

Epidermal growth factor receptor aptamer-conjugated polymer-lipid hybrid nanoparticles enhance salinomycin delivery to osteosarcoma and cancer stem cells

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Abstract. Osteosarcoma is a common childhood bone cancer with a poor survival rate. Osteosarcoma cancer stem cells (CSCs) contribute to the recurrence, drug resistance and metastasis of this disease. Previous evidence suggested that cancer cells are able to spontaneously turn into CSCs, thus it is crucial to simultaneously target osteosarcoma cells and CSCs. Our previous studies have demonstrated that salinomycin preferably eliminated osteosarcoma CSCs. In addition, amplification of the epidermal growth factor receptor (EGFR) is a common genetic aberration in osteosarcoma, and thus EGFR is a promising target in osteosarcoma. The present study aimed to develop EGFR aptamer-conjugated salinomycin-loaded polymer-lipid hybrid nanoparticles (EGFR-SNPs) to target both osteosarcoma cells and CSCs. The results revealed that EGFR was overexpressed in these cells, and that EGFR-SNPs possessed a small size of 95 nm, suitable drug encapsulation efficiency (63%) and sustained drug release over 120 h. EGFR-SNPs targeted EGFR-overexpressing osteosarcoma cells and CSCs, resulting in an enhanced cytotoxic effect compared with non-targeted SNPs and salinomycin. Notably, EGFR-SNPs was able to reduce the osteosarcoma tumorsphere formation rate and proportion of CD133⁺ osteosarcoma CSCs in the osteosarcoma cell lines more effectively compared with SNPs and salinomycin, suggesting that EGFR-SNPs effectively reduced the proportion of osteosarcoma CSCs. In conclusion, the interaction of EGFR aptamers and EGFR is a potential approach to promote the effective delivery of salinomycin to osteosarcoma. The study results suggested that EGFR-SNPs

represents a promising approach to target osteosarcoma cells and CSCs.

Introduction

Osteosarcoma is a common childhood bone cancer that has a poor long-term survival rate (1,2). The recurrence and metastasis of osteosarcoma contribute significantly to the failure of therapies (3,4). Thus, it is critical to develop novel strategies to target osteosarcoma cells that are resistant to conventional treatments (5). Cancer stem cells (CSCs) can lead to cancer recurrence and metastasis, therefore, they serve a pivotal role in cancer therapy (6). Numerous studies have suggested that CSCs are responsible for the initiation, recurrence and metastasis of osteosarcoma (7-9). However, cancer cells may spontaneously turn into CSCs (10,11). Therefore, targeting both CSCs and cancer cells has become a well-defined strategy in cancer therapy (12-15).

Salinomycin has been demonstrated to exert a potent activity against CSCs in various types of cancer (15,16). However, to the best of our knowledge, only two studies exist investigating the anti-CSCs activity of salinomycin in osteosarcoma, including a study by our group (17,18). These studies have demonstrated that salinomycin preferably eliminated osteosarcoma CSCs, whereas its cytotoxic effect towards osteosarcoma cells was not significant (17,18). Thus, it is necessary to improve the cytotoxic effect of salinomycin towards osteosarcoma cells.

Targeted nanoparticles are able to improve the targeting efficiency of chemotherapy drugs to cancer cells overexpressing specific antigens, such as epidermal growth factor receptor (EGFR) (19-21). Aptamers composed of oligonucleic acids have low immunogenicity and are readily available, thus they have been developed as the ligands of targeted nanoparticles (22,23). In addition, EGFR is overexpressed in numerous types of cancer, including osteosarcoma (24-27). Thus, EGFR may be an effective target for osteosarcoma. Although EGFR expression has not been reported in osteosarcoma CSCs, it is hypothesized in the present study that EGFR is overexpressed in osteosarcoma CSCs, since EGFR has been reported to be overexpressed on various types of

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CSCs and contributes to various characteristics of CSCs, including self-renewal (28,29). A study by Esposito *et al.* (30) reported that the EGFR aptamer CL4 was able to specifically bind to EGFR and EGFR-mediated endocytosis may occur. Thus, it is further hypothesized that CL4 may promote effective salinomycin delivery to EGFR-overexpressing osteosarcoma cells.

Polymer-lipid hybrid nanoparticles, which combine nanoparticles of biodegradable polymers and liposomes, are promising drug delivery systems with good stability and biocompatibility, sufficient drug loading and controlled drug-release (21,31). In the present study, EGFR aptamer-conjugated salinomycin-loaded polymer-lipid hybrid nanoparticles (EGFR-SNPs) were constructed to target osteosarcoma cells and CSCs.

Materials and methods

Reagents. Poly(D,L-lactide-co-glycolide) (PLGA; 50:50; Mw 40,000-75,000), coumarin 6, salinomycin sodium, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and all organic reagents of analytical grade were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, B27, insulin-transferrin-selenium (ITS) and TRIzol reagent were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Soybean lecithin and DSPE-PEG(2000)-maleimide were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The thiolated EGFR aptamers (5'-SH-GCCUUAGUAACG UGCUUUGAUGUCGAUUCGACAGGAGGC-3') were provided by Guangzhou Ruibo Biological Technology Co., Ltd. (Guangzhou, China). The anti-EGFR antibody conjugated to fluorescein isothiocyanate (FITC-EGFR) was provided by Santa Cruz Biotechnology, Inc. (1:200, cat. no. sc-120 FITC; Dallas, TX, USA). The CD133 antibody with phycoerythrin (cat. no. 130-080-801; PE-CD133, 1:10) and CD133 MicroBead kit were obtained from Miltenyi Biotec, Inc. (Auburn, CA, USA). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

Cell culture. Two human osteosarcoma cell lines, U2OS and MG63, were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM with 10% FBS, 25 mM hydroxyethyl piperazine ethanesulfonic acid buffer, 100 U/ml penicillin and 100 µg/ml streptomycin in an atmosphere with 5% CO₂ at 37°C.

Expression levels of EGFR and CD133 in osteosarcoma cell lines. The expression levels of two surface markers of the osteosarcoma cell lines, namely EGFR and CD133, were analyzed by flow cytometry. Briefly, the cells were treated with 1 µg/ml FITC-EGFR or PE-CD133 for 30 min at 4°C. Cells were then washed with PBS twice and run on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using the FlowJo v10 (Tree Star, Inc., Ashland, OR, USA). The mean fluorescence intensity of EGFR, expressed as the geometric mean, was analyzed at 520 nm, using the FlowJo v10.

