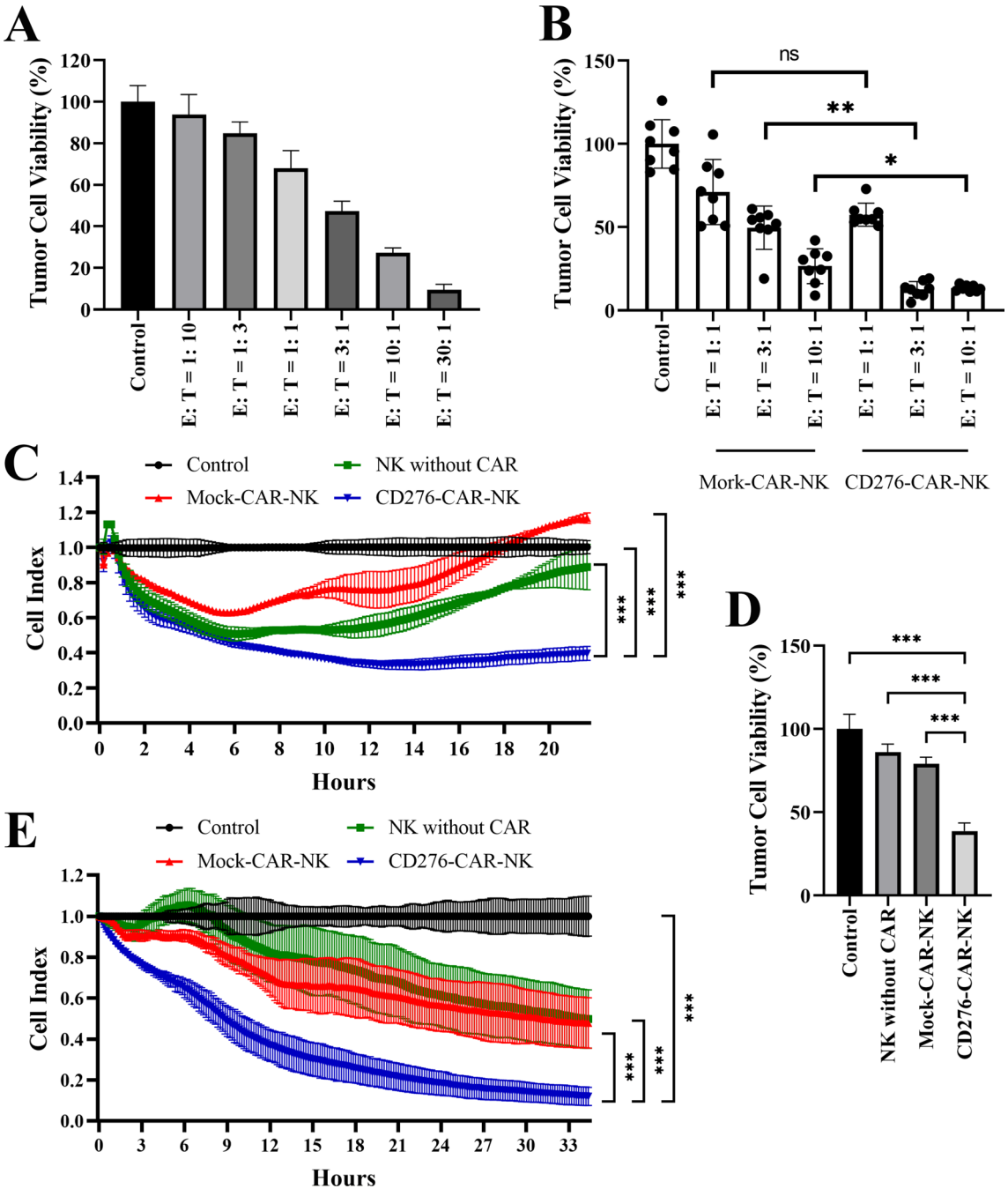

Fig. 3 CD276-CAR-NK lyse OTSCC cell line and primary cancer cells. (**A**) Rate of TSCCa cell lysis by CD276-CAR-NK cells with different E-T ratio. E: number of effective cells, T: number of target cells. (**B**) Rate of PSPCs of OTSCC lysis by CD276-CAR-NK and Mock-CAR-NK cells with different E-T ratio. (**C**) Time curve of different NK cells, including Mock-CAR-NK (Red), NK (green), CD276-CAR-NK (Blue) and control (medium, black), lysing TSCCa cells. (**D**) CCK-8 assay result of co-culturing TSCCa cells with Mock-CAR-NK and CD276-CAR-NK cells. (**E**) Time curve of Mock-CAR-NK (Red), CD276-CAR-NK (Blue) and control (medium, black), lysing PSPCs of OTSCC. The difference with a value of $p < 0.05$ was considered significant and presented as "*", followed, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$

