Life Sciences 227 (2019) 145–152

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Synergistic effects of 7-*O*-geranylquercetin and siRNAs on the treatment of human breast cancer

Jiaxin Zuo[a,1], Yameng Jiang[a,1], Enxia Zhang[a], Yuling Chen[a], Ze Liang[a], Jie Zhu[b], Yinan Zhao[c], Hong Xu[d], Guoliang Liu[a], Jiasi Liu[a], Wei Wang[a], Shubiao Zhang[c,*], Yuhong Zhen[a,**]

[a] College of Pharmacy, Dalian Medical University, Dalian 116044, China
[b] Second Affiliated Hospital of Dalian Medical University, Dalian 116027, China
[c] Key Laboratory of Biotechnology and Bioresources Utilization of Ministry of Education, Dalian Minzu University, Dalian 116600, China
[d] College of Basic Medical Sciences, Dalian Medical University, Dalian 116044, China

ARTICLE INFO

Keywords:
7-*O*-geranylquercetin
Breast cancer
siRNA
Survivin
Interleukin-10

ABSTRACT

Aims: To investigate the antitumor effect of 7-*O*-geranylquercetin (GQ) combining with survivin siRNA (siSuvi) or IL-10 siRNA (siIL-10) to breast cancer.

Main methods: Xenograft tumor model was established by subcutaneously inoculating human breast cancer MCF-7 cells in BALB/c nude mice. Transfection efficiency of siRNA mediated by cationic liposome CDO14 in MCF-7 cells and tumor bearing mice was measured by flow cytometer and living imaging sysytem, respectively. Cell viability was detected using CCK-8 assay. Cell apoptosis was determined by Hoechst33342 staining and AV-PI staining. Tumors bearing mice were administered with GQ by gavage, and/or with liposome CDO14 mediated siRNAs via tail intravenous injection. Expression levels of proteins and cytokines were detected by western blot and ELISA, respectively.

Key findings: Liposome CDO14 could deliver siRNA to tumor effectively. Combination of GQ and siSuvi promoted the antiproliferation and pro-apoptosis effects of GQ or siSuvi to MCF-7 cells, and reduced the level of survivin and raised the level of caspase-7 in cells. GQ combining with siSuvi inhibited the growth of tumor, down-regulated the expression of survivin and up-regulated the expression of caspase-7 in tumor tissue. Similarly, GQ combining with siIL-10 inhibited the growth of tumor, decreased the level of IL-10 and increased the level of TNF-α. These results revealed that GQ enhanced the pro-apoptosis effect of siSuvi on tumor cells and the modulating effect of siIL-10 on tumor microenvironment.

Significances: Synergistic anti-tumor effect of GQ and siRNAs against breast cancer proved that chemical drugs combining with siRNAs is a promising antitumor strategy.

1. Introduction

Breast cancer is one of the most life threatening malignancies to women. Among the various therapeutic strategies for breast cancer, chemotherapy is still the major one owning to its high efficiency and great benefits in breast conserving [1]. However, the efficacy of traditional chemotherapy is limited by multidrug resistance [2]. Furthermore, the high toxicity and multiple side effects of traditional chemotherapeutic drugs seriously affect the life quality of patients [3]. So it is necessary to develop safe and effective drugs or therapeutic methods for the treatment of breast cancer.

7-*O*-geranylquercetin (GQ) designed and synthesized by our group is a novel alkylated derivative of quercetin with higher lipid solubility [4]. Our previous studies have demonstrated that the anti-proliferation and pro-apoptosis effects of GQ were stronger than those of quercetin against human breast cancer cell line MCF-7 [4], human lung cancer cell lines A549 and NCI-H1975 [5], and human gastric cancer cell lines SGC-7901 and MGC-803 [6]. However, GQ has to be administrated at high dosages to achieve anti-tumor efficiency due to the limitation of solubility and bioavailability.

Gene therapy has been paid more attention in recent years [7]. RNA interference (RNAi) is a universal silencing mechanism, which refers to highly efficient and specific degradation of homologous mRNA induced by double-stranded RNA. As the executor of RNAi [8], small interfering

* Correspondence to: S. Zhang, College of Life Science, Dalian Minzu University, 18 Liaohe West Road, Dalian Development Zone, Dalian, China.
** Correspondence to: Y. Zhen, College of Pharmacy, Dalian Medical University, No. 9 Lvshun Southern Road, Lvshunkou District, Dalian, China.
E-mail addresses: zsb@dlnu.edu.cn (S. Zhang), zhenyhwaner@aliyun.com (Y. Zhen).
[1] The first two authors contributed equally to this paper.

https://doi.org/10.1016/j.lfs.2019.04.047
Received 4 January 2019; Received in revised form 1 April 2019; Accepted 18 April 2019
Available online 19 April 2019
0024-3205/

RNA (siRNA) can disrupt cellular pathways by knocking down genes [9]. Although RNAi has a good prospect in the treatment of cancer and other diseases [10,11], its clinical application is still facing great challenges because of the poor stability and low transfection efficiency of siRNA [12]. So, the utilization of efficient transfection vectors is crucial for executing RNAi [13]. CDO14 is a peptide cationic liposome developed by our laboratory. In our previous studies, CDO14 showed higher transfection efficiency and lower toxicity as the carrier of DNA and siRNA compared with the existing cationic liposomes [14].

Survivin, as a member of the inhibitor of apoptosis proteins family [15], is over expressed in tumor tissues [16,17] and related to apoptosis and cell cycle [18]. Interleukin-10 (IL-10) is expressed abnormally in various tumors and plays an important role in immunomodulation of tumor microenvironment [19,20]. Therefore, survivin and IL-10 might be used as the targets of RNAi for tumor therapy.

Due to the different antitumor mechanisms of chemotherapy and gene therapy, their combination may achieve more significant activity and reduce the dosage of chemical drugs, thereby lowering their side effects [21]. In this study, we investigated the synergistic antitumor effects of GQ combining with siSuvi or siIL-10 on human breast cancer in vitro and in vivo, and peptide cationic liposome CDO14 was used as the carrier of siRNA. The research will provide a reference for the combination of chemotherapy drugs and siRNA in cancer therapy.