Isolation of CD133⁺ cells using magnetic-activated cell sorting. The CD133⁺ cells were isolated from the U2OS and MG63 osteosarcoma cell lines using the CD133 MicroBead kit, according to the manufacturer's protocols. Briefly, the cell suspension was collected and centrifuged for 5 min at 1,200 x g at 4°C. The supernatant was removed and 20 µl CD133 microbeads were added, mixed, and incubated for 15 min at 4°C in the dark. Uncombined microbeads were removed using two washes and the pellet was resuspended in 500 µl PBE [phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin and 5 mM ethylenediaminetetraacetic acid] and separated on a magnetic separation column. The effluent from the column contained CD133⁻ cells. The CD133⁺ cells retained by the column were washed with PBE and collected. The CD133 expression of the cells was then analyzed by flow cytometry as described earlier.

Fabrication of polymer-lipid hybrid nanoparticles. EGFR-SNPs were developed by a one-step process of nanoprecipitation (21). Briefly, 2 mg PLGA and 2 mg salinomycin were dissolved in 2 ml acetonitrile, while 1 mg soybean lecithin and 0.3 mg DSPE-PEG(2000)-maleimide were dissolved in a 4% ethanol aqueous solution preheated to 65°C. Under gentle stirring, the acetonitrile solution was added dropwise into the lipid solution. The mixture was then stirred gently for 6 h at 25°C. Next, free drug molecules were removed from the nanoparticles using a dialysis tube (Spectra/Por 6 dialysis membrane; molecular weight cut-off, 1,000; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) in phosphate-buffered saline (PBS; pH 7.4). The nanoparticles were used immediately or concentrated to achieve the desired concentration by the Amicon[®] Ultra-4 centrifugal filter devices (nominal molecular weight limit, 100,000; EMD Millipore, Billerica, MA, USA). The resultant nanoparticles (2 ml) were incubated with 0.5 ml EGFR aptamers (1 mg/ml) for 6 h under magnetic stirring. Subsequently, the unconjugated EGFR aptamers were removed by ultrafiltration, and the remaining solution contained EGFR-SNPs.

EGFR aptamer-conjugated polymer-lipid nanoparticles (EGFR-NPs) were also prepared according to the same procedure without the addition of salinomycin. In addition, coumarin 6-loaded polymer-lipid nanoparticles (C6-NPs) and EGFR aptamer-conjugated C6-NP (EGFR-C6-NPs) were prepared with the same protocol by adding 0.1% (w/w) coumarin 6. Salinomycin-loaded polymer-lipid nanoparticles (SNPs) without EGFR were also prepared.

Characteristics of nanoparticles. The particle size and ζ-potential of nanoparticles were analyzed using a Zetasizer Nano S device (Malvern Instruments, Ltd., Malvern, UK). The morphology of nanoparticles was evaluated by transmission electron microscopy (TEM; Hitachi H-600; Hitachi, Ltd., Tokyo, Japan).

Drug encapsulation efficiency and loading. The salinomycin encapsulation efficiency and loading were evaluated as described previously (17). Briefly, 5 mg nanoparticles were dissolved in dichloromethane, and then evaporation of the dichloromethane solution was performed. Subsequently, methanol was added for high performance liquid chromatography

(HPLC) analysis (L-2000 system; Hitachi) with a reverse phase Diamonsil C-18 column (250x4.5 mm, 5 μ m; Dikma Technologies Inc., Lake Forest, CA, USA). The mobile phase was acetonitrile/deionized water/tetrahydrofuran/phosphoric acid (85/10/5/0.01, v/v), with a flow rate of 1.5 ml/min and a detection wavelength of 210 nm. The salinomycin encapsulation efficacy was calculated as follows: Encapsulation efficiency (%) = (weight of encapsulated salinomycin)/(weight of total salinomycin) x 100%. In addition, the salinomycin drug loading percentage was determined as follows: Drug loading (%) = (weight of encapsulated salinomycin)/(weight of nanoparticles) x 100%. The drug loading of coumarin 6 in the nanoparticles was calculated with a coumarin 6 calibration curve as previously described (17).

In vitro drug release. The *in vitro* release of salinomycin was evaluated in PBS (pH 7.4) alone or in PBS with 10% FBS, as previously described (17). Briefly, nanoparticles suspended in a centrifuge tube (0.5 mg/ml) were placed in an orbital shaker that vibrated horizontally at 80 x g and 37°C for 120 h. The tubes were removed from the shaker at different time-points (1, 2, 4, 12, 24, 48, 72 and 120 h) and centrifuged at 8,000 x g for 40 min at 37°C. The supernatant was then subjected to HPLC analysis as described earlier.

In vitro cellular uptake. After osteosarcoma cells (5x10⁵ cells per well) were seeded in 12-well plates overnight at 37°C, the cells were treated with free coumarin 6 or C6-NP for 2 h at 37°C, with an equivalent concentration of coumarin 6 (15 ng/ml). In the competitive assay, a high concentration of EGFR aptamers (10 μ l, 50 mg/ml) was added to the cells. The pretreatment of EGFR aptamers was performed 30 min before the treatment of EGFR-C6-NPs at 37°C. Following the treatment, the cells were washed and analyzed using flow cytometry.

Alternatively, osteosarcoma cells were seeded (5x10⁵ cells/well) in 12-well plates overnight at 37°C and then treated with salinomycin, SNPs or EGFR-SNPs at a concentration of 25 μ g/ml salinomycin for 4 h. In the competitive assay, a high concentration of EGFR aptamers (10 μ l, 50 mg/ml) was added to the cells. The pretreatment of EGFR aptamers was performed 30 min prior to the treatment of EGFR-C6-NPs at 37°C. Next, the cells were harvested by adding 1 ml methanol, sonicated for 1 min and centrifuged at 10,000 x g for 10 min at 25°C. The salinomycin content in the supernatant was measured by HPLC. The cellular protein concentration was examined by a BCA protein assay kit (Shanghai Biyuntian Biotechnology Co., Ltd., Shanghai, China). Finally, the percentage of intracellular uptake of salinomycin was calculated as follows: Salinomycin intracellular uptake (%) = Intracellular salinomycin concentration/intracellular protein concentration x 100%.

