Engineered extracellular vesicles with polypeptide for targeted delivery of doxorubicin against EGFR-positive tumors

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Received April 7, 2024; Accepted September 13, 2024

DOI: 10.3892/or.2024.8813

Abstract. Lack of effective tumor-specific delivery systems remains an unmet clinical challenge for the employment of chemotherapy using cytotoxic drugs. Extracellular vesicles (EVs) have recently been investigated for their potential as an efficient drug-delivery platform, due to their good biodistribution, biocompatibility and low immunogenicity. In the present study, the formulation of GE11 peptide-modified EVs (GE11-EVs) loaded with doxorubicin (Dox-GE11-EVs), was developed to target epidermal growth factor receptor (EGFR)-positive tumor cells. The results obtained demonstrated that GE11-EVs exhibited highly efficient targeting and drug delivery to EGFR-positive tumor cells compared with non-modified EVs. Furthermore, treatment with Dox-GE11-EVs led to a significantly inhibition of cell proliferation and increased apoptosis of EGFR-positive tumor cells compared with Dox-EVs and free Dox treatments. In addition, it was observed that treatment with either free Dox or Dox-EVs exhibited a high level of cytotoxicity to normal cells, whereas treatment with Dox-GE11-EVs had only a limited effect on cell viability of normal cells. Taken together, the findings of the present study demonstrated that the engineered Dox-GE11-EVs can treat EGFR-positive tumors more accurately and have higher safety than traditional tumor therapies.

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Key words: engineered extracellular vesicles, EGFR-positive cancer, chemotherapy, targeted drug delivery, colorectal cancer, glioma

Introduction

Currently, chemotherapy remains the key approach in the treatment of cancer. However, off-target adverse effects often occur in healthy organs resulting from the lack of specificity of chemotherapeutic drugs (1). Classical chemotherapeutic drugs exert their effects through inducing DNA damage either directly or indirectly. However, the systemic administration of these agents has been shown to exhibit poor specificity and high toxicity, and to induce drug resistance, thereby restricting their clinical use (2). Therefore, novel approaches to enhance drug permeability and selectivity would improve the effectiveness of chemotherapeutics for patients with cancer.

Extracellular vesicles (EVs) are nano-sized membrane vesicles (40-160 nm in diameter) that are secreted by numerous cell types, such as human embryonic kidney cells (3). EVs are suitable as a promising platform for cancer treatment due to their low immunogenicity and toxicity compared with synthetic nano-formulations, such as polymers, dendrimers or liposomes (4). EVs may also be less immunogenic compared with other artificial delivery vehicles, as they can be derived from the patient's own cells. Another potential advantage of EVs is that their size is sufficiently small, thus they may evade phagocytosis via the mononuclear phagocyte system, which

facilitates their extravasation through tumor vessels and subsequent diffusion in tumor tissues (5). EVs are also promising vehicles for brain-drug delivery due to their ability to cross the blood-brain barrier (BBB) (6). Collectively, these attributes render EVs an ideal vehicle for delivering chemotherapeutic drugs in cancer therapy.

In numerous human tumors of epithelial origin, the expression of epidermal growth factor receptor (EGFR) is increased, and therefore EGFR could be a target for chemotherapeutic drug delivery (7). As the EGFR ligand, epidermal growth factor (EGF), is strongly mitogenic and neoangiogenic; an alternative ligand is required for cancer treatment (8). The GE11 peptide (amino-acid sequence YHWYGYTPQNVI), which binds specifically to EGFR and has markedly less mitogenicity compared with EGF (9), has been used in EGFR-targeted polymersomal and micellar drug delivery systems against tumors (10-12). Therefore, the GE11 peptide is likely to be superior to EGF in terms of targeting EGFR-positive tumors.

In the present study, 293T cells were engineered to stably express a well-characterized exosomal membrane protein CD63 fused with GE11 peptide. The results obtained showed that GE11-EVs could efficiently deliver doxorubicin to EGFR-positive tumors, which consequently inhibited cell proliferation and increased apoptosis of the EGFR-positive tumor cells. Furthermore, the data suggested that the Dox-GE11-EVs formulation has limited cytotoxicity towards normal cells. Taken together, this investigation has demonstrated that GE11-EVs is a potential drug-delivery vehicle for chemotherapeutic agents that have high tumor selectivity and are lowly toxic to normal tissues.

Materials and methods

Cell lines and cell culture. HCT116 (cat. no. CCL-247EMT), A172 (cat. no. CRL-1620) SW620 (cat. no. CCL-227), RKO (cat. no. CRL-2577) and U-87MG (cat. no. HTB-14) cell lines were obtained from the American Type Culture Collection (ATCC). NCM460 (cat. no. NCM460) were purchased from Guangzhou Xinyuan Biotechnology Co., Ltd. HA1800 (cat. no. JNO-H01443) cell lines were purchased from GuangZhou Jennio Biotech Co., Ltd. SF763 (cat. no. CL-0437), 293T (cat. no. CL-0005) and U251 (cat. no. CL-0237) cell lines were purchased from Wuhan Pricella Biotechnology Co., Ltd. HCT116, SF763, A172, SW620, RKO, U-87MG, U251, NCM460, HA1800 and 293T cells were cultured in either DMEM (Corning, Inc.) or RPMI-1640 medium (Corning, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1X penicillin/streptomycin (HyClone; Cytiva) and maintained at 37˚C in an atmosphere of 5% CO_2 in a humidified tissue-culture incubator. The U-87MG (https://web.expasy.org/cellosaurus/CVCL_0022) was certified by STR as an ATCC version, as detailed in the STR certification report.

Lentiviral production and infection. GE11 peptide was subcloned into the lentiviral plasmid. The lentiviral plasmids pLV-CMV-MCS-EGFP-3 x FLAG-IRES-Puro vector were used and obtained from OBiO Technology (Shanghai) Corp., Ltd. Lentivirus were produced in 293T cells using the third-generation lentiviral system (Guangzhou iGenebio Biotechnology Co., Ltd). A total of $1x10^6$ 293T cells were inoculated in 10-cm culture dish 24 h before transfection, 5 μg lentiviral construct, 5 μg lentiviral envelope and package plasmid (both from Shanghai Obio Technology Co., LTD.) were co-transfected into 293T cells (mixing ratio of lentiviral envelope and packaging plasmid: 1:1). After transfection for 24 h (37˚C, 5% CO_2), fresh medium was replaced. After 48 h, culture medium containing recombinant lentivirus was collected and filtered with a 0.45-μm filter. Then the cells were transfected with 5 MOI for 72 or 96 h and selected in medium containing 4 μg/ml puromycin for 1 week. Finally, cells were maintained in medium containing 0.1 μg/ml puromycin medium. Surviving pools were subjected to the indicated subsequent experiments.

