A novel nitidine chloride nanoparticle overcomes the stemness of CD133 + EPCAM + Huh7 Hepatocellular Carcinoma Cells for liver cancer therapy

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Research Article

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Abstract

Background: Stemness of $CD133^{+}EPCAM^{+}$ hepatocellular carcinoma cells ensures cancer resistance to apoptosis ,which is a challenge to current liver cancer treatments. Here we discovered that a novel nitidine chloride nanoparticle (TPGS-FA/NC, TPGS-FA: folic acid modified D-α-tocopheryl polyethylene glycol 1000 succinate , NC: nitidine chloride) targeted Huh7 human hepatocellular carcinoma and promoted its apoptosis in mice and cells. CD133 expression regulates AQP3 expression, promoting to increase the stemness properties of hepatoma cells. Importantly, AQP3 is association with stimulation and nuclear translocation of STAT3 with a increasing expression level of CD133 .

Methods: Cell viability was assessed by MTT and colony assays.TPGS-FA/NC nanoparticles were assayed by using confocal microscopy targeting Huh7 Hepatocellular Carcinoma Cells . A sphere culture technique was used to enrich cancer stem cells (CSC) and sort the $CD133^{+}EPCAM^{+}$ Huh 7 cells by magnetic-activated cell sorting assay. The proteins were examined by immunohistochemistry and western blotting assay.

Result: TPGS-FA/NC nanoparticles reduced the $CD133^{+}EPCAM^{+}$ Huh7 cells numbers in vitro. Furthermore, its time-dependently suppressed the AQP3/CD133/STAT3/JAK signaling pathways. Nitidine chloride nanoparticles therapy prevented and treated hepatocellular carcinoma in mice without adverse effects.

Conclusions: TPGS-FA/NC is shown to a promising and safe drug against liver cancer therapy via AQP3/STAT3/CD133 axis.

Background

Multiple drugs have been used broadly in liver cancer therapy, but their water-insolubility and toxicity have raised serious concerns [**1,2**]. Nitidine chloride has been developed in the past two decades due to its promise pharmacological action. Nitidine chloridepromises therapeutic efficiency but often faces challenges due to potential organ damage, hypersensitivity, and neurotoxicity**[3,4]**.

The cancer stem cells(CSC) are identified as stem cell properties,which revealed the existence of CSC in HCC [**5**,**6**] .$CD133^{+}EpCAM^{+}$phenotype precisely represented the characteristics of CSC in Huh7 cells [**7**–**11**] . Currently some chemotherapeutic drugs primarily inhibit the growth of differentiated tumor cells with no impact on CSC[12-**13**] . Cancer stem cells (CSCs) maintain the stemness to ensure their survival and growth, and becoming resistant to current treatments[**14-16**] . The intrinsic pathway of $CD133^{+}$Huh7 cells is regulated by the AQP3proteinin the progression and metastasis of several malignant tumors[**17-20**] . Furthermore, Nek2is the criticalregulatorofthe centrosome ,makinghepatocellular carcinomamoreresistanceto current treatments[21].

Based on the understanding thathepatocellular carcinomacontain functionalAQP3andNek2is mutated or highly expressed , these survival mechanisms can be overcome by the pharmacological action

ofAQP3/STAT3/CD133 pathway degradation and Nek2 inhibition. Furthermore, Our findings investigate the combination of direct AQP3/STAT3/CD133 pathway and Nek2 protein inhibition as a novel nitidine chloride nanoparticle therapeutic strategy that can promote cancer stem cells apoptosis in hepatocellular carcinoma.we addressed the nitidine chloride nanoparticle therapeutic potential in vitro and in vivo