2. Materials and methods

2.1. Cell line and cell culture

Human breast cancer cell line MCF-7 was purchased from Shanghai Cell Biology Institute of Chinese Academy of Sciences (Shanghai, China). MCF-7 cells were cultured in DMEM medium containing 10% FBS at 37 °C under a humidified atmosphere of 5% CO_2.

2.2. Animals

Female BALB/c-nude mice of age 4–6 weeks were purchased from SPF animal center of Dalian Medical University, China [animal certificate SCXK (Liaoning) 2010–0002]. Mice were fed under specific pathogen free (SPF) condition and all the experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Dalian Medical University.

2.3. Reagents

Survivin siRNA (siSuvi), IL-10 siRNA (siIL-10) and negative control siRNA (siN·C.) were designed and synthesized by GenePharma (China). SiSuvi, sense: 5′-GAAUUAACCCUUGGUGAAUTT-3′, antisense: 5′-AUU CACCAAGGGUUAAUUCTT-3′; siIL-10, sense: 5′-GGUGAAGACUUUCU UUCAATT-3′, antisense: 5′-UUGAAAGAAAGUCUUCACCTT-3′; siN.C., sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense: 5′-ACGUGACA CGUUCGGAGAATT-3′; Cy5-siRNA and FAM-siRNA represent siN.C. labeled by cyanine5 (Cy5) and carboxyfluorescein (FAM) respectively. GQ [22] and CDO14 [14] were synthesized in our lab according the reported method. CCK-8 was purchased from Biotool (China). Hoechst 33342 was purchased from Solarbio (China). ECL chemiluminescence self-development kit was purchased from Biyun Institute of Biotechnology (China). Enzyme linked immunosorbent assay (ELISA) Kits for mouse tumor Necrosis Factor-α (TNF-α) and interleukin-10 (IL-10) were purchased from Langton Biology (China). DMEM medium, fetal bovine serum and trypsin were purchased from Gibco (USA). Matrigel Basement Membrane matrix was purchased from BD Bicscience (USA) Rabbit antibodies to human survivin and GAPDH, horseradish peroxidase-conjugated goat anti-rabbit antibody were purchased from Proteintech (USA). All other chemicals were of the highest purity available.

2.4. Liposome and lipoplexes preparation

Liposome CDO14 and lipoplexes of CDO14 with siRNAs were prepared according to our previous report method [14]. Briefly, cationic lipid CDO14 and neutral lipid DOPE (molar ratio1:1) were dissolved in chloroform in a glass vial and dried under a stream of nitrogen, then placed in a vacuum overnight. The dry lipid was resuspended in distilled water at 55 °C and sonicated to form liposome. To prepare lipoplexes, cationic liposome CDO14 was mixed with siRNAs in RPMI1640 at needed weight ratios and incubated for 20 min at room temperature.

2.5. Transfection of siRNA in vitro

The transfection efficiency of FAM-siRNA in MCF-7 cells mediated by liposome CDO14 at different conditions was detected by flow cytometry. MCF-7 cells (5×10^4 cells per well) were seeded in 24-well plates and incubated at 37 °C for 16–24 h until approximately 50% cell density was attained. FAM-siRNA (1.5 μg, 500 μL) was transfected by liposome CDO14 at various liposome/siRNA (N/P) weight ratios (2,1, 3:1 and 4:1) for 6 h. Similarly, FAM-siRNA was transfected into MCF-7 cells by liposome CDO14 at a N/P ratio of 3:1 for 6, 8 and 10 h. After transfection, the cells were washed with PBS, then trypsinized and resuspended in PBS. Cell suspension was determined by a FACS-Calibur flow cytometer (BD Biosciences, Franklin, NJ, USA).

2.6. Transfection of siRNA in vivo

The transfection efficiency of Cy5-siRNA and FAM-siRNA in tumor-bearing mice was detected by living imagine. MCF-7 cells (1×10^7 cells/mL) were mixed with matrigel at a N/P ratio of 3:1 and then subcutaneously inoculated near the 4th mammary gland of female BALB/c nude mice to form xenograft. For about 20 days, until the volumes ($V = ab^2/2$, a: the maximum length of the transplanted tumor, b: the maximum transverse diameter of the transplanted tumor) reached to approximately 150 mm^3, the 3:1 complex of CDO14 and Cy5-siRNA or FAM-siRNA (0.5 mg/kg) was administered by tail intravenous injection. The dynamic distribution of siRNA in the mice was monitored by an in-vivo imaging system (Maestro, Woburn, MA, USA) at various time.

2.7. Cell viability assay

To evaluate the proliferation inhibition effect of GQ combining with siSuvi on MCF-7 cells, the cell viability was measured by CCK-8 assay as described in previous report [23,24]. MCF-7 cells (5×10^3 cells per well) were seeded in 96-well plates and incubated at 37 °C for 18 h. SiN.C. and siSuvi were complexed with liposome CDO14 at a N/P ratio of 3:1 for 20 min. GQ was dissolved in dimethyl sulfoxide (DMSO) and diluted with the medium to desired concentration. And the concentration of DMSO was kept below 0.1% in treatment groups. DMSO at the same concentration with that in GQ group was used as vehicle control. Then, the cells were treated with siN.C. (0.3 μg, 100 μL), siSuvi (0.3 μg, 100 μL), GQ (20 μM) and GQ (20 μM) combining with siSuvi (0.3 μg, 100 μL) for 24, 48, 72 and 96 h. After the incubation, CCK-8 (10 μL per well) was added and incubated for 1 h at 37 °C. The absorbance at 450 nm was monitored by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Hoechst33342 staining assay

GQ and complexes of liposome and siRNAs were prepared as described in 2.7. MCF-7 cells (2×10^5 cells per well) were seeded in 24-well plates for 18 h and treated with siN.C. (1.5 μg, 500 μL), GQ (20 μM), siSuvi (1.5 μg, 500 μL), GQ (20 μM) combining with siSuvi (1.5 μg, 500 μL) for 48 h. The cells were stained with Hoechst33342 solution (250 μL) at 37 °C for 30 min, and then photographed using an