Cell proliferation assays. The cytotoxic effect of the nanoparticles against the osteosarcoma cells was evaluated using the CCK-8 assay (17). Briefly, cells were seeded (1x10⁴ cells/well) in 96-well plates overnight at 37°C. The cells were then incubated with various concentrations in a tenfold dilution manner (500, 166.67, 55.56, 18.52, 6.17, 2.06, 0.69, 0.23, 0.07 and 0.02 μ g/ml) of drugs (EGFR-NPs, SNPs, salinomycin and EGFR-SNPs) at 37°C. After 72 h, the cell viability was

evaluated according to the protocol provided in the CCK-8 kit. IC₅₀ was calculated by curve fitting using GraphPad 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed as following a previously described protocol (17). Total RNA was extracted from the cells using TRIzol[®] Reagent (Thermo Fisher Scientific, Inc.). The concentration and purity of total RNA samples were evaluated using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RNA integrity was assessed by running the total RNA on a denaturing 1% agarose gel. Using the Reverse Transcription System kit (Promega Corporation, Madison, WI, USA), the first-strand complementary DNA was reverse transcribed from RNA. The temperature protocol of reverse transcription was as follows: 70°C for 10 min, 42°C for 15 min, 95°C for 5 min, and 4°C for 5 min. SYBR[™] Green PCR Master Mix (Thermo Fisher Scientific, Inc.) and a Light Cycler (Hoffman-La Roche Ltd., Basel, Switzerland) was used to perform PCR. The messenger RNA (mRNA) expression levels, which were normalized against β -actin, were calculated and expressed as 2^{- $\Delta\Delta$ Cq} (32). The sequence of the primers were as follows: β -actin forward, 5'-CGTGGACATCCGTAAGA CC-3' and reverse, 5'-ACATCTGCTGGAAGGTGGAC-3'; and EGFR forward, 5'-TCCTCTGGAGGCTGAGAAAA-3' and reverse, 5'-GGGCTCTGGAGGAAAAGAAA-3'. After 2 min of denaturation at 95°C, 40 PCR cycles were performed with 3 sec denaturation at 95°C, 10 sec annealing at 55°C and 25 sec extension at 72°C.

Effect of nanoparticles on the proportion of CSCs within the osteosarcoma cells. Subsequent to seeding in 12-well plates overnight at 37°C at a density of 5x10⁴ cells/well, the osteosarcoma cells were treated with 40 μ g/ml nanoparticles (EGFR-NPs, SNPs, or EGFR-SNPs, at an equivalent concentration of 3 μ g/ml salinomycin) or 3 μ g/ml salinomycin or left untreated (control group). At 24 h after treatment, the cells were washed and incubated with fresh medium at 37°C for 72 h. The cells were manipulated by the following two approaches. First, the cells were subsequently collected and cultured in ultra-low adherent 96-well dishes (500 cells/well) to obtain tumorspheres in a stem cell-conditioned culture medium (containing 200 μ l DMEM-F12, 20 ng/ml bFGF, 20 ng/ml EGF, 1X B27 and 1X ITS). The number of the tumorspheres was counted after 7 days. Cells were then trypsinized, and the proportion of CD133⁺ cells was analyzed by flow cytometry.

Statistical analysis. Data are expressed as the mean \pm standard deviation of a least three independent experiments. A direct comparison between two groups was conducted by Student's non-paired t-test, while one-way analysis of variance with the Dunnett's or Newman-Keuls post hoc test was used to compare the mean values of three or more groups. P-value <0.05 was considered to indicate a statistically significant difference.

Results

CD133 and EGFR expression levels in osteosarcoma cells. The CD133 expression of the two osteosarcoma cell lines, U2OS

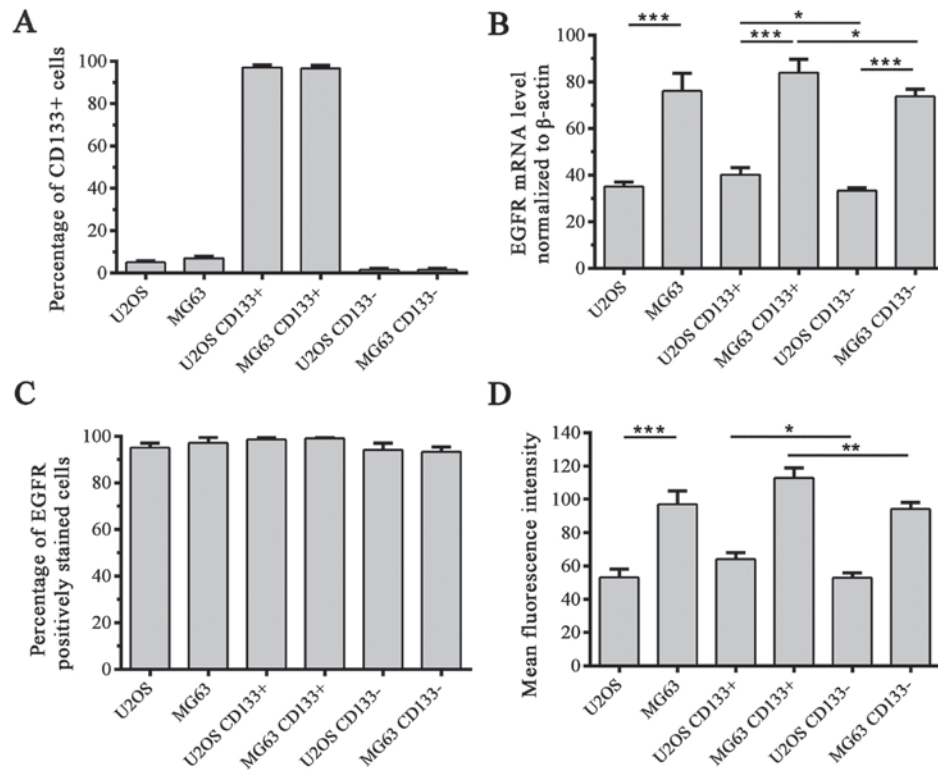


Figure 1. CD133 and EGFR expression in osteosarcoma cell lines analyzed by flow cytometry. (A) Percentage of CD133 positively stained cells in osteosarcoma cells. (B) Reverse transcription-quantitative polymerase chain reaction analysis of EGFR mRNA level in osteosarcoma cells normalized to β -actin. (C) Percentage of EGFR positively stained cells and (D) mean fluorescence intensity of EGFR protein expression in osteosarcoma cells. Data are expressed as the mean \pm standard deviation (n=3). * P <0.05, ** P <0.01 and *** P <0.001. EGFR, epidermal growth factor receptor.

and MG63, was examined by flow cytometry (Fig. 1A). Prior to isolation by magnetic sorting, the proportion of CD133⁺ cells in the osteosarcoma cells was only 5-7%. Following CD133 magnetic-activated cell sorting, the proportion of CD133⁺ cells in the sorted U2OS CD133⁺ and MG63 CD133⁺ cells was >95%, whereas the proportion of CD133⁺ cells in the sorted CD133⁻ cells was <2%.