Cell counting kit-8 (CCK-8) assay. The CCK-8 assay kit of Dojindo Molecular Technologies, Inc. was used to evaluate cell viability, and the manufacturer's protocol was precisely followed. Briefly, 96-well plates were seeded with 5,000 cells per well, which were allowed to adhere overnight. The cells were subsequently exposed to different concentrations of Dox, Dox-EVs or Dox-GE11-EVs for 24 or 48 h. Subsequently, DMEM containing 10% CCK-8 solution was added to each well, and the cells were incubated at 37˚C for 2 h. A microplate reader (Synergy H1; BioTeke Corporation) was used to evaluate the optical density of each well at a wavelength of 450 nm. The cytotoxicity of Dox, Dox-EVs or Dox-GE11-EVs to the cell lines was also assessed.

Polymerase chain reaction. RNA extraction was performed using TRIzol®, following the manufacturer's protocol (Thermo Fisher Scientific, Inc.). Subsequently, 2 μg of RNA was used to generate cDNA with oligo (dT) and RevertAid™ Reverse Transcriptase (Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. Subsequently, PCR amplification of the cDNA was performed to detect whether the transfection of the target plasmid was successful. For PCR, 2X Phanta Flash Master Mix (Dye Plus) (P520; Vazyme Biotech Co., Ltd.) was used. PCR conditions were as follows: first step, 95˚C for 5 min; second step, 95˚C 3 for 0 sec; 58˚C for 30 sec, and 72˚C for 1 min (30 cycles); third step, 72˚C for 5 min. The primer sequences used for PCR are provided in Table SI.

Western blotting. To extract protein from cells, M-PER buffer (Thermo Fisher Scientific, Inc.) containing 1X protease inhibitor cocktail (Topscience Biotech Co., Ltd.) was used, and the mixture was incubated on ice for 5 min. The concentration of extracted protein was quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). SDS-PAGE was performed on 20 μg of the extracted protein using either 10 or 15% gels, and the proteins were subsequently transferred to PVDF membranes (MilliporeSigma). The membranes were then blocked with 5% skimmed milk powder at room temperature for 1 h, followed by incubation with primary antibodies overnight at 4˚C. The primary antibodies included antibodies against CD63 (1:1,000; cat. no. 59286; Santa Cruz Biotechnology, Inc.), GFP (1:1,000; cat. no. 2956; Cell Signaling Technology, Inc.), ALG-2-interacting protein X (Alix; 1:1,000; cat. no. 2171; Cell Signaling Technology, Inc.), GM130 (1:1,000; cat. no. 12480; Cell Signaling Technology,

Inc.), EGFR (1:1,000; cat. no. 4267; Cell Signaling Technology, Inc.), histone H2A.X (1:1,000; cat. no. 7631; Cell Signaling Technology, Inc.), phospho-histone H2A.X (1:1,000; cat. no. 9718; Cell Signaling Technology, Inc.), caspase-3 (1:1,000; cat. no. 9662; Cell Signaling Technology, Inc.), cleaved caspase-3 (1:1,000; cat. no. 9664; Cell Signaling Technology, Inc.), α-tubulin (1:10,000; cat. no. ARG65693; Arigo Biolaboratories Corp.), β-actin (1:10,000; cat. no. ARG65683; Arigo Biolaboratories Corp.) and GAPDH (1:10,000, cat. no. ARG65680; Biolaboratories Corp.). Goat anti-rabbit IgG (1:10,000; ARG65680; Arigo Biolaboratories Corp.) or goat anti-mouse IgG (1:10,000; ARG65680; Arigo Biolaboratories Corp.) were used for peroxidase-conjugated secondary antibodies at room temperature for 1 h. Western blotting images were captured using a ChemiDoc XRS$^+$ system (Bio-Rad Laboratories, Inc.). The gray value of the protein bands was analyzed by ImageJ software (version:1.53; National Institutes of Health).

Isolation and characterization of EVs from 293T-GE11 cells. Isolation of EVs was performed by culturing 293T-GE11 cells in serum-free medium at 37˚C for 72 h, followed by differential centrifugation to remove cell debris and large vesicles. The supernatant was first centrifuged at 300 x g for 10 min at 4˚C, then at 2,000 x g for 10 min and 10,000 x g for 30 min. EVs were collected after being sedimented for 75 min at a speed of 100,000 x g. The protein concentration of the EVs was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.), and they were subsequently resuspended in cold PBS. All procedures were performed at 4˚C. EVs were characterized using transmission electron microscopy (TEM; Philips Healthcare) and nanoparticle tracking analysis (NTA; Malvern Panalytical, Ltd.). The sample solution was added to the carbon-coated copper grids of TEM, fixed with 2% paraformaldehyde at 37˚C for 20 min, washed with PBS and placed in 1% glutaraldehyde for 5 min, then transferred to ultrapure water for 2 min, and placed in 1% uranol acetate solution for 5 min. Finally, the grids were dyed with uranyl acetate for 10 min, washed with distilled water, and then examined under 80 kV voltage after air drying. EVs were lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc.) containing 1X protease inhibitor cocktail (Topscience Biotech Co., Ltd.) for 5 min on ice. The expression levels of the exosomal proteins CD63 and Alix were subsequently determined using western blot analysis, as aforementioned.