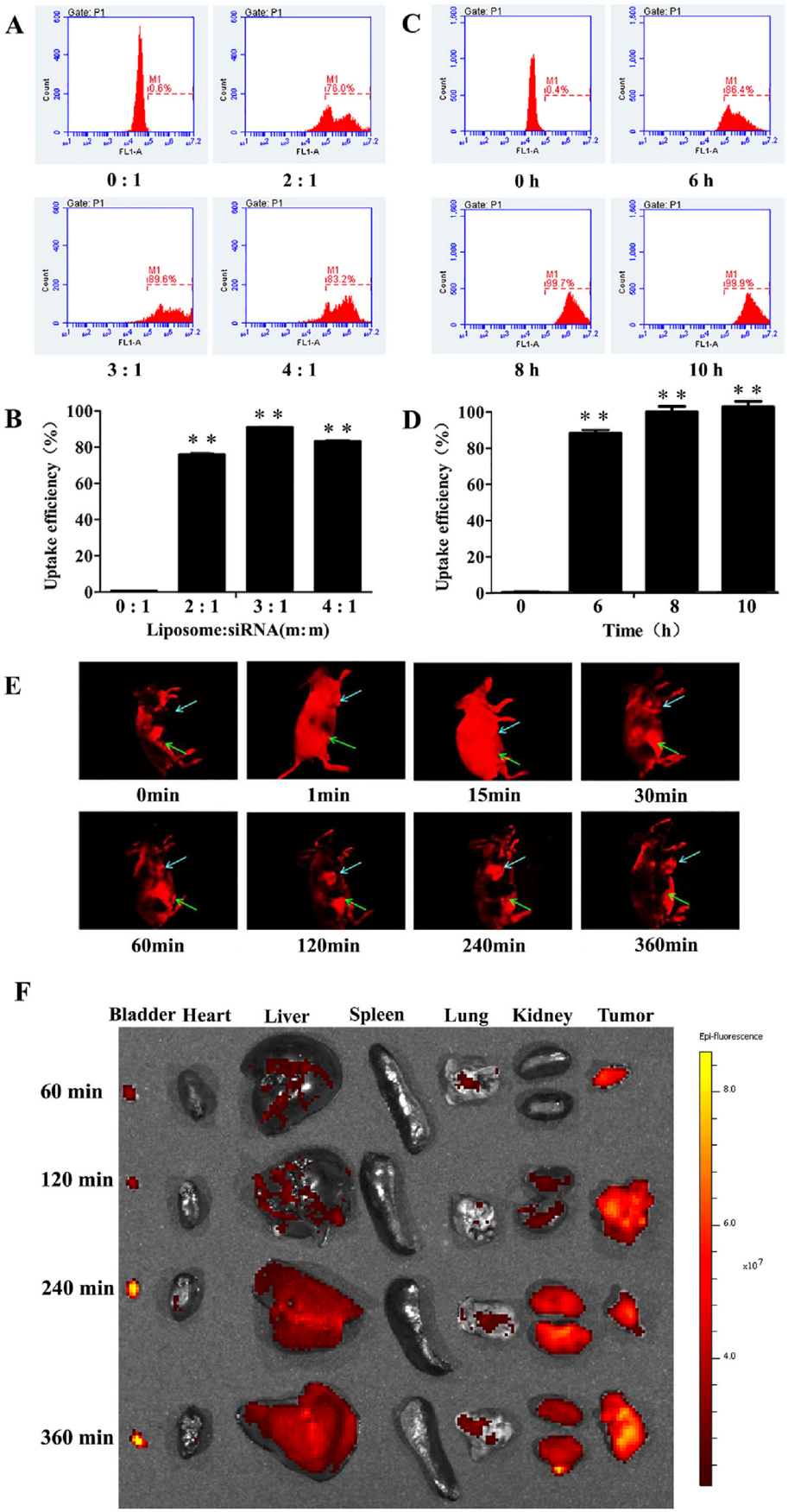

Fig. 1. Transfection efficiency of siRNA mediated by liposome CDO14 in cells and mice. (A, B) FAM-siRNA was transfected into MCF-7 cells by liposome CDO14 at various N/P ratios for 6 h. (C, D) FAM-siRNA was transfected into MCF-7 cells by liposome CDO14 at a N/P ratio of 3:1 for 6, 8 and 10 h. (E) Cy5-siRNA was transfected into the mice using liposome CDO14 as delivery via tail vein injection, and the dynamic distribution of siRNA in the mice was monitored using the in-vivo imaging system at different time. The fluorescence in liver and tumor was indicated by green arrow and blue arrow respectively. (F) FAM-siRNA was transfected into the mice using liposome CDO14 as delivery via tail vein injection, and the dynamic distribution of siRNA in the mice was monitored using the in-vivo imaging system at different time. The bar indicated the bare density of fluorescence. All data represent the means ± SD of three independent experiments. $^{**}p < 0.01$, comparing with the bare FAM-siRNA transfecting group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

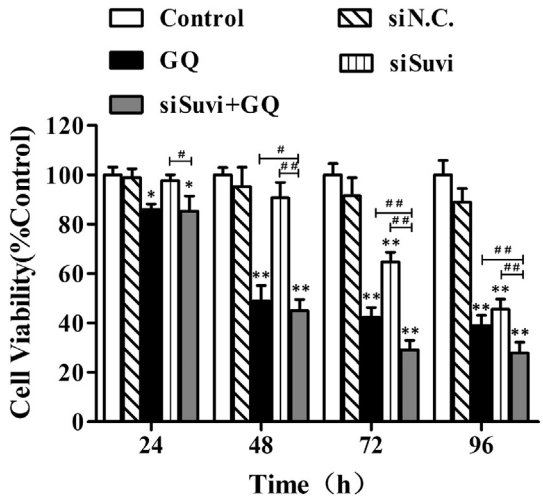

Fig. 2. Combination of GQ and siSuvi inhibited the proliferation of MCF-7 cells. MCF-7 cells (5×10^3) were treated with siN.C.(0.3 μg), GQ (20 μM), siSuvi (0.3 μg) and GQ (20 μM) combining with siSuvi (0.3 μg) for 24, 48, 72 and 96 h. The cell viabilities were measured using CCK-8 assay. All data represent the means ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, comparing with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, comparing with combination group.

inverted fluorescence microscope (100×) (Olympus, Hataya, Japan).