An RT-qPCR assay was conducted to examine the EGFR mRNA levels in osteosarcoma cells (Fig. 1B). The results demonstrated that the EGFR mRNA level was significantly increased in MG63 cells compared with U2OS cells prior to CD133 sorting (P <0.001). Following CD133 sorting, the expression of EGFR mRNA was significantly increased in MG63 CD133⁺ cells compared with U2OS CD133⁺ cells (P <0.001) and the EGFR mRNA level was significantly increased in MG63 CD133⁻ cells compared with U2OS CD133⁻ cells (P <0.001). Notably, the EGFR mRNA level was significantly increased in CD133⁺ cells compared with CD133⁻ cells in both the MG63 and U2OS cell lines (P <0.05). Next, the EGFR protein expression in the osteosarcoma cells was investigated by examining the EGFR positive staining and mean fluorescence intensity of cells (Fig. 1C and D). EGFR was observed to be highly expressed in the unsorted, CD133⁺ and CD133⁻ cells, with almost 100% of the osteosarcoma cells expressing EGFR at different mean fluorescent intensities (Fig. 1C). To further analyze the EGFR protein expression among the different osteosarcoma cells, the EGFR mean fluorescence intensity was used as an evaluation index (Fig. 1D). The EGFR mean fluorescence intensity of MG63

cells was significantly increased by 2-fold compared with that of U2OS cells (P <0.001). Notably, a positive correlation was identified between CD133 and EGFR expression levels. The EGFR expression of U2OS CD133⁺ cells was significantly higher as compared with that of U2OS CD133⁻ cells (P <0.05), and a similar trend was observed in the MG63 cells (P <0.01; Fig. 1B and D).

Characteristics of nanoparticles. SNPs and EGFR-SNPs were prepared using the one-step process of nanoprecipitation illustrated in Fig. 2A. The nanoprecipitation method involved a simple approach to encapsulate salinomycin in polymer-lipid hybrid nanoparticles. The characteristics of the different nanoparticles are presented in Table I. The size of the nanoparticles was ~90 nm with a narrow polydispersity (PDI) of <0.15, indicating a homogeneous distribution of nanoparticles. In addition, the nanoparticles had relatively high negative ζ -potential ~-20 mV. The encapsulation efficiency of nanoparticles was ~65%, while their drug loading was ~8%, suggesting that nanoprecipitation effectively encapsulated salinomycin in the polymer-lipid hybrid nanoparticles (Table I). Furthermore, the morphology of the nanoparticles was examined by the TEM, and they exhibited a spherical structure and smooth surface (Fig. 2B). The *in vitro* release of salinomycin from SNPs and EGFR-SNPs was subsequently examined in PBS and in PBS with 10% FBS at 37°C (Fig. 2C). The two types of nanoparticles demonstrated faster release in PBS with 10% FBS as compared with that in PBS, with a significant difference in drug release observed after 48, 72 and 120 h (P <0.05).

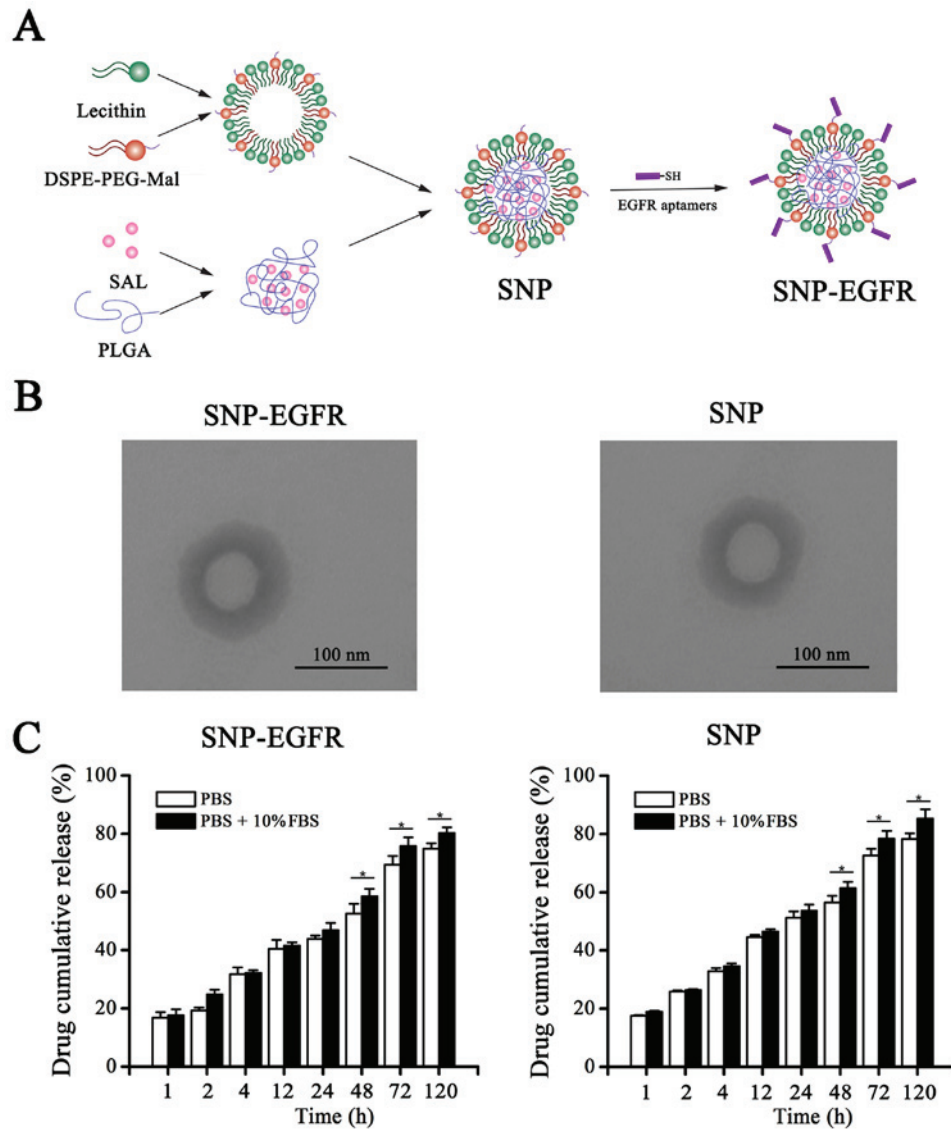


Figure 2. Preparation, morphology and drug release of nanoparticles. (A) Preparation procedure of nanoparticles. (B) Transmission electron microscopy image of nanoparticles (bar, 100 nm). Magnification, $\times 100,000$. (C) Cumulative salinomycin release from the nanoparticles in PBS or in PBS with 10% FBS. The two groups at different time points were compared by Student's nonpaired t-test. Data are expressed as the mean \pm standard deviation ($n=3$). * $P<0.05$. EGFR, epidermal growth factor receptor; SAL, salinomycin; PLGA, poly (D,L-lactide-co-glycolide); PBS, phosphate-buffered saline; FBS, fetal bovine serum; SNPs, SAL-loaded polymer-lipid hybrid nanoparticles; EGFR-SNPs, EGFR aptamer-conjugated SNPs.