Loading chemotherapeutic drug and loading capacity. In the present study, a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.) was used to determine the total protein content of EVs. To prepare Dox-loaded EVs, 150 μg/ml Dox (in PBS) and 150 μg/ml GE11-EVs (in PBS) were mixed, sonicated using a Scienta-IID sonic dismembrator (Scientz, https://www.scientz.com/index.php?m=search&scope=product&keyword=%20sonic%20dismembrator), and incubated at 37˚C for 1 h (13). The sonication conditions were as follows: 20% amplitude; and eight cycles of 4 sec pulse and 2 sec pause for each cycle, with a 3 min cooling period on ice. To ensure the integrity of the EV membranes, the solution was further incubated at 37˚C for 1 h. Subsequently, Dox-loaded EVs vesicles were obtained by centrifugation at 100,000 x g at 4˚C for 75 min, and free Dox was collected by washing with cold PBS. The concentration of non-loaded Dox in the supernatant was measured using high-performance liquid chromatography (HPLC; Agilent 1100; Agilent Technologies, Inc.) and Agilent 5 HC-C18 column (250x4.6 mm); the 10-μl supernatant was analyzed by chromatography at 25˚C. The mobile phase consisted of acetonitrile and ultrapure water containing 0.1% trifluoroacetic acid (TFA), and the flow rate was 1 ml/min. The concentration of unsupported Dox in the supernatant was determined by measuring the absorbance at 254 nm, and calculated according to the established Dox standard curve. The drug loading efficiency (LE) and encapsulation efficiency (EE) of Dox-GE11-EVs were determined using the following formulas: $LE=M_{Dox\text{-}loaded}/(M_{GE11\text{-}EVs} + M_{Dox\text{-}loaded})$, and $EE=M_{Dox\text{-}loaded}/M_{Dox\text{-}initial}$, where $M_{GE11\text{-}EVs}$ is the gross mass of GE11-EVs employed for the preparation of Dox-GE11-EVs, and $M_{Dox\text{-}loaded}$ is the mass of final loaded Dox in GE11-EVs, which was calculated by subtracting the amount of unloaded Dox in the supernatant from $M_{Dox\text{-}initial}$. Fluorescence co-localization of PKH67-labeled GE11-EVs with Dox was investigated using confocal laser scanning microscopy (CLSM) analysis, with a confocal laser scanning microscope (Leica SP8; Leica Microsystems GmbH). PKH67 labeling was performed by incubating GE11-EVs with 10 μM PKH67 (MINI67; Sigma-Aldrich; Merck KGaA) for 10 min at room temperature, and subsequently 2 ml of 1% BSA (w/v, in PBS) was added to stop the reaction, followed by washing with cold PBS and ultracentrifugation (100,000 x g for 75 min) to collect the PKH67-labeled GE11-EVs (14). The rhodamine B-labeled Dox was obtained from Xi'an ruixi Biological Technology Co., Ltd. The specific operation of Dox packaging was as follows: 10 mg doxorubicin was weighed out and dissolved in 2 ml of dimethyl fumarate (DMF), and rhodamine B-isothiocyanate (1.1 eq.) and triethylamine (3.0 eq.) were added to dissolve the mixture completely. The reaction was allowed to proceed at room temperature for 30 min, after which a large amount of ethanol: acetone (1:1 solution precipitation) was poured in. The dissolution precipitation was then repeated twice, followed by centrifugation to collect the product and vacuum drying to obtain RB-adriamycin.

Cellular uptake of the EVs/GE11-EVs. The HCT116, DLD1, RKO, SW620, SF763, A172, U-87MG and U251 cells were seeded in confocal dishes at a density of $2x10^5$ cells per dish. The GE11-EVs were then incubated at room temperature with 10 μM PKH67 for 10 min. Subsequently, the labeled EVs/GE11-EVs were incubated with the aforementioned cells at a concentration of 0.1 μg/μl for 24 h. Paraformaldehyde (PFA; 4%; cat. no. P0099; Beyotime Institute of Biotechnology) was subsequently added to the aforementioned cells for 20 min and permeabilized with 0.5% Triton-100 (in PBS; cat. no. T8787; Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. After counterstaining with TRITC-Phalloidin (cat. no. P1951; Sigma-Aldrich; Merck KGaA) for 60 min and Hoechst 33342 (cat. no. B2261; Sigma-Aldrich; Merck KGaA) for 20 min at room temperature, the cellular uptake of EVs/GE11-EVs was observed using confocal laser scanning microscopy (CLSM) (15,16), where EVs served as the negative control.

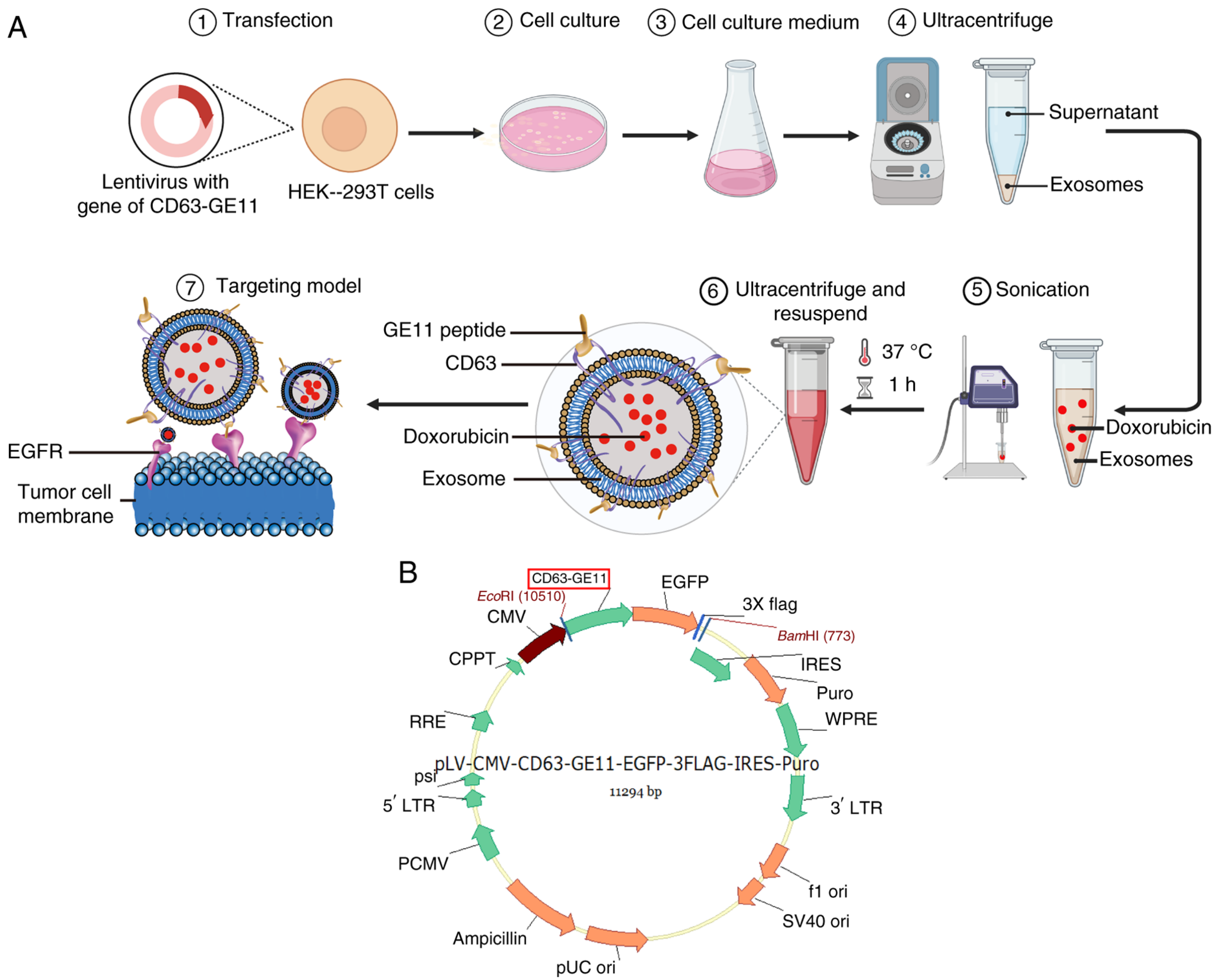

Figure 1. Extraction diagram of Dox-GE11-EVs and plasmid profile of CD63-GE11. (A) Diagram of preparation of Dox-GE11-EVs and the target model. (B) Construction of recombinant plasmid carrying CD63-GE11. Dox, doxorubicin; EVs, extracellular vesicles.