2.9. Annexin V-PI staining assay

MCF-7 cells (2×10^5 cells per well) were seeded in 6-well plates overnight and treated with siN.C. (1.5 μg, 500 μL), GQ (20 μM), siSuvi (1.5 μg, 500 μL), GQ (20 μM) combining with siSuvi (1.5 μg, 500 μL) for 48 h. The cells were collected and stained with Annexin V-PI as described by the manufacturer. The labeled cells were then analyzed using a FACS-Calibur flow cytometer (BD, Franklin, NJ, USA).

2.10. Tumor inhibition in mice

The xenograft tumor model was established as described in 2.6. Fifteen tumor-bearing mice (18–22 g) were averagely divided into 5 groups which were treated with PBS, siN.C., GQ, siSuvi and GQ combining with siSuvi, respectively. Another 18 tumor-bearing mice were averagely divided into 6 groups and treated with PBS, siN.C., GQ, siIL-10, GQ combining with siIL-10, and GQ combining with siSuvi and siIL-10, respectively. The 3:1 complex of CDO liposome and siRNAs (0.5 mg/kg) were administered by tail intravenous injection, and GQ (18 mg/kg) was administered by gavage. The mice were treated for 7 times at an interval of 2 days, and the tumor volumes were measured with vernier calipers and calculated. The mice were sacrificed at 15th day. The tumors were removed from the bodies and photographed and kept at −20 °C for western blot and ELISA assay.

2.11. Western blot assay

The expression levels of survivin and caspase-7 in MCF-7 cells and tumor tissue were measured by western blot. MCF-7 cells were seeded in 6-well plates (2×10^5 cells per well) for 24 h and treated with the above reagents for 48 h. The cells were harvested and directly lysed with ice-cold lysis buffer containing 1% PMSF. Lysates were centrifuged at 12,000 *g* for 10 min at 4 °C and supernatants containing total cellular proteins were collected and stored at 80 °C until use. Tumor tissue obtained from tumor inhibition test were homogenized and lysed with ice-cold lysis buffer containing 10% PMSF. The lysates were centrifuged and kept as above.

The protein samples (30 μg per lane) were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mL/L Tween-20) containing 5% (w/v) nonfat milk powder and then incubated with appropriate primary antibodies overnight at 4 °C. Finally, the membranes were exposed to horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. Protein bands were visualized by BeyoECL Plus reagents and imaged using a BioSpectrum Gel Imaging System (UVP, USA). Densitometry of the signal bands was analyzed using LabWorks software (UVP, USA). Human GAPDH was used as an internal control.

2.12. ELISA test

The expression levels of IL-10 and TNF-α in tumor were detected by ELISA. Tumor tissue was homogenized in PBS (pH 7.4) and centrifuged at 3500*g* and 4 °C for 15 min. The supernatants were collected and then subjected to ELISA according the introduction of the producer.

2.13. Statistical analysis

The data were presented as means ± SD. Data were analyzed using SPSS 21.0 (SPSS, Chicago, USA) by ANOVA test and Tukey's multiple comparison test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Liposome CDO 14 mediated siRNA transfection in MCF-7 cells and tumor-bearing mice

In order to optimize the transfection condition of siRNA mediated by liposome CDO14 in MCF-7 cells, the transfection efficency at different time and N/P ratios were detected by flow cytometer. After 6 h of transfection, the uptake rates of FAM-siN.C. were 76%, 89.6% and 83.2% at the N/P ratios of 2:1, 3:1 and 4:1, respectively (Fig. 1A and B). To further explore the optimal transfection time, FAM-siRNA were transfected into MCF-7 cells by CDO14 at 3:1 N/P ratio for various time. The uptake rate of siRNA was about 86.4% at 6 h of transfection, and increased to 99.7% at 8 h. After transfection for 10 h, the uptake rate was 99.9%, which means that siRNA was almost completely ingested (Fig. 1C and D). The results indicated that liposome CDO14 could transfect FAM-siRNA into MCF-7 cells efficiently.

Cy5-siRNA was transfected into tumor-bearing mice by liposome CDO14 via tail vein injection, and the dynamic distribution of siRNA in the mice was monitored by in-vivo imaging system (Fig. 1E). The results showed that red fluorescence could be found in mice after 1 min of administration and distributed throughout the body at 15 min. After 30 min of injection, the fluorescence in the body weakened, but obvious fluorescence was found in enterocoelia and tumor. The fluorescence in enterocoelia reached to maximum at 60 min, and then the fluorescence accumulated at liver (green arrow) from 120 min to 360 min. The fluorescence in tumor (blue arrow) gradually increased from 60 min to 240 min and kept strong at 360 min. The distribution of FAM-siRNA in the organs of the mice was also tested. The image showed that siRNA accumulated at liver, kidney, bladder and tumor at 4–6 h of injection (Fig. 1F). These results suggested that liposome CDO14 could deliver siRNA to tumor site.

3.2. Combination of GQ and siSuvi inhibited the proliferation of MCF-7 cells

CCK-8 assay showed that siN.C. had no effect on the proliferation of MCF-7 cells, but siSuvi markedly inhibited the proliferation of MCF-7 cells after transfection for 72 h and 96 h. After MCF-7 cells were treated with 20 μM of GQ for 24 h, the cell viability was about 85.5% and then reduced to 48.9%, 42.4% and 38.9% at 48 h, 72 h and 96 h, respectively. When the cells were treated with 20 μM of GQ and 0.15 μg of