The cumulative drug release of the nanoparticles reached $\sim 80\%$ at 120 h.

In vitro cellular uptake. Flow cytometry was conducted to evaluate the *in vitro* cellular uptake using coumarin 6 as a fluorescent tracing marker (Fig. 3A and B). The mean fluorescence intensity of the C6-NPs-treated group was significantly higher compared with that of the free coumarin 6-treated group in U2OS CD133⁺ and CD133⁻ cells ($P<0.05$), suggesting that nanoparticles facilitated the uptake of free drugs (Fig. 3A). In addition, the mean fluorescence intensity of the EGFR-C6-NPs-treated group was significantly higher in comparison with that of the C6-NPs-treated group in U2OS CD133⁺ and CD133⁻ cells ($P<0.01$), whereas the mean fluorescence intensity of the EGFR-C6-NPs-treated group was significantly decreased following pretreatment with EGFR aptamers ($P<0.01$). Since pretreatment with EGFR aptamers decreased the mean

Table I. Characterization of nanoparticles.

Parameter	SNP	EGFR-SNPs
Size (nm)	89.6 \pm 9.5	95.6 \pm 7.3
ζ -potential (mv)	-21.6 \pm 5.8	-26.4 \pm 4.1
Polydispersity	0.12 \pm 0.05	0.11 \pm 0.03
Drug loading (%)	7.8 \pm 3.5	8.9 \pm 2.1
Encapsulation efficacy (%)	66.7 \pm 8.2	63.1 \pm 7.3

Data are expressed as the mean \pm standard deviation ($n=3$). SNP, salinomycin-loaded polymer-lipid hybrid nanoparticles; EGFR-SNPs, epidermal growth factor receptor aptamer-conjugated SNP.

fluorescence intensity of the EGFR-C6-NPs-treated group, the targeting molecule of EGFR-C6-NPs is the same as EGFR

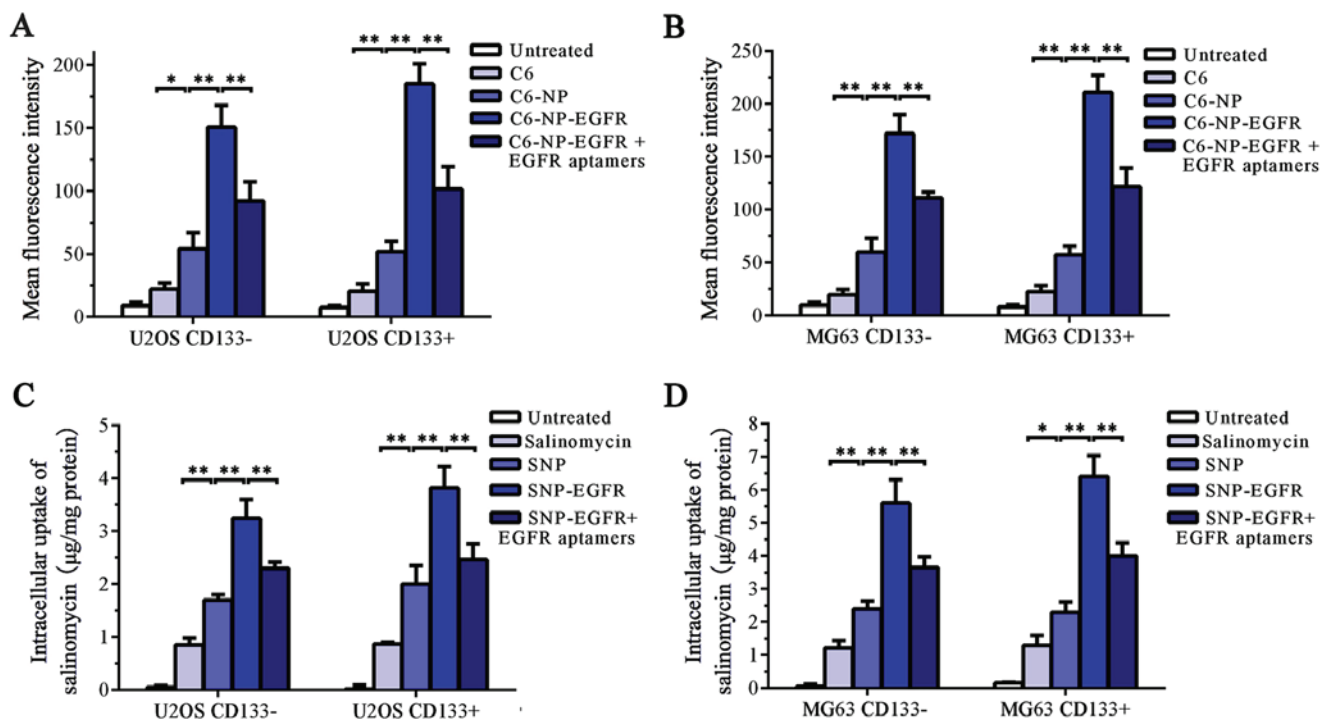


Figure 3. *In vitro* cellular uptake of nanoparticles in CD133⁺ and CD133⁻ osteosarcoma cells. The *in vitro* cellular uptake of nanoparticles was evaluated by flow cytometry in (A) U2OS and (B) MG63 cells, and by high-performance liquid chromatography in (C) U2OS and (D) MG63 cells. The intracellular uptake of salinomycin (%) was calculated as follows: Intracellular salinomycin concentration/intracellular protein concentration \times 100%. In the competitive assay, a high concentration of EGFR aptamers (50 mg/ml) was added prior to the treatments. The comparisons among the groups were conducted by one-way analysis of variance with the Newman-Keuls post hoc test. Data are expressed as the mean \pm standard deviation ($n=3$). * $P<0.05$ and ** $P<0.01$. EGFR, epidermal growth factor receptor; C6, coumarin 6; SNPs, salinomycin-loaded polymer-lipid hybrid nanoparticles; EGFR-SNPs, EGFR aptamer-conjugated SNPs.

aptamers. Thus, EGFR-C6-NPs are dependent on their conjugated EGFR-aptamers to increase the uptake of nanoparticles in osteosarcoma cells. It is hypothesized that the mechanism by which aptamers promote the uptake of nanoparticles is explained as follows. Following the binding of the aptamers on the nanoparticles to cells, the targeted nanoparticles are efficiently internalized via receptor-mediated endocytosis, whereas non-targeted nanoparticles are only taken up in a non-specific manner which, is not efficient. Similar results were achieved in the MG63 cells (Fig. 3B).