Methods

Materials

TPGS-FA/NC was synthesized in our laboratory and dissolved in DMSO. DMEM was purchased from Life Technologies (AB & Invitrogen)(Gibco, Suzhou, China). Fetal bovine serum (FBS) was purchased from Gemini (Gemini Calabasas, CA, USA). Huh7 was purchased from Procell Life Science & Technology Co. Ltd. on July 11, 2019 (identification number: CL-0120, Wuhani, China).Recombinant human bFGF (bFGF) and MTT were purchased from Beijing Solarbio Science & Technology Co., Ltd.(Solarbio,Beijing, China) and recombinant human 1epidermal growth factor (EGF) were acquired from Shanghai Yuanye Biotechnology Corporation (Yuanye ,Shanghai, China). were purchased from Procell Science&Technology Co.,Ltd.(Procell,Wuhan, China) .B27 (×50) were purchased from Thermofish Scientific(Thermofish ,waltham, USA). DMEM/F-12, insulin-Transferrin-Selenium (ITS×100), L-glutamine (×100) were purchased from Procell Science&Technology Co.,Ltd.(Procell,Wuhan, China). Anti-CD133 (AC133)-phycoerythrin (PE) and anti-CD326 (EpCAM)-allophycocyanin (APC) antibodies and isotype-matched mouse anti-IgG1-PE and anti-IgG1-APC were purchased from MiltenyiBiotec (North Rhine-Westphalia, Germany). Anti-phosphoSTAT3 (Tyr705), STAT3, JAK1, JAK2, AQP3, EpCAM , NEK2 were purchased from the Beijing Solarbio Science & Technology Co., Ltd. (Solarbio ,Beijing, China). Anti-CD133 and anti-GAPDH were purchased from the Procell Science&Technology Co.,Ltd.(Procell,Wuhan, China).Anti-rabbit secondary antibodies were purchased from Thermo Fisher Scientific Science&Technology Co.,Ltd.(Thermo Fisher Scientific, Shanghai, China). DAPI was obtained from Shanghai Beyotime Biotechnology Co. Ltd. (Beyotime ,Shanghai, China). TheiFluor TM 647 phalloidin iFluor™were purchased from Yeasen Biotechnology Co., Ltd(Yeasen,Shanghai, China). 5-fluorouracil (5-Fu) was purchased from MedChemExpress (MCE, Monmouth Junction, NJ, USA).

Cell culture

Huh7 cells were cultured in DMEM 10% FBS containing 10% FBS , 100 U/mL penicillin, and 50 mg/mL streptomycin at 37 °C in a humidified 5% CO2 incubator.

Tumor sphere formation assay and flow cytometric analysis

Primary sphere cells were obtained by culturing Huh7 cells in sphere-forming conditioned DMEM/F12,supplemented with FGF (20 ng/mL), EGF (20 ng/mL), B27 (1×), and L-glutamine (1×) in 6-well ultra-low attachment plates The primary sphere cells (1×10^3cells/well) were incubated with or without TPGS-FA/NC for 7d. The second and third passages of the cells were grown for 7 d in the absence of TPGS-FA/NC. To examine TPGS-FA/NC effects on the subpopulation of cells that expressed

EpCAM and CD133, cells were incubated with anti-AC133-PE and anti-EpCAM-APC antibodies and analyzed by flow cytometry. Isotype-matched mouse anti-IgG1-PE and anti-IgG1-APC were used as controls.

Confocal microscopy imaging

Huh7 cells cells were seeded on glass cover-slips and cultured at 37 °C overnight.Rhodamine B isothiocyanate 540labeled TPGS-FA/NC were incubated with cells at a final concentration of 100 nM for 4h at 37 °C. After washing twice with PBS buffer, cells were fixed with 4% formaldehyde and washed again, followed by treatment with 0.1% Triton X-100 in PBS buffer for 5min and subsequent cytoskeleton staining with iFluor TM 647 phalloidin iFluor™ for 30 min at room temperature. containing DAPI for cell nucleus staining and assayed onLeicaSP8confocal microscope (LeicaCorp.).

Western blotting

Cells were lysed in RIPA lysis buffer with PMSF and protease in-hibitors. Total protein lysates were boiled with loading sample buffer containing 8% SDS-PAGE. Separated proteins were transferred onto PVDF membranes. PVDF membrane blots were blocked in 10% skimmed milk for 0.5-1h at room temperature, washed in Tris-buffered saline with Tween 20 (TBS-T) and incubated overnight at 4℃ with rabbit anti-phosphoSTAT3 (Tyr705), anti-STAT3, anti-JAK1, anti-JAK2, anti-AQP3 CD33, anti-GAPDH. Anti-rabbit IgG was used as the second antibody.