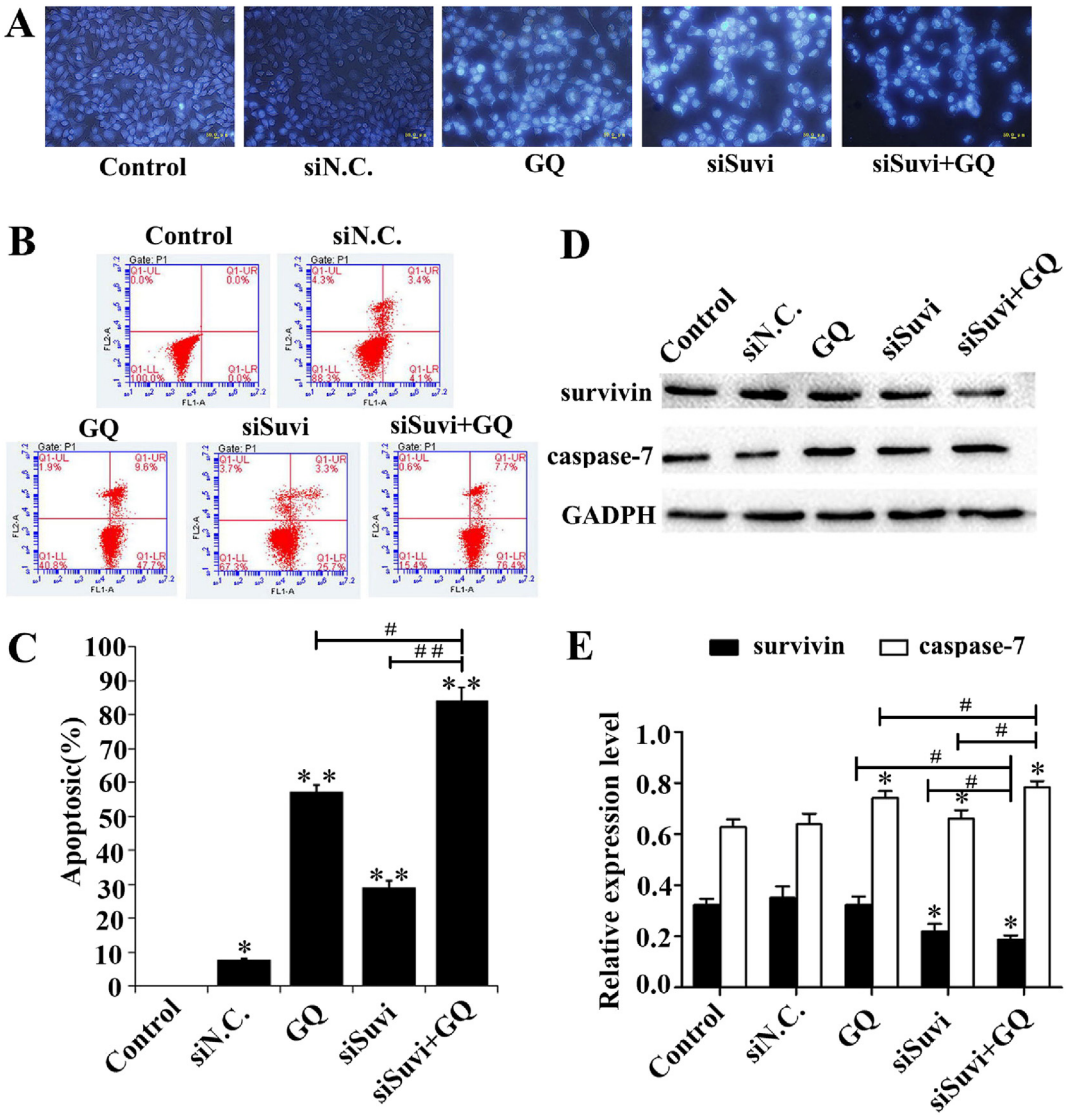

Fig. 3. Combination of GQ and siSuvi promoted apoptosis of MCF-7 cells. (A) MCF-7 cells (2×10^5) were treated with siN.C. (1.5 μg), GQ (20 μM), siSuvi (1.5 μg), GQ (20 μM) combining with siSuvi (1.5 μg) for 48 h, and then stained with Hoechst33342. The morphology of the cells was observed using fluorescence microscope (100×). (B, C) MCF-7 cells were treated as above for 24 h. The treated cells were stained with AV and PI, and analyzed by flow cytometry. (D, E) MCF-7 cells were treated as above for 48 h. Total cell lysates were subjected to western blot to detect the expression levels of survivin, caspase-7 and GADPH. All data represent the means ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, comparing with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, comparing with combination group.

siSuvi together for 24 h, the cell viability was about 85.4% and then reduced to 45.0%, 29.2% and 27.8% at 48 h, 72 h and 96 h, respectively (Fig. 2). These results indicated that combination of GQ and siSuvi promoted the antiproliferation effect of GQ or siSuvi to MCF-7 cells.

3.3. Combination of GQ and siSuvi promoted the apoptosis of MCF-7 cells

Hoechst33342 stain showed that the morphology of siN.C-treated cells did not change obviously compared with that of control cells. The cells treated with GQ or siSuvi showed strong bright blue fluorescence and decreased cell density. When the cells were treated with GQ and siSuvi simultaneously, nuclear shrinkage and nucleolysis appeared and cell density reduced greatly (Fig. 3A). The result demonstrated that both GQ and siSuvi could induce cell apoptosis, and the combination of GQ and siSuvi has stronger effect than the single treatment. Similar result was obtained in AV-PI staining assay. The apoptosis rate of the combination group of siSuvi and GQ (84.1%) was significantly higher than those of the groups treated with GQ (57.3%) or siSuvi (29%) alone (Fig. 3B and C). To further estimate the effect of GQ combining with siSuvi on apoptosis, we detected the expression of apoptosis-related protein, survivin and caspase-7, in MCF-7 cells by western blot. It was found that the level of survivin decreased, while the level of caspase-7 increased significantly after treated with siSuvi. In the MCF-7 cells treated with GQ alone, the level of caspase-7 increased obviously, but the level of survivin didn't decrease compared with the control group. When cells were treated with GQ and siSuvi together, down-regulation of survivin and up-regulation of caspase-7 were more remarkable (Fig. 3D and E). These results proved that combination of GQ and siSuvi enhanced the pro-apoptosis effects of GQ or siSuvi in MCF-7 cells.