The quantity of internalized salinomycin in osteosarcoma cells was measured by an HPLC assay (Fig. 3C and D). The salinomycin uptake in the SNP-treated group was significant higher as compared with that of the free salinomycin-treated group in the U2OS CD133⁺ and CD133⁻ cells ($P<0.05$; Fig. 3C). The salinomycin concentration in the EGFR-SNPs-treated group was also significantly higher compared with that of the SNPs-treated group in U2OS CD133⁺ and CD133⁻ cells, whereas the salinomycin uptake of the EGFR-SNPs-treated group was significantly reduced upon pretreatment with EGFR aptamers ($P<0.01$). Similar results were obtained in the MG63 cells (Fig. 3D). Thus, EGFR-SNPs may promote salinomycin delivery to osteosarcoma cells.

CCK-8 assay. A CCK-8 assay was performed to evaluate the effect of nanoparticles and salinomycin on the proliferation of osteosarcoma cells (Fig. 4). The blank nanoparticles conjugated with EGFR aptamers (namely EGFR-NPs) did not exhibit significant cytotoxicity, whereas salinomycin, SNPs

and EGFR-SNPs demonstrated dose-dependent cytotoxicity on the osteosarcoma cells. The half maximal inhibitory concentration (IC_{50}) values are presented in Table II.

In U2O2 cells, salinomycin exhibited an increased cytotoxic effect towards CD133⁺ cells compared with that in CD133⁻ cells (7.46 ± 2.77 vs. 15.32 ± 4.21 $\mu\text{g}/\text{ml}$; $P<0.05$), suggesting that salinomycin preferably eliminated the osteosarcoma CSCs. By contrast, the cytotoxic effect of SNPs did not differ significantly from that of salinomycin in the U2O2 CD133⁺ and CD133⁻ cells ($P>0.05$). As shown in Table II, the IC_{50} value of EGFR-SNPs (2.12 ± 1.23 $\mu\text{g}/\text{ml}$) was significantly lower in comparison with that of SNPs (8.21 ± 2.33 $\mu\text{g}/\text{ml}$; $P<0.05$) and salinomycin (7.46 ± 2.77 $\mu\text{g}/\text{ml}$; $P<0.05$) in U2O2 CD133⁺ cells. In addition, the IC_{50} value of EGFR-SNPs (5.99 ± 1.26 $\mu\text{g}/\text{ml}$) was significantly lower compared with that of salinomycin (15.32 ± 4.21 $\mu\text{g}/\text{ml}$; $P<0.05$) and SNPs (18.23 ± 6.32 $\mu\text{g}/\text{ml}$; $P<0.05$) in U2O2 CD133⁻ cells. Taken together, EGFR-SNPs was 3.9- or 3.5-folds more effective than SNPs or salinomycin in U2OS CD133⁺ cells, respectively, and 3.0- or 2.5-folds more effective compared with SNPs or salinomycin in U2OS CD133⁻ cells, respectively. Similar results were obtained for the IC_{50} values in MG63 cells. In the MG63 CD133⁺ cells, EGFR-SNPs was 3.4- or 3.2-folds more effective in comparison with SNPs or salinomycin, respectively. In MG63 CD133⁻ cells, EGFR-SNPs was 4.1- or 3.7-folds more effective compared with SNPs or salinomycin, respectively. Thus, these results suggested that EGFR-SNPs possessed an increased cytotoxic effect towards CD133⁺ and CD133⁻ osteosarcoma cells compared with SNPs and free salinomycin.

Table II. IC₅₀ values of salinomycin and nanoparticles in osteosarcoma cells at 72 h.

IC ₅₀ (μg/ml)	U2OS		MG63	
	CD133 ⁺	CD133 ⁻	CD133 ⁺	CD133 ⁻
Salinomycin	7.46±2.77 ^a	15.32±4.21	11.63±4.98 ^a	25.62±6.35
SNP	8.21±2.33	18.23±6.32	12.15±3.92	28.76±6.74
EGFR-SNPs	2.12±1.23 ^{b,c}	5.99±1.26 ^{d,e}	3.61±1.04 ^{b,c}	6.98±1.23 ^{d,e}
NP-EGFR	>500.0	>500.0	>500.0	>500.0

Data are expressed as the mean ± standard deviation (n=3). The difference between groups was compared using analysis of variance followed by a Newman-Keuls post hoc test. ^aP<0.05, salinomycin in CD133⁺ cells vs. salinomycin in CD133⁻ cells; ^bP<0.05, EGFR-SNPs vs. SNPs in CD133⁺ cells; ^cP<0.05, EGFR-SNPs vs. salinomycin in CD133⁺ cells; ^dP<0.05, EGFR-SNPs vs. SNPs in CD133⁻ cells; ^eP<0.05, EGFR-SNPs vs. salinomycin in CD133⁻ cells. IC₅₀, half maximal inhibitory concentration; EGFR, epidermal growth factor receptor; NP, nanoparticles; SNP, salinomycin-loaded polymer-lipid hybrid NP; EGFR-SNPs, EGFR aptamer-conjugated SNP.