Immunohistochemistry (IHC)

AQP3/CD133/EPCAM/NEK2 expression was analyzed in paraffin-embedded specimens obtained from nude mice tumor tissue. Tissue sections were incubated with anti-AQP3 (1:100,Solarbio), anti-CD133 (1:100,Solarbio), anti-EPCAM (1:100,Solarbio), and anti-NEK2 (1:100,Solarbio) overnight at 4°C. Then, the sections were incubated with biotinylated goat anti-rabbit IgG as a secondary antibody (Zhongshan Kit, China) for 30 min at 37°C. The specimens were assessed three times.

In vivo biodistribution assay.

Rhodamine B isothiocyanatelabeled TPGS-FA/NC (2mg.kg^{-1} , NC per body weight) were systemically administered via the tail vein into Huh7 tumor bearing mice. PBS-injected mice were used as fluorescence negative controls. The whole-body imaging of mice was conducted at 8h using an IVIS system (XMRS) with excitation at 535 nm and emission at 694 nm. The mice were sacrificed at 8h post-injection by the inhalation of CO_2 followed by cervical dislocation, and major organs were collected and subjected to fluorescence imaging for the assessment of biodistribution profiles. The fluorescence imaging datas of average radiant efficiency ([ps^{-1}cm^{-2}sr^{-1}] [μWcm^{-2}]$^{-1}$) were quantitative by IVIS system (XMRS) program.

Magnetic-activated cell sorting assay.

Determine cell number,Centrifuge cell suspension at 300×g for 10 minutes , Aspirate supernatant completely. Resuspend cell pellet in300μLof buffer per 5×10⁷total cells. Add 100μL of FcR Blocking Reagent per 5×10⁷total cells and mix well. Add 100μL of EpCAM microbeads per 5×10⁷total cells. Mix well and incubate for 30minutes in the refrierator (2–8°C. Wash cells by adding 5–10mL of buffer per 5×10^7 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely and suspend up to 10^6cells in 500μL buffer, proceed to magnetic separation , EpCAM Huh7 cells were collected. Followed above methods, EpCAM Huh7 cells were sorted after CD133 microbeads incubation. $EpCAM^+$and $CD133^+$Huh7 cells were collected by magnetic separation.

In vivo tumor inhibition by TPGS-FA/NC nanoparticles.

Freshly sorted $CD133^+EpCAM^+$cells were collected in sterile DMEM without FBS. 200μL cell suspension, mixed with matrigel (BD Biosciences, CA) (1:1), was subcutaneously injected into each BALB/c nude mice,which were randomly divided into four groups (n=5 biologically independent animals). Samples were administrated by i.v. injection in a total of 5 doses (4mg kg^{-1} , NC per body weight) every other day. Tumor volume, calculated as (length×$width^2$)/2, and mouse weight were monitored every other day.Data were statistically analyzed by two-tailed unpaired t-test and presented as mean ± SD; *p < 0.05; **p < 0.01; ***p < 0.001.

Statistics. Statistical differences were evaluated using two-tailed unpaired t-test with GraphPad software, and statistically significant differences are denoted as *p< 0.05, **p<0.01, and ***p <0.001. No adjustments were made for multiple comparisons.

Results

TPGS-FA/NC inhibited cell proliferation and targeted the Huh7 cells

Huh7 cells (2×10^3cells/well) were seeded into 96-well plates and treated with TPGS-FA/NC (0–120 μg/mL) for 24, 48, and 72h (Fig.**1**). Cell proliferation was assessed using MTT in a concentration- and time-dependent manner.To evaluate nanoparticles targeting tumor capability, the RhodamineB isothiocyanate 540 fluorophore was attached to TPGS-FA. Confocal microscope imaging showed that TPGS-FA/NC nanoparticles entered the Huh7 cells in vitro, compared with the control groups (Fig. **2**).