3.4. Combination of GQ and siRNA inhibited tumor growth in mice

After proving the anti-tumor effect of GQ combining with siSuvi in vitro, we then investigated the synergetic anti-tumor effect of GQ and siRNA on xenograft in mice. It was found that the tumor volumes of the mice treated with GQ, siSuvi or GQ combining with siSuvi were

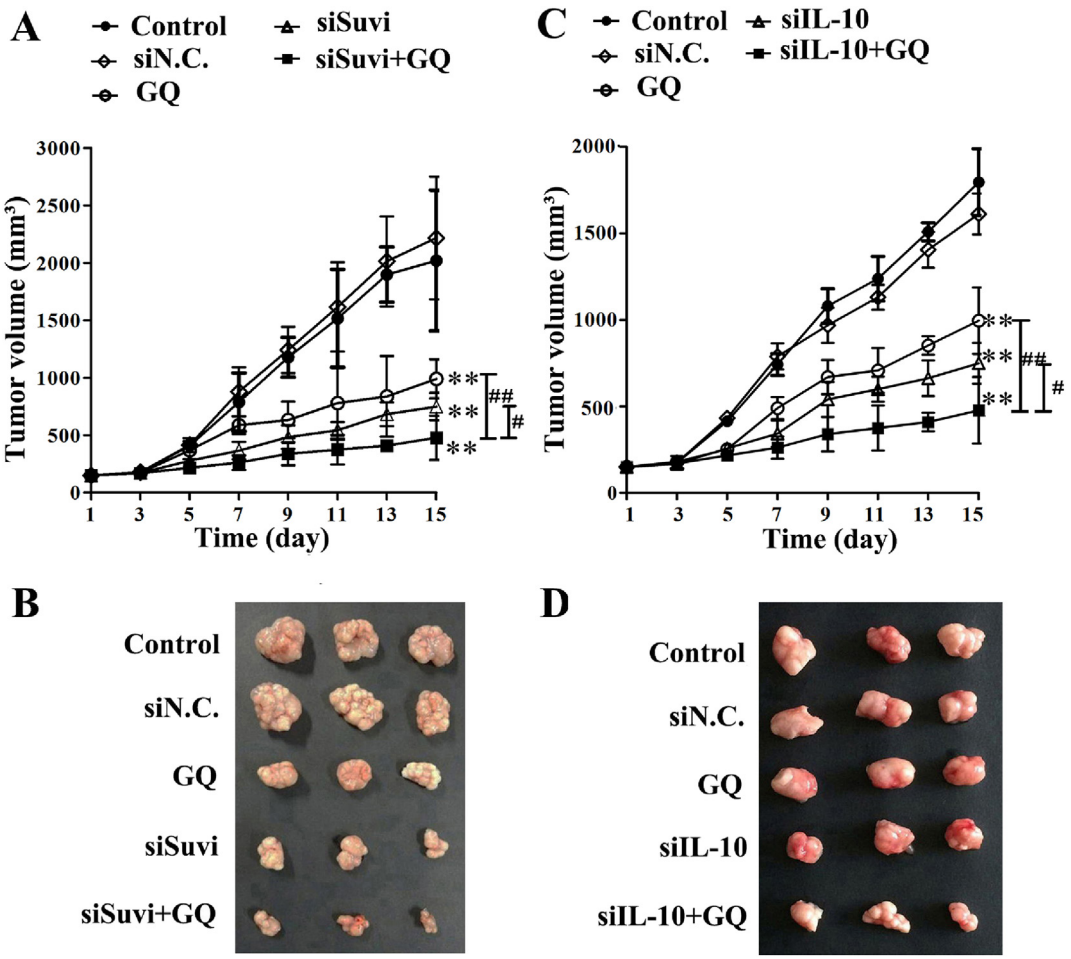

Fig. 4. Inhibition effect of GQ combining with siRNA on xenograft of MCF-7 cells in mice. Tumor-bearing mice were transfected with siN.C., siSuvi or siIL-10 (0.5 mg/kg) by liposome CDO14 (1.5 mg/kg) via tail vein injection or treated with GQ (18 mg/kg) by gavage for 7 times at an interval of 2 days. (A, C) Volumes of tumor in mice during the treatment. (B, D) Tumors removed from the mice after treatment. All data represent the means ± SD of three independent experiments. $^{*}p < 0.05$, comparing with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, comparing with combination group.

significantly lower than those in siN.C. group and the control group, and GQ combining with siSuvi showed strongest inhibit effect to the tumor (Fig. 4A and B). Similarly, GQ combining with siIL-10 could markedly inhibit the growth of tumor (Fig. 4C and D). These results indicated that combination of GQ and siRNA enhanced the antitumor effect of GQ or siRNA in vivo.

3.5. Combination of GQ and siRNA affected the expression of apoptosis-related proteins and cytokines in tumor tissue

Expression levels of two apoptosis-related proteins, survivin and caspase-7, in tumor tissue were detected by western blot. The results showed that the level of survivin decreased and the level of caspase-7 increased significantly in siSuvi transfection group compared with control group and siN.C. transfection group. But the expression level of survivin didn't decrease while that of caspase-7 increased in GQ treatment group. When mice were treated with GQ and siSuvi together, the down-regulation of survivin and up-regulation of caspase-7 were more remarkable (Fig. 5A and B).

To evaluate the effect of GQ combining with siIL-10 on tumor microenvironment, the expression levels of IL-10 and TNF-α in tumor tissues were detected by ELISA. The results showed that GQ or siIL-10 alone decreased the level of immunosuppressive factor IL-10 (Fig. 5C) and increased the level of TNF-α (Fig. 5D). However, siSuvi did not affect the expression of IL-10 and TNF-α. GQ combining with siIL-10 showed much stronger regulating effect on cytokines in tumor than GQ or siIL-10 alone.

The above results revealed that siSuvi played an antitumor effect by promoting apoptosis while siIL-10 played an antitumor role by regulating immune factors in tumor microenvironment. GQ has dual functions of promoting apoptosis and immunomodulation. GQ and siRNA could inhibit the growth of tumor or regulate the tumor microenvironment synergistically.