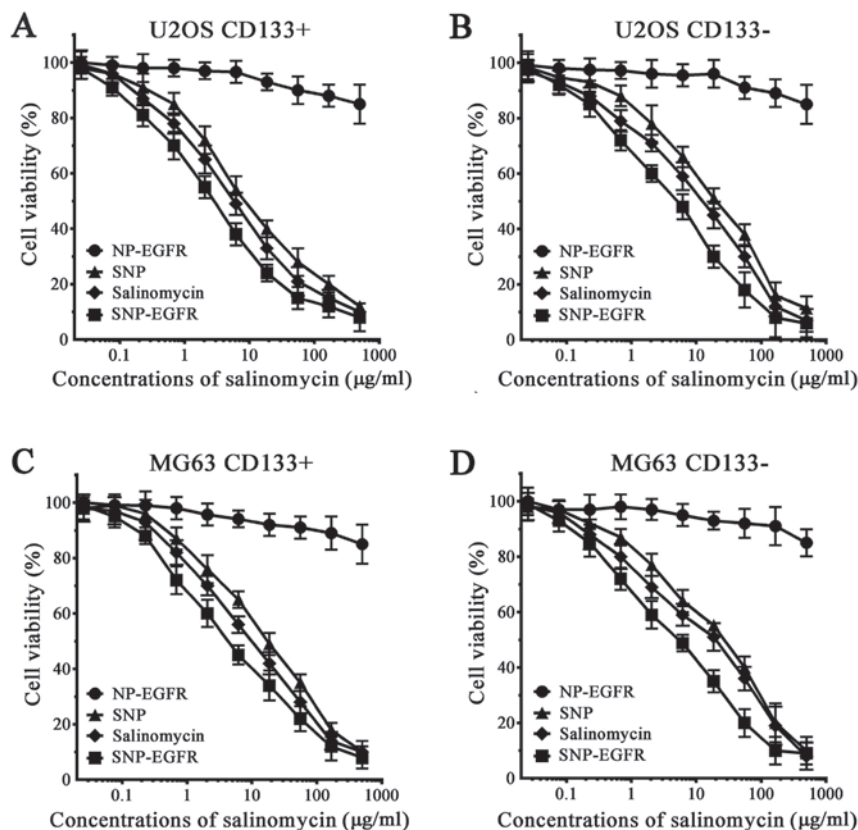


Figure 4. Cell proliferation evaluated by the Cell Counting Kit-8 assay in (A) U2OS CD133⁺, (B) U2OS CD133⁻, (C) MG63 CD133⁺, and (D) MG63 CD133⁻ cells. Data are expressed as the mean ± standard deviation (n=3). EGFR, epidermal growth factor receptor; NPs, nanoparticles; SNPs, salinomycin-loaded polymer-lipid hybrid NPs; EGFR-SNPs, EGFR aptamer-conjugated SNPs.

Efficacy of nanoparticles on the proportion of CSCs in osteosarcoma cells. The tumorsphere formation rate has been reported to be correlated with the proportion of CSCs (12,13). In Fig. 5A, salinomycin was observed to significantly reduce the number of U2OS tumorspheres relative to the untreated control group (P<0.05). Notably, EGFR-SNPs resulted in a 2-fold decrease in the number of U2OS tumorspheres relative to the untreated control, and resulted in much fewer tumorspheres compared with salinomycin (P<0.05) and SNPs (P<0.01). Similar results were obtained in the MG63 cells

(Fig. 5B), in which EGFR-SNPs resulted in a 5-fold decrease in the number of tumorspheres relative to the untreated control, and in much fewer tumorspheres compared with salinomycin (P<0.01) and SNPs (P<0.01). Consistently, EGFR-SNPs significantly decreased the proportion of CD133⁺ cells in the U2OS and MG63 cells as compared with the salinomycin (P<0.05) and SNPs (P<0.01) treatments (Fig. 6A). According to these results, EGFR-SNPs exhibited an enhanced therapeutic efficacy towards osteosarcoma CSCs within the osteosarcoma cell lines compared with SNPs and salinomycin.

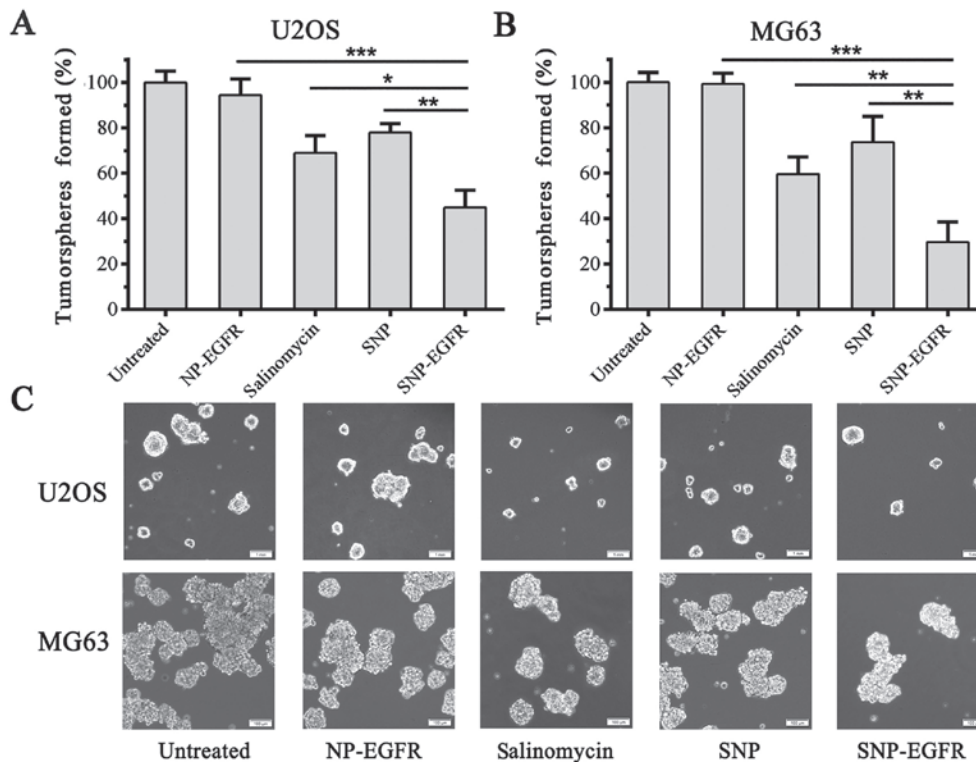


Figure 5. Effect of salinomycin or nanoparticles on the proportion of cancer stem cells within the osteosarcoma cell population, as reflected by the tumorsphere formation ability. Tumorsphere formation assay of the (A) U2OS and (B) MG63 cells. (C) Representative images from all groups in the tumorsphere formation assay under conventional light microscope. Magnification, x400. Comparison between two groups was conducted by one-way analysis of variance with the Dunnett's post hoc test. Data are expressed as the mean \pm standard deviation (n=4). *P<0.05, **P<0.01 and ***P<0.001. EGFR, epidermal growth factor receptor; NPs, nanoparticles; SNPs, salinomycin-loaded polymer-lipid hybrid NPs; EGFR-SNPs, EGFR aptamer-conjugated SNPs.

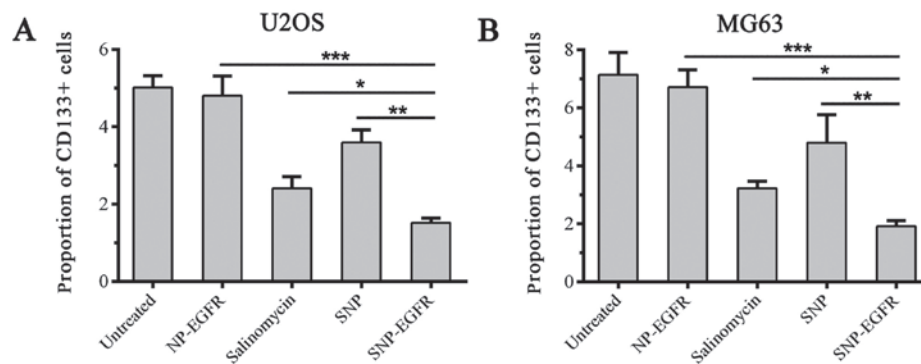


Figure 6. Effect of the nanoparticles on the proportion of cancer stem cells within the (A) U2OS and (B) MG63 osteosarcoma cell population, as reflected by the proportion of CD133⁺ cells. Comparisons among two groups were performed by one-way analysis of variance with the Dunnett's post hoc test. Data are expressed as the mean \pm standard deviation (n=4). *P<0.05, **P<0.01 and ***P<0.001. EGFR, epidermal growth factor receptor; NPs, nanoparticles; SNPs, salinomycin-loaded polymer-lipid hybrid NPs; EGFR-SNPs, EGFR aptamer-conjugated SNPs.