was that the residual OTSCC cells would regrow up if the lysis (at the E: T ratio of 3: 1) was incompletely finished (Fig. 3C). We considered that it should be one of the reasons for explaining why OTSCC being easier to relapses in clinic, due to the limit capability of immunity system inside of the patients. On the other hand, CD276-CAR-NK cells had potential to inhibit the reproliferating of OTSCC cells based on their high-efficient recognition reaction (Fig. 3C). Moreover, raising the quantities of the CD276-CAR-NK cells in treatment (at the E: T ratio of

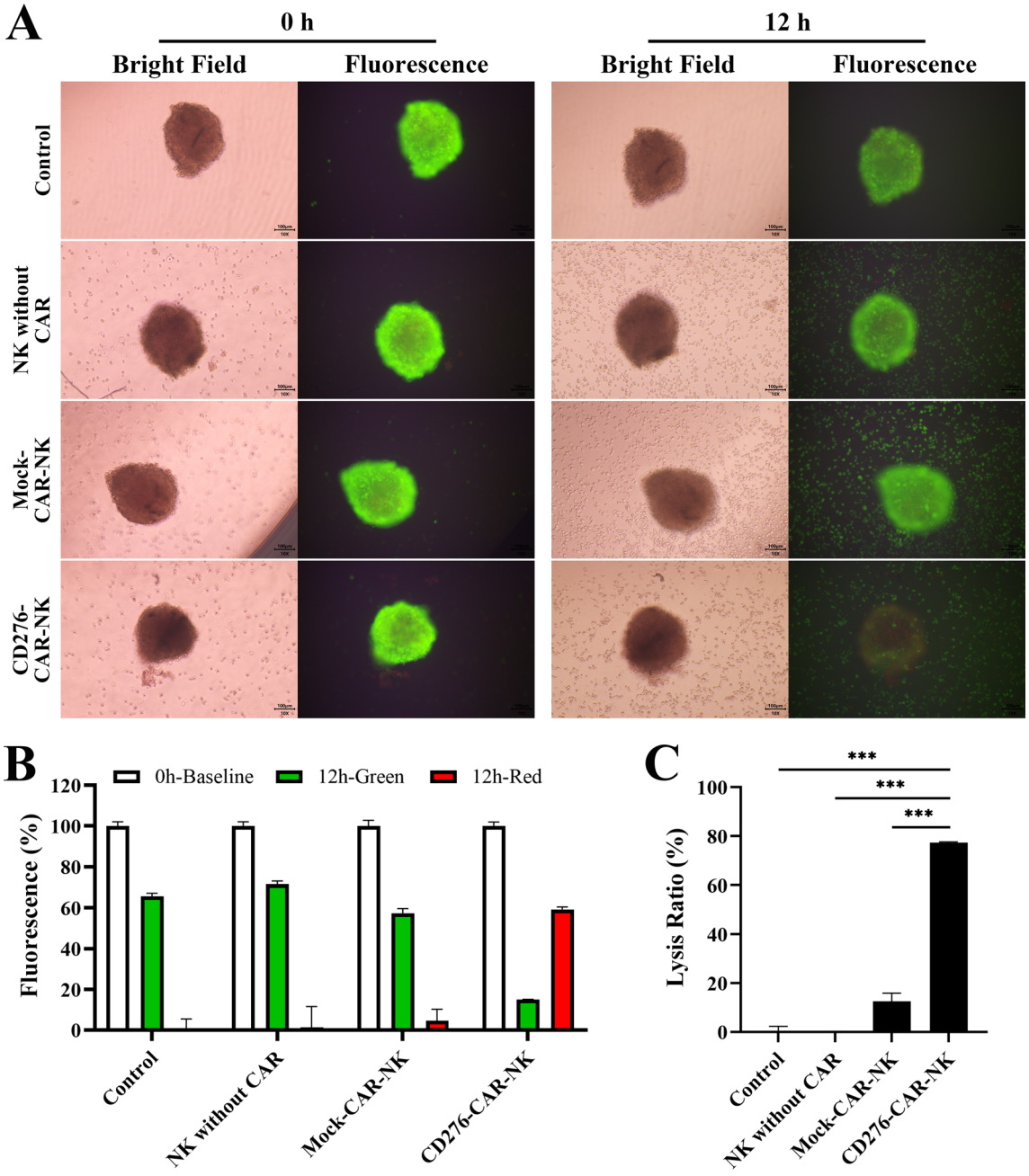

Fig. 4 Detection of CD276-CAR-NK lysing OTSCC organoids. **(A)** bright field and fluorescent images of organoids after treatment with CD276-CAR-NK cells, Mock-CAR-NK cells, NK cells or medium as control. Organoids presented as lager spherical form with green fluorescence, NK cells presented as smaller spherical form around the Organoid. The photographs were taken at the beginning of hour 0 to the lysis finishing of hour 12. **(B)** Quantification of fluorescence intensity of organoids, indicating the cells viability in organoids. **(C)** Calculation of lysis ratio of organoid after treatment with different NK cells

10: 1) was helpful in eliminating the OTSCC cells totally, preventing its regrowth (Fig. 3E).

We did not choose CDX mice model as the in vivo experimental object in consideration of the inappropriate growing environment of OTSCC cells in the groin of mice. Instead, we generated the OTSCC-PSO from the tumor tissues of the patients to observe the process of CAR-NK cells killing OTSCC organoids straightly, in which the organoid being regarded as another valuable approach to translational study recently [19–21]. The PSOs were stained by fluorescence (green light for living cells and red light for the dead) in advance and then co-cultured with different kind of NK or CAR-NK cells for 12 h. From the photographs in bright field, we could observe that CD276-CAR-NK cells had clustered, being contacting and attacking the OTSCC-PSO (Fig. 4A).

Meanwhile, the photographs in fluorescent field showed that the OTSCC-PSO the in CD276-CAR-NK group was mostly in red, indicating that the OTSCC-PSO basically being killed by the CD276-CAR-NK cells (Fig. 4A).

The NK cells differentiated from UCBCs without CAR or with non-relevant CAR were used as the control groups in the experiments. As for the morphology of the UCBC-NK cells, we actually observed multiple kinds of cell states with different stimulating conditions. That the differentiation process with co-culturing of IKT as the feeding cells would bring UCBC-NK cells to a rapid proliferation status without cluster formation, otherwise, the process without IKT cells would initiate a slow proliferation of UCBC-NK cells in cluster formation (Supplementary Fig. 1). While, there was inadequate evidence to prove the significant disparity of lysis efficiency on cells between the two kinds of statuses.