TPGS-FA/NC reduced hepatic cancer stem-like cells

To investigate whether TPGS-FA/NC suppressed hepatic CSCs, we enriched the hepatic CSC populations in the Huh7 cell lines using the sphere culture technique. The flow cytometric analysis demonstrated that the EpCAM+/CD133+ cells accounted for 82.0% of the Huh7 sphere cells, respectively. TPGS-FA/NC (10、20 and 40μg/mL) potently reduced the fraction of EpCAM+/CD133+ cells (Fig. **3a**).

TPGS-FA/NC inhibited hepatoma cell proliferation and colony formation

HCC cells (1×10^3cells/well) were treated with or without TPGS-FA/NC in 6-well ultra-low attachment microplates and allowed to grow for 17 to 21 days. The TPGS-FA/NC treatment inhibited Huh7 cell proliferation and also markedly reduced the number of colonies in the clonogenic assays (Fig. **3b**).

TPGS-FA/NC suppressed the AQP3 /CD133/STAT pathways

TPGS-FA/NCreduced the protein expression levels of JAK1, JAK2, pY705-STAT3, STAT3 (Fig .**4**). Furthermore, TPGS-FA/NC reduced the AQP3 protein expression,which suppressed the expression of activated STAT3 (pY705-STAT3) (Fig. **4**). Then, in vivo experiment, we tested the CD133 and AQP3 expression levels in sections of nude mice subcutaneous tumors by IHC, results showed TPGS-FA/NC downregulated AQP3 and CD133 protein levels (Fig. **5**).

TPGS-FA/NC impaired NEK2/CD133/EpCAM signaling of HCC

The protein levels of NEk2,CD133 and EpCAM were determined in cells and nude nice treated with and without TPGS-FA/NC. TPGS-FA/NC successfully reduced protein expression levels of NEk2, CD133 and EpCAM in HCC. In vivo experiment, we tested the CD133 and AQP3 expression levels in sections of nude mice subcutaneous tumors by IHC, results showed TPGS-FA/NC downregulated NEk2, CD133 and EpCAM protein levels (Fig. **5**).

In vivo significant inhibition of tumor by TPGS-FA/NC nanoparticles

Tumor quantitative biodistribution and targeting of the TPGS-FA/NC were assessed , which were injected through the tail vein in vivo. Those images of mice 8h post-injection showed that the TPGS-FA/NC nanoparticles markly accumulated in tumor, with low or no accumulation in brain, heart,spleen. (Fig. 6a). Quantitative analysis of the organ images showed strongly tumor accumulation. (Fig. 6b). After injecting with TPGS-FA/NC at a dose of 4 mgkg −1 (NC per mouse weight) every 2 days for a total of five dosages, the results revealed a inhibitory capability in vivo as administration by tumor volumes, whereas control group (Fig. 6c). The specific tumor inhibition was further confirmed from the tumors harvested after 2-week post injections (Fig. 6d).Those nanoparticles were biocompatible , which showed no obvious organ toxicity over two-week post injections (Fig. 7).

Discussion

In this study, TPGS□D-α-tocopheryl polyethylene glycol 1000 succinate□is a very safe biocom-patible and safe agent that can efficiently for use as a drug solubilizer[**23**,**24**]**.** Previously, we reported the folic acid modified D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS-FA) as a potential carrier for controlled delivery [**25**].

Our studies suggest thatpopulations of hepatic cancer stem cells self-renewal capacities with expressed biomarkers (EpCAM and CD133). TPGS-FA/NC demonstrated markedly decreased the positive EpCAM/CD133 cell spheres population , and numbers and sizes .The fact that our studies in vivo reveal

atherapeutic effificacy with adose of 4 $mgkg^{-1}$ treatment for 14d significantly inhibited Huh7 xenograft tumor growth and overall a safe profile in organ , is noteworthy. CD133 represents Liver cancer stem cells (LCSCs) marker with promoting HCC proliferation and invasion[**26**-**28**].Previous evidence has shown that high expression levels of AOP3 exerted carcinogenic and therapy resistance with promoting hepatocellular carcinoma formation[**29**].Importantly, our work addresses a promising chemotherapeutic drug delivery by using theTPGS-FA with thecapabilities of nitidinechloride, tumor targeting for liver cancer therapy.