The cells (HCT116, DLD1, RKO, SW620, SF763, A172, U-87MG and U251) were seeded at a density of 3×10^5 cells per well in 6-well plates. At room temperature, the GE11-EVs were then incubated with 10 μM PKH26 (MINI26; Sigma-Aldrich; Merck KGaA) for 10 min. Subsequently, the labeled EVs/GE11-EVs were incubated with the aforementioned cells at a concentration of 0.1 μg/μl for 24 h. Finally, the uptake of EVs/GE11-EVs by the cells was evaluated using a flow cytometer (BD LSRFortessa™ X-20; BD Biosciences) and analyzed with FlowJo 10.0 software.

Terminal deoxynucleotidyl transferase mediated nick-end-labeling (TUNEL) assay. For TUNEL assays, 2×10^5 cells per well were seeded in 6-well plates for 24 h. Subsequently, cells were treated with PBS, Dox, Dox-EVs and Dox-GE11-EVs for 24 h. The cells were fixed in 4% PFA (cat. no. P0099; Beyotime Institute of Biotechnology) for 20 min, and permeabilized using 0.5% Triton-100 (in PBS; cat. no. T8787; Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. Thereafter, the extent of apoptosis was assessed using the Colorimetric TUNEL Apoptosis Assay Kit (cat. no. C1098; Beyotime Institute of Biotechnology), following to the manufacturer's protocol (17-20). Images were captured using an inverted fluorescence microscope (DMi8; Leica Microsystems) to observe apoptosis after the various treatments.

Quantification and statistical analysis. GraphPad Prism 8.0 (GraphPad Software, Inc.; Dotmatics) was used for all data analysis. Comparisons between different groups were evaluated using the Student's t-test (unpaired, for two groups) or one-way ANOVA, followed by Dunnett's post hoc test. All data are shown as the mean ± SD, and data values are presented of at least three biological replicates. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Establishment and characterization of 293T-GE11 cells. Surface engineering aims to increase the local concentration of EVs at the tumor site, thereby reducing toxicity and maximizing therapeutic efficacy. Considering that GE11 peptide specifically binds to EGFR, GE11 peptide was fused to the exosomal membrane localization protein CD63 in 293T cells via infection with lentiviral vector (Fig. 1A and B). The expression efficiency of CD63-GE11 was subsequently

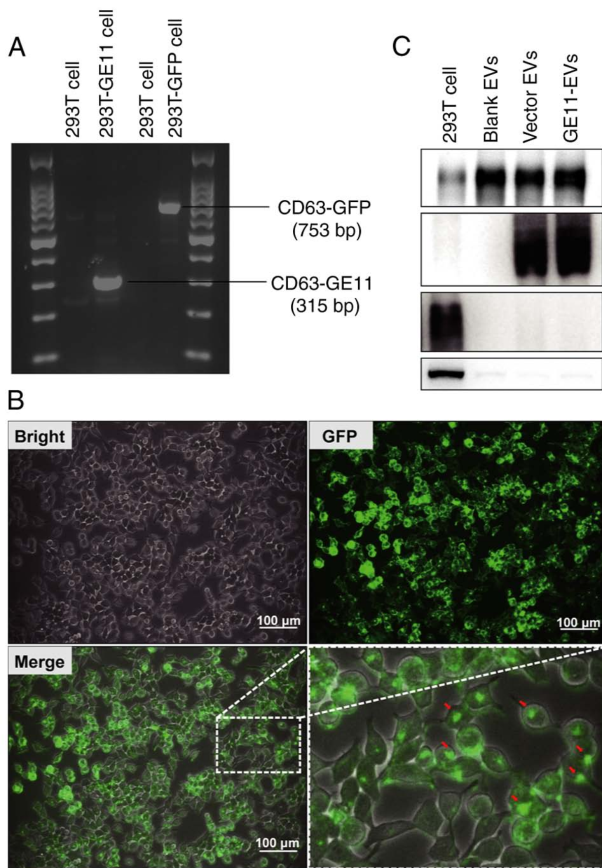

Figure 2. Establishment and characterization of HEK-293T-GE11 cells. (A) Nucleic Acid Gel Electrophoresis analysis of CD63-GFP fragment and CD63-GE11 fragment in 293T cells, and 293T-transfected cells. (B) Representative fluorescence images of GFP in 293T-GE11 cells. Scale bar, 100 μm. (C) Western blot analysis of CD63, GFP, GM130 and β-actin expression in 293T cells, blank EVs, EVs and GE11-EVs. EVs, extracellular vesicles.

verified using nucleic acid gel electrophoresis (Fig. 2A) and fluorescence detection (Fig. 2B). The EVs were also extracted from the culture medium of 293T-GE11 cells and verified by western blotting (Fig. 2C). The sequences of CD63-GE11 and CD63-GFP are respectively provided in Tables SII and SIII. Taken together, all these procedures confirmed the successful establishment of engineered 293T cells to produce GE11-EVs.

Preparation and characterization of Dox-GE11-EVs. GE11-EVs was isolated from the culture medium of 293T-GE11 cells through precisely following a previously reported sequential ultracentrifugation method (Fig. 1A) (21). Western blotting revealed the presence of more enriched positive exosomal markers (CD63 and Alix) in EVs compared with cell lysate (22), whereas negative exosomal markers (GM130 and β-actin) were not detected (Fig. 3A). Based on TEM and Nanoparticle Tracking Analysis (NTA), the GE11-EVs exhibited a characteristic 'saucer-like' morphology (Fig. 3B) (6), with a peak particle size of 128.8±0.6 nm (Fig. 3C). Collectively, these data demonstrated that the isolated extracellular vesicles were EVs.

On the basis of a standard curve of Dox using HPLC, the drug LE and EE values of GE11-EVs loaded with Dox were separately calculated as 7.26±2.3 and 7.85±2.7%, respectively (Fig. 3D). The characteristic 'saucer-like' morphology of EVs was maintained even after drug loading via sonication, as shown in Fig. 3E. The particle size of GE11-EVs loaded with Dox was found to be 136.4±2.1 nm using NTA analysis, which was larger than the particle size reported for compared with GE11-EVs (Fig. 3F). As revealed by CLSM analysis, overlapped fluorescence of Dox (Rhodamine-labeled, and in red) and GE11-EVs (PKH67-labeled, and in green) was observed, indicating that the EVs had encapsulated the chemotherapeutic agents (Fig. 3G) (23).