4. Discussion

Our previous study had shown that GQ could inhibit the proliferation and promote the apoptosis of MCF-7 cells while it had no effect on the proliferation of normal breast epithelial MCF-10 cells [4]. The present study proved that GQ had inhibition effect on the xenograft of MCF-7 cells in mice and indicated that GQ combining with siRNA had stronger effects to MCF-7 cells both in vitro and in vivo compared with GQ or siRNA individually. In addition, we evaluated the antiproliferation effect of GQ combining with siSuvi to triple negative breast cancer MDA231 cells and found that both siSuvi and GQ inhibited the proliferation of MDA231 cells, their combination promoted the inhibition effect of either of them (S-Fig. 1).

Survivin, as an apoptosis inhibitor, is over expressed in almost all human tumors [15,17]. It not only inhibits the activation of apoptosis executers caspase-3 and casepase-7 [18,25,26], but also contributes to tumor resistance against chemotherapeutic drugs [16,27]. Preclinical studies showed that disrupting survivin by using a survivin-specific

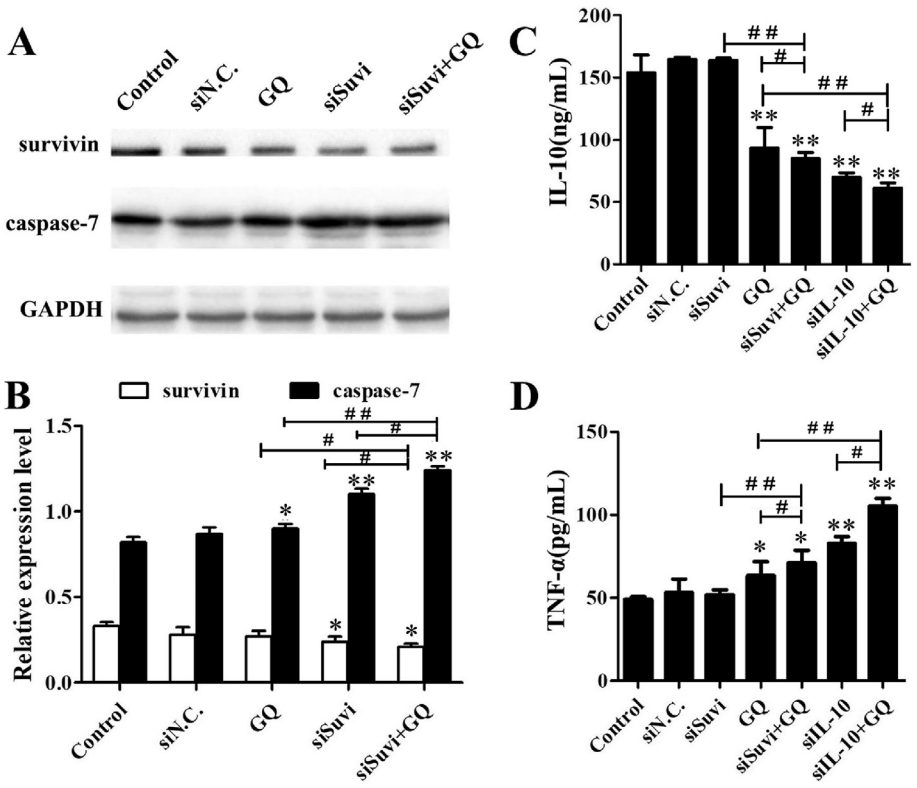

Fig. 5. Effects of GQ combining with siRNA on the expression of proteins and cytokines in tumor tissue. (A, B) The expression levels of survivin and caspase-7 in tumor tissue were detected by western blot. (C, D) The expression levels of IL-10 and TNF-α in tumor tissue were detected by ELISA. All data represent the means ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, comparing with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, comparing with combination group.

shRNA cocktail can significantly inhibit tumor cells both in vitro and in vivo [28,29]. RNAi has been utilized for depressing survivin in tumor cells [13,30]. Li reported that both mRNA and protein levels of survivin in MCF-7 cells were down regulated by siSuvi RNA transfection [31]. Similarly, we found that siSuvi could inhibit the proliferation of MCF-7 cells and the growth of xenograft in mice, indicating that RNAi against survivin is an effective method for breast cancer treatment. This study also demonstrated that silencing survivin gene decreased the expression of survivin and increased the expression of caspase-7 in MCF-7 cells and tumor tissues. Our previous study had documented that GQ induced apoptosis of MCF-7 cells via a caspase-independent Endo G-mediated mitochondria pathway [22]. These suggested that GQ and siSuvi induced apoptosis of MCF-7 cells via different pathways, which may led to their synergetic antitumor effect.

High levels of immune suppressive cytokines, such as TGF-β and IL-10, play an important role in the formation of tumor microenvironment [19,20,32]. The effect of IL-10 on promoting cancer growth and metastasis had been reported [33]. Pradhan's study indicated that silencing IL-10 gene could inhibit B cell lymphoma and affect cytokine balance in dendritic cells [34]. IL-10 has a strong inhibitory effect on the activity of antigen-presenting cells (APCs) which can secrete many kinds of cytokine [35]. Among them, TNF-α is considered as an endogenous tumor killer and takes a crucial part in the process of antitumor [36]. Previous studies have demonstrated that IL-10 suppressed the production of TNF-α in human cells [37,38]. In this study, we found that siIL-10 inhibited the growth of tumor in mice, decreased the level of IL-10 and increased the level of TNF-α, which indicated that siIL-10 could inhibit breast cancer by regulating tumor microenvironment. Furthermore, TNF-α has been reported to increase the production of reactive oxygen species (ROS) in human leukemia U937 cells which may be important mediators triggering cell life or death [39,40]. Our previous study had found that GQ could promote ROS generation in gastric cancer cells [6] and lung cancer cells [5] and then induce apoptosis. So, we speculated that combination of GQ and siIL-10 augmented ROS generation in tunmor cells and in microenvironment, which may led to their synergetic antitumor effect.

A growing number of studies have confirmed that administrating drugs and siRNA simultaneously would be more effective than treating the cancer cells with either siRNA or drugs alone [30]. Sisuvi can significantly enhance the sensitivity of MCF-7 cells to epirubicin or paclitaxel and promote cell apoptosis in vitro [41]. Although the combination of immunotherapy and chemotherapy has been implemented in the treatment of many types of cancer [19,42], the combining use of siIL-10 and chemical drugs has not been reported. In this study, siIL-10 and GQ showed synergetic effects on inhibiting the growth of tumor in mice and the expression of TNF-α in tumor microenvironment.