Conclusion

TPGS-FA/NC significantly inhibit Huh7 cellular proliferation and colony formation. TPGS-FA/NC suppressed the AQP3/CD133/STAT3 pathway with reducing expression of phosphorylation STAT3, its upstream factor (AQP3) and two downstream signaling molecules (JAK1 and JAK2) . In this study, Our findings demonstrate TPGS-FA/NC therapeutic effects in vitro and in vivo. The work offer preclinical proof-of-concept for the treatment of a new intravenously bioavailable AOP3 inhibitor, nitidine chloridenanoparticles, which may provide a broad therapeutic effect in liver cancer.

Declarations

Ethics Approval and Consent to Participate

This study was approved by Ethics Committee of Guangxi University for Chinese medicine, all procedures reporting in this study on the animals were carried in accordance with the ARRIVE guidelines, and the study was carried out in accordance with the relevant guidelines and regulations. Huh 7 human hepatocellular carcinoma line did not require ethics approval for their use. Informed consent was therefore not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during the present study are included in this article. supplementary data are present in the supplemental materials. Additional data related to this paper can be requested from the author (2742657328@qq. com).

Competing interests

The authors declare that they have no competing interests.

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Authors'contribution

D. L. and H.Z. and T.L. and F.D. designed the main manuscript and D.L. and Q.Z. and Y.Z. prepared the experiments and prepared figures 1-7. All authors reviewed the manuscript.

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Figures

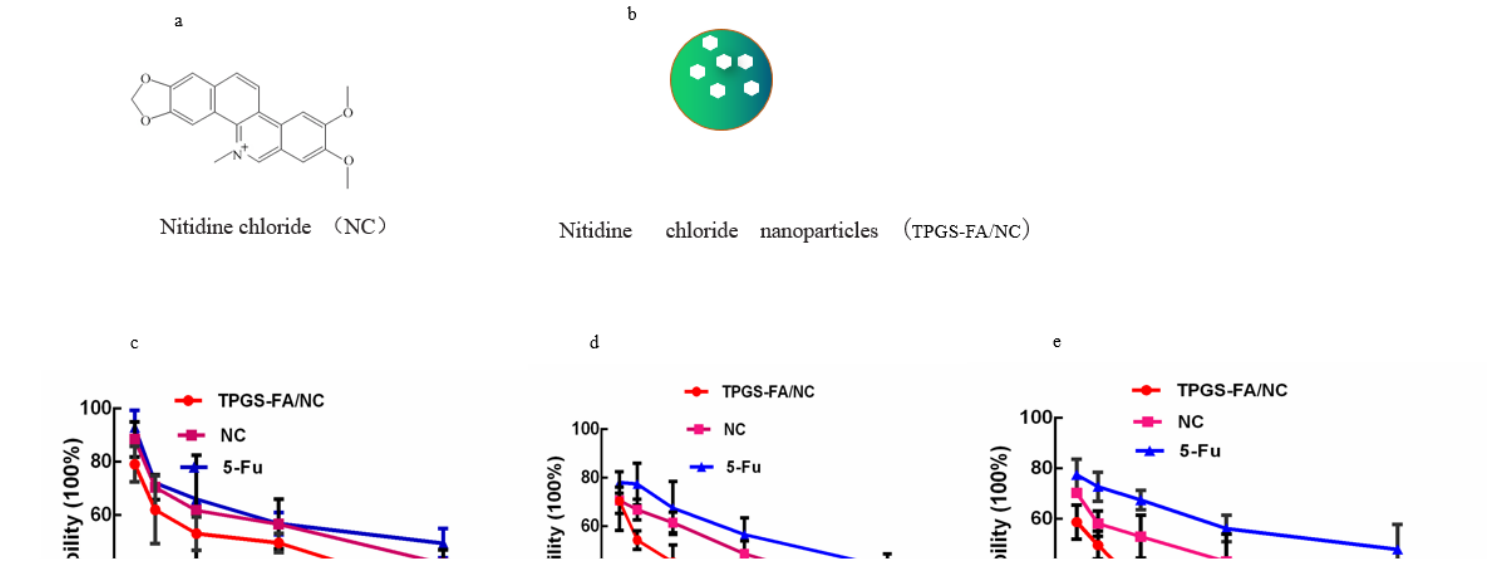


Figure 1

The effect of TPGS-FA/NC on Huh7 cell proliferation and colony formation. (a) Chemical structure of NC (b) TPGS-FA/NC nanoparticles. (c-e) TPGS-FA/NC inhibited Huh7 cell proliferation as determined by the MTT assay in 24, 48 and 72h. *n*=3. **P*<0.05 *vs* control.