EGFR-dependent uptake of GE11-EVs by colorectal cancer in vitro. Cellular uptake is well known to be a prerequisite for drug delivery. Previous studies have shown that EGFR overexpression occurs frequently in colorectal cancer (CRC) (24,25). To explore the cellular uptake of GE11-EVs, the protein level of EGFR was first detected in eight CRC cell lines (Fig. 4A). These data revealed that the majority of CRC cell lines exhibited high EGFR levels, with the exception of two CRC cell lines (RKO and SW620). For this reason, two EGFR-positive CRC cell lines (HCT116 and DLD1) and two EGFR-negative CRC cell lines (RKO and SW620) were chosen to investigate the cellular uptake of GE11-EVs. A BCA assay was used to measure the total protein content of EVs, and equivalent EVs were respectively added to the aforementioned cells. CLSM analysis revealed that the uptake of GE11-EVs in EGFR-positive CRC cell lines was significantly higher compared with that in the EVs treatment group, whereas there was no significant difference in the uptake of EVs and GE11-EVs in the EGFR-negative CRC cell lines (Fig. 4B), and flow cytometric analysis was subsequently performed, which showed the same results (Fig. 4C). Moreover, a quantitative analysis was conducted (Fig. 4D), and it was evident that the uptake of GE11-EVs in EGFR-positive CRC cell lines was significantly higher than that in the EVs treatment group. This suggests that EGFR-positive CRC cells can effectively recognize and bind to GE11-EVs.

EGFR-dependent uptake of GE11-EVs by glioma in vitro. Glioma poses a significant challenge to chemotherapy due to inadequate tumor targeting and the presence of the BBB, resulting in a high mortality rate and poor prognosis (26,27). It was previously demonstrated that EVs can cross BBB as natural nanocarriers for delivery of drug to brain (23). The protein level of EGFR in eight glioblastoma (GBM) cell lines was first detected (Fig. 5A) (24). The present data indicated that four of eight cell lines exhibited high EGFR levels. On this basis, two EGFR-positive GBM cell lines (SF763 and A172) and two EGFR-negative GBM cell lines (U251 and U-87MG) were chosen to investigate the cellular uptake of GE11-EVs. The BCA assay was used to measure the total protein content of EVs and equivalent EVs were respectively added to the aforementioned cells. CLSM and flow cytometric analysis revealed that the uptake of GE11-EVs in EGFR-positive GBM cell lines was significantly higher than the EVs treatment group. Nevertheless, there was no obvious difference between the uptake of EVs and GE11-EVs in EGFR-negative GBM cell lines (Fig. 5B-D). Although the ability of GE11-EVs to bind to cells in EGFR-negative cells U-87MG was stronger than that of EVs, it was far less than that of EGFR-positive

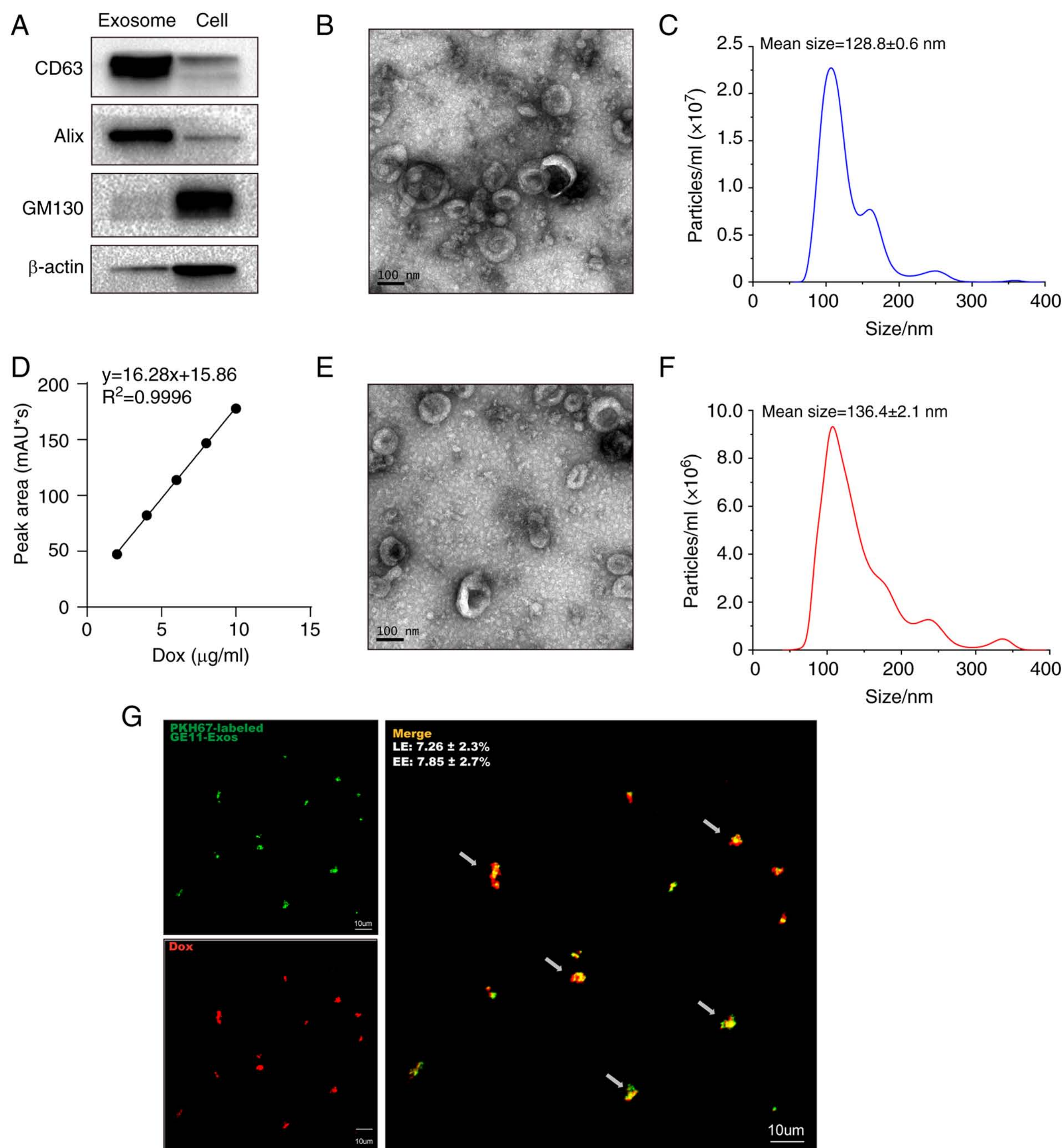

Figure 3. Preparation and characterization of Dox-GE11-EVs. (A) Western blot analysis of CD63, Alix, GM130 and β-actin expression in 293T cell and EVs. (B) TEM images of EVs. Scale bar, 100 nm. (C) Size distribution analysis of EVs using NTA. (D) Standard curve of Dox using high-performance liquid chromatography. (E) TEM images of Dox-GE11-EVs. Scale bar, 100 nm. (F) Size distribution analysis of Dox-GE11-EVs using NTA. (G) Confocal images and co-localization analysis between Dox (red) and PKH67-labeled GE11-EVs (green). Scale bar,10 μm. Dox, doxorubicin; EVs, extracellular vesicles; TEM, transmission electron microscopy; LE, drug loading efficiency; EE, encapsulation efficiency.