Discussion

In the present study, EGFR was overexpressed in osteosarcoma CSCs and cells. Thus, the present study developed EGFR-SNPs in order to promote the effective delivery of salinomycin to EGFR-overexpressing osteosarcoma CSCs and cells.

Small-molecule inhibitors (such as erlotinib) and antibodies (such as cetuximab) targeting EGFR have been demonstrated to achieve superior therapeutic efficacy in cancer (33). The amplification and mutation of the EGFR gene are characteristic genetic abnormalities detected in osteosarcoma; thus, patients

with osteosarcoma have been included in phase I clinical trials of EGFR inhibitors (26,27). Furthermore, abrogation of EGFR phosphorylation in osteosarcoma led to growth inhibition, whereas EGFR overexpression promoted cancer cell motility and invasion (34). However, the EGFR expression in osteosarcoma CSCs has not previously been investigated to the best of our knowledge. In the present study, it was demonstrated that EGFR was not only overexpressed in osteosarcoma cells, but also in osteosarcoma CSCs, and the EGFR expression of osteosarcoma CSCs was significantly higher in comparison with that in osteosarcoma cells. The finding that EGFR

was overexpressed in osteosarcoma CSCs is consistent with previous studies demonstrating that EGFR serves a fundamental role in the regulation of stemness and tumorigenesis of CSCs in various types of cancer (28,29,35,36). Therefore, it is hypothesized that EGFR may represent a promising biomarker for targeting both CSCs and cancer cells.

In the present study, pretreatment with EGFR aptamers decreased the mean fluorescence intensity of the EGFR-C6-NPs-treated group, which indicated that the targeting molecule of EGFR-C6-NPs is the same as that of EGFR aptamers. Thus, EGFR-C6-NPs are dependent on their conjugated EGFR-aptamers to increase the uptake of nanoparticles in osteosarcoma cells. It is hypothesized that the mechanism by which aptamers promote the uptake of nanoparticles is as follows. Following the binding of aptamers on the nanoparticles to cells, the targeted nanoparticles are efficiently internalized via receptor-mediated endocytosis, whereas non-targeted nanoparticles are taken up in a non-specific manner which, is not efficient. Therefore, the interaction of EGFR and EGFR aptamers achieves effective salinomycin delivery to EGFR-overexpressing osteosarcoma CSCs and cells and the existence of EGFR aptamers was pivotal to guarantee the targeting efficacy of EGFR-SNPs to osteosarcoma cells. HPLC and flow cytometry performed in the current study confirmed that EGFR-SNPs was able to efficiently target EGFR-overexpressing osteosarcoma CSCs and cells. Notably, EGFR-SNPs demonstrated an enhanced cytotoxic effect compared with non-targeted SNPs and salinomycin in EGFR-overexpressing osteosarcoma CSCs and cells. Furthermore, EGFR-SNPs was able to reduce the osteosarcoma tumorsphere formation rate and the proportion of CD133⁺ osteosarcoma CSCs in the osteosarcoma cell lines more effectively when compared with SNPs and salinomycin. The preferable elimination of osteosarcoma CSCs by EGFR-SNPs is attributable to the higher EGFR expression of osteosarcoma CSCs and the anti-CSC activity of salinomycin. Thus, the present results confirmed that EGFR-SNPs increased the therapeutic efficacy of salinomycin against EGFR-overexpressing osteosarcoma. Nevertheless, the relatively broad expression of EGFR in humans may decrease the targeting efficacy of EGFR-SNPs in osteosarcoma, and may pose potential damage to normal tissues. However, considering that a large amount of reagents have been developed to target EGFR (33), it is hypothesized that the side effects EGFR-SNPs may not be severe.

The safety issue of nanomedicines is important for their clinic use (37). In contrast to inorganic nanoparticles that do not degrade and may pose potential damage to the human body, the components of EGFR-SNPs consist of lecithin, PLGA, DSPE-PEG(2000), aptamers and salinomycin, which are materials or biocompatible reagents approved by the Food and Drug Administration (21). Aptamers are oligonucleotide ligands demonstrating high-affinity binding to molecular targets. Notably, pegaptanib sodium, an RNA aptamer directed against vascular endothelial growth factor-165, is the first aptamer approved for use in humans (38). A pilot clinical trial of salinomycin achieved partial breast cancer regression of tumor metastasis without severe side effects, and a phase I/II clinical trial of salinomycin will be conducted (39). The nanoparticles investigated

in the present study are also expected to have a satisfactory safety in clinical use.

The prepared EGFR-SNPs in the current study used only salinomycin to target the osteosarcoma CSCs and cells. This may be regarded as an advantage of the study, since researchers usually utilized two drugs to realize targeting CSCs and cancer cells (15). For instance, Ke *et al* (40) developed the co-delivery of thioridazine and doxorubicin using polymeric micelles for targeting lung CSCs and cancer cells. Similarly, Zhang *et al* (41) developed paclitaxel and salinomycin-loaded micelles to target breast CSCs and cancer cells. Compared with the two-drug strategy, the one-drug strategy developed in the present study possesses two advantages: Firstly, the co-delivered two drugs in single nanoparticles require optimization of their loading ratio to avoid antagonism, whereas the one-drug strategy does not require optimization (13,42); in addition, loading two drugs at a specified ratio is challenging, which is not required in the nanoparticles of the present study (13,42).

In conclusion, the results of the present study suggested that the antitumor mechanism of EGFR-SNPs involves the efficient accumulation of EGFR-SNPs in osteosarcoma cancer due to the long circulation and the enhanced permeability and retention effect of PEGylated nanoparticles. This is followed by specific binding to and internalization in EGFR-overexpressing osteosarcoma CSCs and cells, resulting in cell death.

In conclusion, the present study first utilized EGFR-targeted polymer-lipid nanoparticles to increase the efficacy of salinomycin in osteosarcoma CSCs and cells. EGFR-SNPs was observed to promote the efficient delivery of salinomycin to the EGFR-overexpressing osteosarcoma CSCs and cells, resulting in significant cytotoxic effects. Thus, the use of EGFR-SNPs represents a promising approach to treat osteosarcoma, and these nanoparticles may provide further insights on the treatment of osteosarcoma in the future.

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