Figure 2

In vitro Huh7 cells binding of TPGS-FA/NC nanoparticles, shown by confocal microscopy (blue: nucleus; green: cytoskeleton; red: TPGS-FA/NC nanoparticles. Scale bar: 50 μm for original images, and 10 μm for magnified image).

Figure 3

The effect of TPGS-FA/NC on hepatic cancer stem-like cells. a. TPGS-FA/NC reduced the population of EpCAM+/CD133+ cells in the spheres treated with TPGS-FA/NC for 48h. b. TPGS-FA/NC reduced the sizes Huh7 primary spheres (magnification,×400).

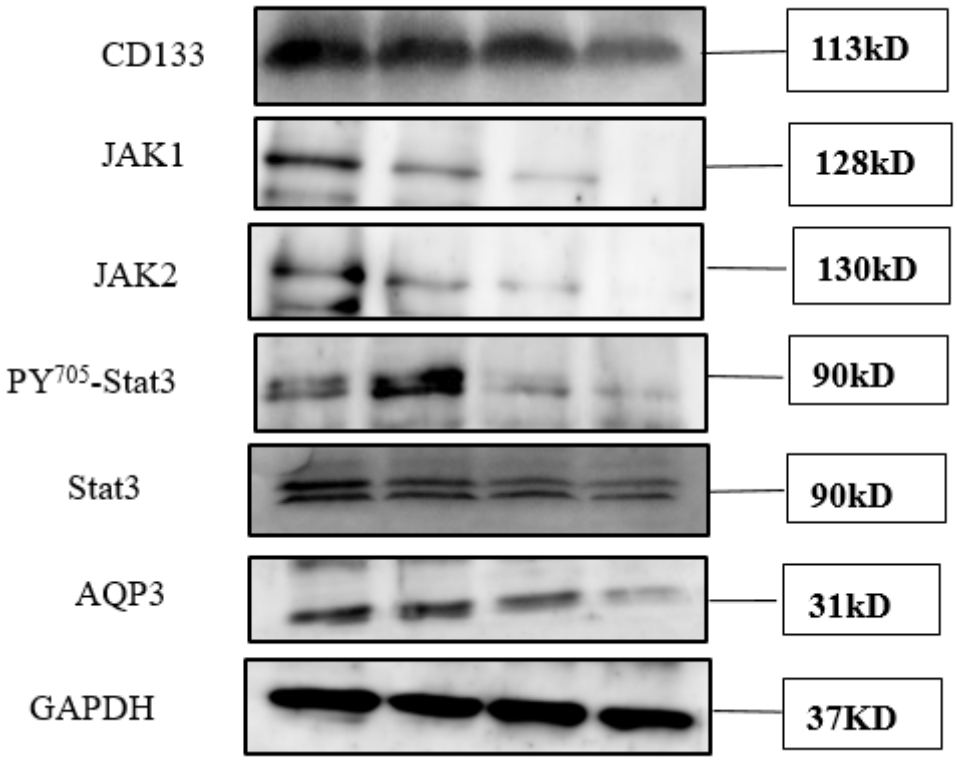
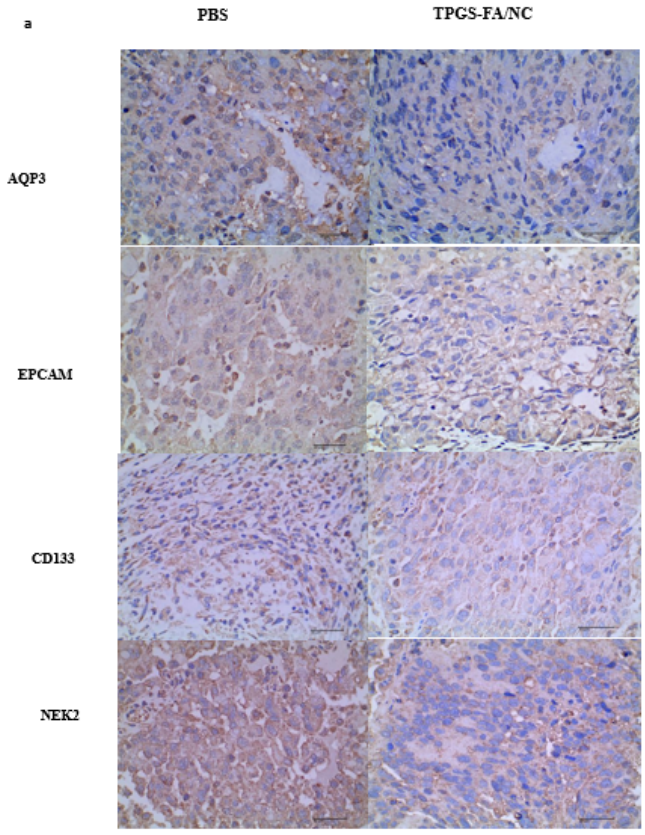