cells. Therefore, these data confirmed that GE11-EVs can be effectively uptaken by EGFR-positive GBM cells.

Dox-GE11-EVs promote anticancer efficiency to EGFR-positive cells and limit cytotoxicity to normal cells. Doxorubicin aims to cause lethal damage to cellular structures, mainly DNA, which, in turn, triggers apoptosis (28,29). On the basis of previous data, two EGFR-positive cell lines (HCT116 and SF763) were chosen; additionally, two normal cell lines (NCM460 and HA1800) were also selected to evaluate the anticancer efficiency of Dox-loaded engineering EVs. Different concentrations of Control, EVs and GE11-EVs were respectively added to the aforementioned cells. CCK-8 assay showed that Dox-GE11-EVs was more effective to inhibit the cell proliferation (28,29); GE11-EVs showed significantly decreased cell proliferation inhibition in two normal cell lines compared with the Control and EVs groups (Fig. 6A). Western blotting demonstrated that GE11-EVs significantly increase DNA damage and apoptosis in EGFR-positive cells, but not in normal cells (Fig. 6B). Finally, TUNEL staining revealed that GE11-EVs promote apoptosis of EGFR-positive tumor cells (Fig. 6C). Collectively, these data confirmed that the

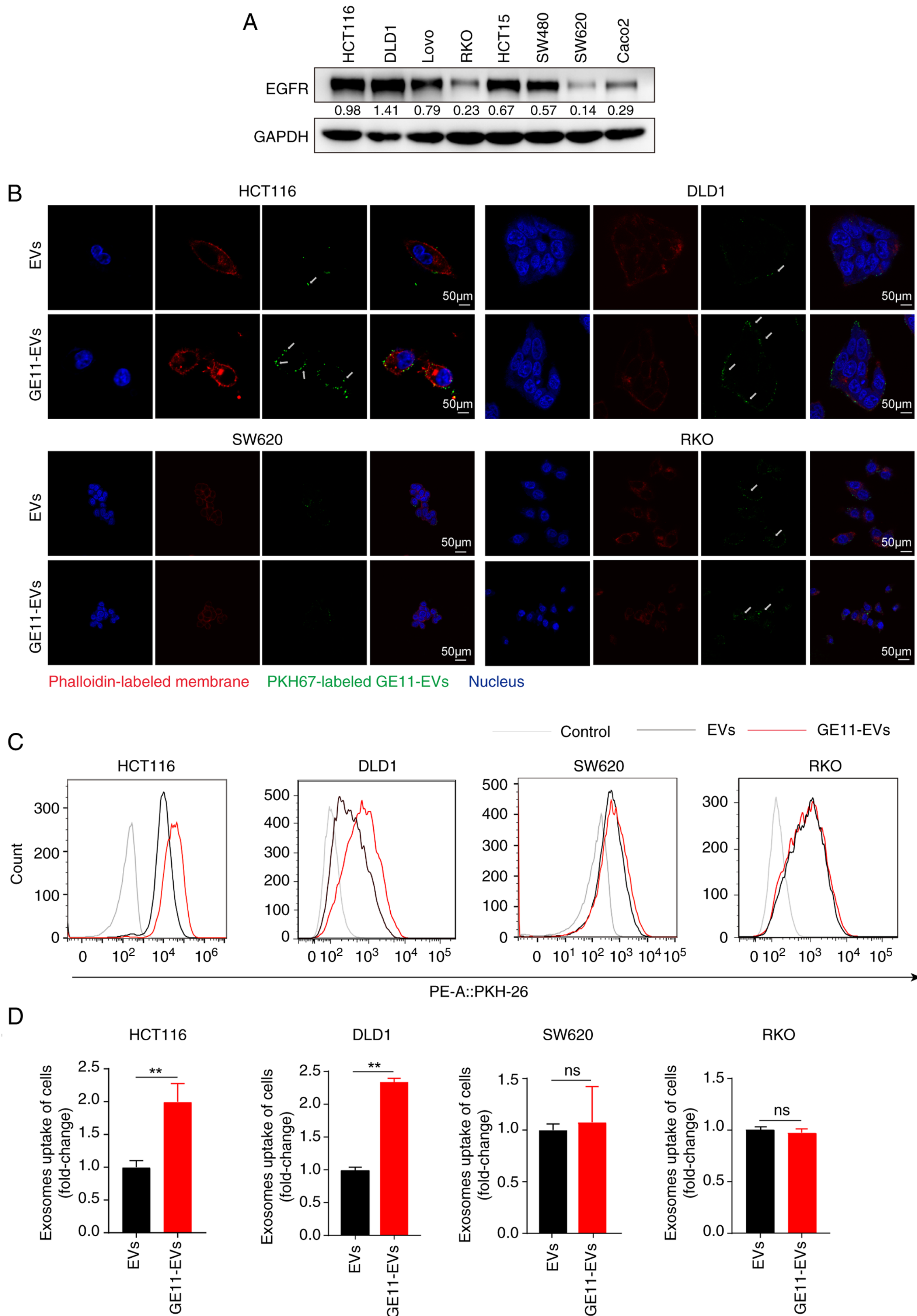

Figure 4. Cellular uptake of GE11-EVs in CRC cells *in vitro*. (A) Western blot analysis of EGFR expression in eight CRC cell lines (HCT116, DLD1, Lovo, RKO, HCT15, SW480, SW620 and Caco2). (B) Representative confocal images of the cellular uptake of GE11-EVs in CRC cells. Scale bar, 50 μm. (C) Flow cytometric analysis of the cellular uptake of GE11-EVs in CRC cells. The control group was treated with PBS. (D) Quantitative flow cytometric analysis of the cellular uptake of GE11-EVs in CRC cells. **P<0.01 (unpaired Student's t-test). EVs, extracellular vesicles; CRC, colorectal cancer; ns, not significant.