Originally, we aimed to find a promising target for the CAR-NK therapy against OTSCC. CD271, which had been reported on neuroblastoma [22], breast cancer [23], melanoma [24] and prostate cancer [25], can be regard as a novel target too. However, Due to the lack of evidence that CD271 had been easily constructed on CAR-NK or CAR-T cells, we finally chose CD276, which had been reported successfully constructed and applied on CD276-CAR-T cells [26, 27], as the feasible CAR to the CAR-NK cells. These data of comparison were to prove that CD276-CAR-NK would be a promising strategy against OTSCC but not to prove that CD276-CAR-NK was the best strategy among all the CAR-NK or CAR-T therapies. On the other hand, we are very willing to explore the potential of CD271 to the OTSCC in our future work.

In conclusion, we identified CD276 as a novel potential target in human OTSCC, and successfully constructed NK cells, resourced from umbilical cord blood, with CD276-CAR. Both in vitro and ex vivo experiment results confirmed the efficacy of the CD276-CAR-NK cells, which provided a decent approach to the OTSCC management in clinic for the future.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-13973-9.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 2

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Not applicable.

Author contributions
Conceptualization: SL, TG, FT, WL, HZ, XZ; Data curation: SL, TG, FT, HZ, YY, JH, PZ, WH; Formal analysis: JH, PZ, WH, TW, HL; Funding acquisition: DC, WL, HZ, XZ; Investigation: SL, TG, TF, SC, PD, ZD, DC; Methodology: JH, PZ, HL, TF, SC; Project administration: SL, TG,; Software: TG, PZ, TW; Resources: SL, HZ, XZ; Supervision: PD, ZD, WH,; Writing– original draft: SL, TG; Writing– review & editing: WL, HZ, XZ, SL, TG, FT. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The experimental protocols involving human biological samples were reviewed and approved by the Institutional Review Board (IRB) of Xuchang Central Hospital (Approval No. HREC-2023-10-002). This study strictly complied with the ethical principles set forth in the Declaration of Helsinki (2013 revision) and the Chinese National Health Commission's Ethical Guidelines for Biomedical Research Involving Human Subjects (2023 version). Written informed consent was obtained from all participants after detailed explanation of the research purpose, specimen usage, and potential risks.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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