Figure 4

TPGS-FA/NC nanoparticles regulates CD133 and PY705-STAT3 protein by AQP3 proteins Expression of (DMSO、TPGS-FA/NC:10μg/mL、20μg/mL、40μg/mL)



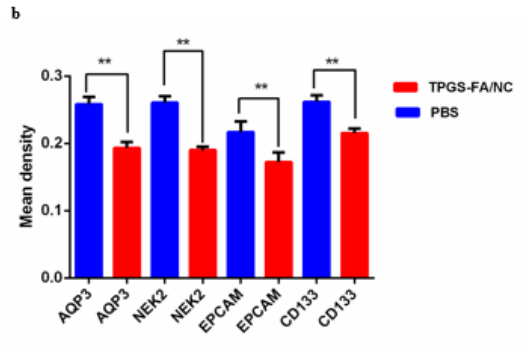


Figure 5

a. The protein of AQP3 , NEK2,EPCAM, and CD133 (×400, 40μm) were mainly located in cytomembrane according to IHC in Huh7 cells. b.The protein expression of AQP3 , NEK2,EPCAM, and CD133 (×200) were mainly located in cytomembrane according to IHC in 25 HCC mice bearing Huh7 xenograft specimens （**p < 0.01）

Figure 6

a. Representative organ images showing specific tumor targeting of rhodamine B isothiocyanate labeled TPGS-FA/NC nanoparticles 8 h post-injection into mice bearing Huh7 xenograft (T: tumor, Li: liver , H: heart, L: lung, K:kidney, S: spleen, and B:brain; Color scale: radiant efficiency, [p s^{-1} cm^{-2} sr^{-1}] [μWcm^{-2}]$^{-1}$). V b. Quantitative analysis of biodistribution in tumors and normal organs, quantified from the organ images. Intravenous treatment of nude mice bearing orthotopic Huh7 xenografts with TPGS-FA/NC nanoparticles (red) and control groups (turquoise: NC, fuchsia:5-Fu, blue: PBS) every other day for a total of five injections (4 mg kg−1,NC per body weight, indicated by arrows). c. Mice body weight was monitored during the time course of treatments (n =5 biologically independent animals, statistics was calculated by two-tailed unpaired t-test presented as mean ±SD, *p < 0.05, **p < 0.01, ***p<0.001 p = 4.3×10^{-3},3.4×10^{-3} and 5.0×10^{-4} comparing TPGS-FA/NC to NC ,5-Fu and PBS, respectively). d. Representative images of liver cancer tumors harvested from mice after treatments *p < 0.05, **p < 0.01, ***p<0.001; p=0.01, 8×10−4,and 2×10−4 comparing TPGS-FA/NC to NC, 5-Fu, and PBS, respectively. Source data are provided as a Source Data file.

Figure 7

HE stained in 20 HCC mice bearing Huh7 xenograft specimens

Supplementary Files

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- Supplementarymaterial.pdf