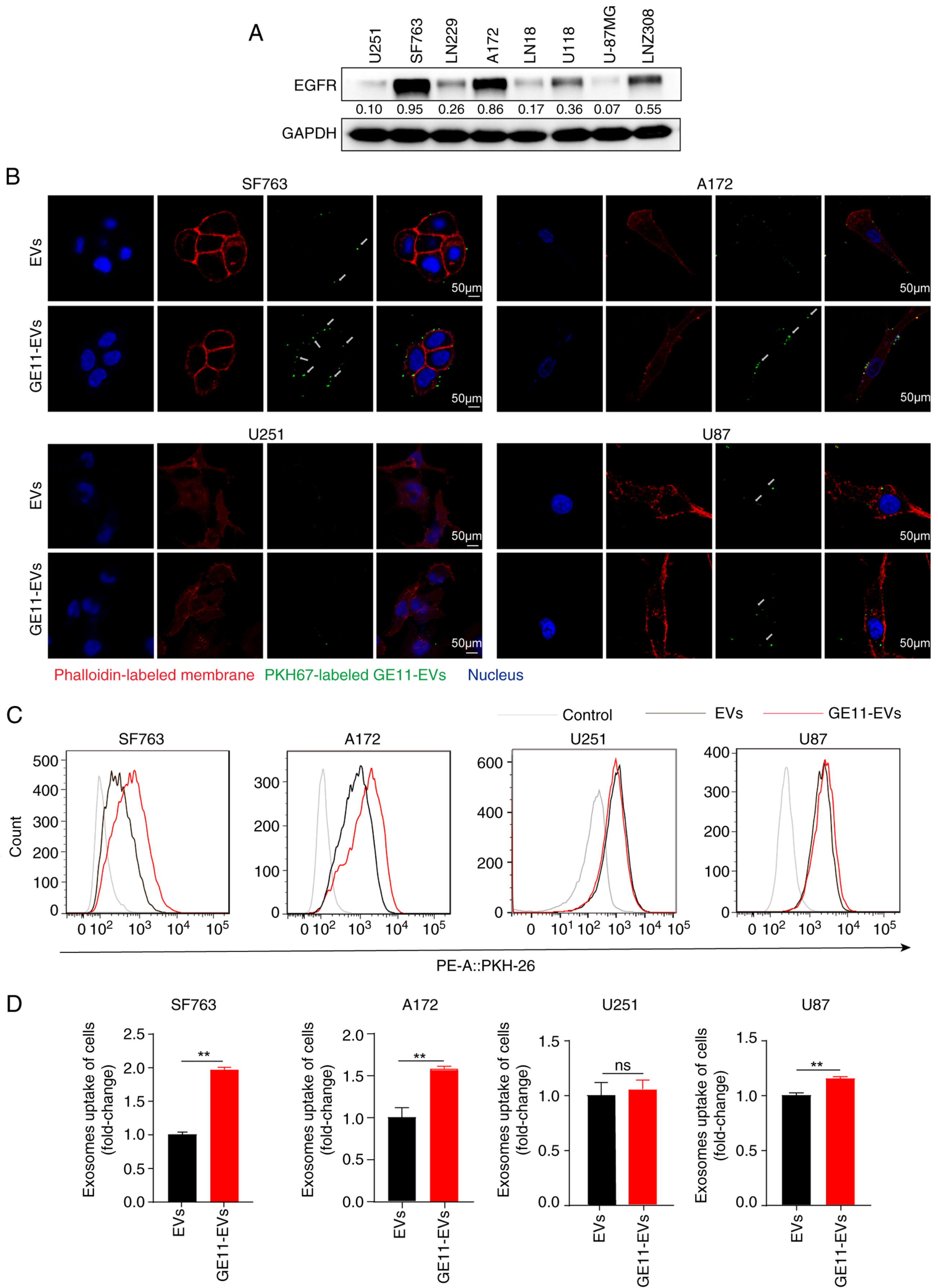

Figure 5. Cellular uptake of GE11-EVs in GBM cells *in vitro*. (A) Western blot analysis of EGFR expression in eight GBM cell lines (U251, SF763, LN229, A172, LN18, U118, U-87MG and LNZ308). (B) Representative confocal images of the cellular uptake of GE11-EVs in GBM cells. Scale bar, 50 μm. (C) Flow cytometric analysis of the cellular uptake of GE11-EVs in GBM cells. The control group was treated with PBS. (D) Quantitative flow cytometric analysis of the cellular uptake of GE11-EVs in GBM cells. **P<0.01 (unpaired Student's t-test). EVs, extracellular vesicles; GBM, glioblastoma; ns, not significant.

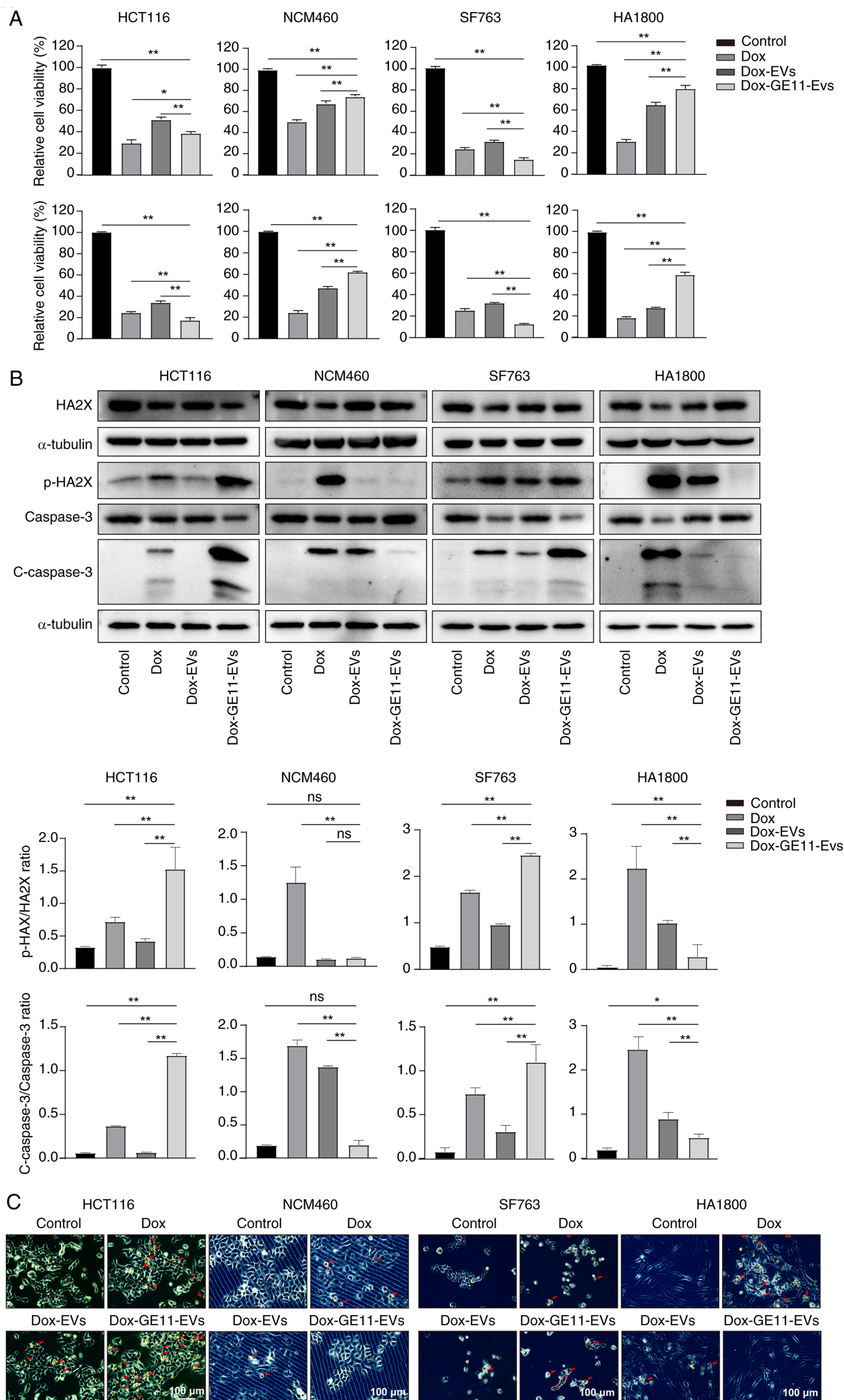

Figure 6. Dox-GE11-EVs promote anticancer efficiency to EGFR-positive cells and limit cytotoxicity to normal cells. (A) Viability was examined by Cell Counting Kit-8 assays in EGFR-positive and normal cells with DOX from different sources for 24 (up) or 48 h (down). The Dox concentration used by HCT116 and NCM460 was 2.0 μg/ml, Dox concentration used by SF763 and HA1800 was 0.25 μg/ml. $^{*}P<0.05$ and $^{**}P<0.01$ (One-way ANOVA). (B) Western blot analysis of p-H2AX and Cleaved Caspase-3 expression in EGFR-positive and normal cells with different treatments for 24 h. Control group was not treated. (C) TUNEL staining of apoptotic cells in EGFR-positive and normal cells with different treatments for 24 h. Scale bar, 100 μm. Control group was not treated. Dox, doxorubicin; EVs, extracellular vesicles; ns, not significant.

Dox-loaded engineering EVs (Dox-GE11-EVs) can selectively target EGFR-positive cells and promote anticancer efficiency without harming normal cells.

Discussion

In the field of tumor therapy, chemotherapeutic drugs are widely used, and some new traditional Chinese medicine therapies have been proposed (30,31), but most of the anticancer drugs used in clinical practice have certain toxicity, which can damage normal tissues while fighting cancer. Therefore, in order to reduce the damage of conventional anticancer drugs to normal tissues, it is particularly important to construct drug delivery vehicles with targeted tumor cells. However, current synthetic carrier systems, for instance, viruses, lipid nanoparticles, or polymeric nanoparticles, which have shown some success in drug delivery, are hindered by their intrinsic undesirable properties, including immune activation as foreign particles and potential toxicity. Nevertheless, EVs have been proven to minimize these potential adverse effects and offer multiple advantages over other carrier systems. EVs are biocompatible and can be easily obtained from biological cells and tissue samples, reducing immune effects arising from the use of synthetic materials. The therapeutic moiety is encapsulated in the EVs, providing stability and improving bioavailability (32). Furthermore, targeting ligands can be attached to the membrane surface of EVs to enable the delivery of biologics to preferred sites of interest.

The clinical use of doxorubicin, a key player in the treatment of numerous neoplastic diseases, has been largely limited by its dose-dependent cardiac toxicity, including cardiomyopathy and congestive heart failure (33). To overcome these obstacles in chemotherapy based on doxorubicin, a targeted drug delivery system is required. Previous studies have shown that EVs can be used for the targeted delivery of chemotherapeutic agents against neoplastic diseases (23,34).

On the basis of the aforementioned, 293T cells were selected as the producer cell line for EVs sourcing and a small EGFR ligand, GE11 peptide, was introduced onto CD63 (exosomal membrane protein) to promote the selectivity of EVs for EGFR-positive tumor cells. After Dox loading, the present data indicated that the engineered EVs (Dox-GE11-EVs) promoted anticancer efficiency to EGFR-positive cells and limited cytotoxicity to normal cells. However, previous studies showed the average 2.35% drug loading rate of EVs (35-37); Dox-GE11-EVs have a higher drug loading rate (7.26%). Briefly, this engineered EVs-targeted delivery system loaded with doxorubicin may serve as a potent platform for delivering chemotherapeutic agents to EGFR-positive tumor cells. Relevant online databases can be also used to understand the signature proteins of cancer and target them by constructing EVs that can bind to their signature proteins (38).

Although numerous preclinical studies have investigated the use of EVs for therapeutic delivery, few of them have progressed to clinical trials. Multiple demerits hinder the full potential of EVs-based drug delivery, including the need for clear and definite guidelines regarding EVs isolation methods and their characteristics. Additionally, the safety and toxicology of EVs-based therapeutics in humans are also generally unknown. Further clinical studies on EVs therapeutics, which focus on safety and toxicology in humans, can bridge these gaps and pave the way for EVs-based cancer therapy.

Acknowledgements

The authors would like to thank Professor Mong-hong Lee (The Sixth Affiliated Hospital of Sun Yat-sen University) for the technical assistance.

Funding

The present study was supported by the Science and Technology Program of Guangzhou (grant no. 202201010057), the NSFC Incubation Project of Guangdong Provincial People's Hospital (grant no. KY0120220046), the Guangdong Basic and Applied Basic Research Foundation (grant no. 2022A1515110674), the Medical Scientific Research Foundation of Guangdong (grant no. B2022067), the National Natural Science Foundation of China (grant no. 62301245) and the Guangdong Natural Science Funds of Distinguished Young Scholar (grant no. 2021B1515020067).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YY and FW wrote the manuscript, obtained and analyzed data. YL, RC, XW and JC performed the experiments and data analysis. RW, YH, XL and HZ designed the study and revised the manuscript. All authors read and approved the final version of the manuscript. YY, FW and RW confirm the authenticity of all raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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