Although both in vitro and in vivo experiments showed that GQ combining with siSuvi and siIL-10 had better anti-tumor effect than either of them, the difference in administration ways of GQ and siRNA brought enormous inconvenience to treatment. Our study indicated that siRNA could be efficiently delivered by cationic liposome CDO14 to tumor via tail intravenous injection. But GQ with low water solubility has to be given by gavage. Liposomes with lipid bilayer structure can be used as good carriers of lipid-soluble drugs [30,43]. Some drug carrying liposomes have been used to tumor therapy in clinic, such as doxorubicin liposome and paclitaxel liposome [44]. Therefore, we had tried to load GQ and siRNA with cationic liposome CDO14 at the same time, so as to achieve co-delivery and make collaborative therapy easier to implement. However, the drug loading capacity of cationic liposome is relatively lower compared with neutral liposomes, which will be the next step for us to solve.

5. Conclusion

The present study demonstrated that GQ combining with siSuvi or siIL-10 had synergistic effect to breast cancer. GQ enhanced the pro-apoptosis effect of siSuvi to cancer cells and the regulation effect of siIL-10 to tumor microenvironment. Our findings highlight that GQ combined with siRNA is a promising strategy for the treatment of breast cancer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.047.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Yuhong Zhen and Shubiao Zhang designed research;Yameng Jiang, Guoliang Liu, Enxia Zhang and Ze Liang peformed research; Jie Zhu, Wei Wang and Yuling Chen analyzed the data; Yinan Zhao, Hong Xu and Jiasi Liu contributed new analytical tools and reagents; Jiaxin Zuo and Yuhong Zhen wrote the paper.

Acknowledgements

This research was supported by the National High-tech R&D Program (863 Program) of China (Project No. 2014AA020707), the National Natural Science Foundation of China (Project No. 21776044/21606041/21503015), the Fundamental Research Funds for the Central Universities and the Natural Science Foundation of Liaoning Province China (Project No. 20170540233).

References

[1] M. Vergine, P. Scipioni, S. Garritano, M. Colangelo, A. Dipaolo, G. Livadoti, A. Maturo, M. Monti, Breast-conserving surgery after neoadjuvant chemotherapy in patients with locally advanced cancer. Preliminary results, G. Chir. 34 (2013) 254–256.
[2] M. Filipits, G. Pohl, M. Rudas, O. Dietze, S. Lax, R. Grill, R. Pirker, C.C. Zielinski, H. Hausmaninger, E. Kubista, H. Samonigg, R. Jakesz, Clinical role of multidrug resistance protein 1 expression in chemotherapy resistance in early-stage breast cancer: the Austrian Breast and Colorectal Cancer Study Group, J. Clin. Oncol. 23 (2005) 1161–1168.
[3] R.S. Kerbel, G. Klement, K.I. Pritchard, B. Kamen, Continuous low-dose anti-angiogenic/metronomic chemotherapy: from the research laboratory into the oncology clinic, Ann. Oncol. 13 (2002) 12–15.
[4] H. Liao, X.R. Bao, J. Zhu, J. Qu, Y. Sun, X.D. Ma, E.X. Wang, X. Guo, Q. Kang, Y.H. Zhen, *O*-alkylated derivatives of quercetin induce apoptosis of MCF-7 cells via a caspase-independent mitochondrial pathway, Chem. Biol. Interact. 242 (2015) 91–98.
[5] E.X. Wang, B.Y. Zou, L. Shi, L.Y. Du, Y.Y. Zhu, Y.M. Jiang, X.D. Ma, X.H. Kang, C.Y. Wang, Y.H. Zhen, L.D. Sun, 7-*O*-geranylquercetin-induced autophagy contributes to apoptosis via ROS generation in human non-small cell lung cancer cells, Life Sci. 180 (2017) 102–113.
[6] Y.Y. Zhu, Y.M. Jiang, L. Shi, L.Y. Du, X.D. Xu, E.X. Wang, Y. Sun, X. Guo, B.Y. Zou, H.X. Wang, C.Y. Wang, L.D. Sun, Y.H. Zhen, 7-*O*-Geranylquercetin induces apoptosis in gastric cancer cells via ROS-MAPK mediated mitochondrial signaling pathway activation, Biomed. Pharmacother. 87 (2017) 527–538.
[7] G. Kseniya, S.W. Mark, Therapeutic siRNA: principles, challenges, and strategies, Yale J. Biol. Med. 85 (2012) 187–200.
[8] E. Ashihara, E. Kawata, T. Maekawa, Future prospect of RNA interference for cancer therapies, Curr. Drug Targets 11 (2010) 345–360.
[9] J.C. Burnett, J.J. Rossi, RNA-based therapeutics-current progress and future prospects, Chem. Biol. 19 (2012) 60–71.
[10] P.D. Zamore, B. Haley, Ribo-gnome: the big world of small RNAs, Science 309 (2005) 1519–1524.
[11] Y.K. Oh, T.G. Park, siRNA delivery systems for cancer treatment, Adv. Drug Deliver. Rev. 61 (2009) 850–862.
[12] K. Gao, L. Huang, Non-viral methods for siRNA delivery, Mol. Pharm. 6 (2009) 651–658.
[13] F. Takeshita, T. Ochiya, Therapeutic potential of RNA interference against cancer, Cancer Sci. 97 (2006) 689–696.
[14] Y.N. Zhao, S.B. Zhang, Y. Zhang, S.H. Cui, H.Y. Chen, D.F. Zhi, Y.H. Zhen, S.F. Zhang, L. Huang, Tri-peptide cationic lipids for gene delivery, J. Mater. Chem. B 3 (2015) 119–126.
[15] D.C. Altieri, Validating survivin as a cancer therapeutic target, Nat. Rev. Cancer 3 (2003) 46–54.
[16] S. Fukuda, L.M. Pelus, Survivin, a cancer target with an emerging role in normal adult tissues, Mol. Cancer Ther. 5 (2006) 1087–1098.
[17] G. Ambrosini, D.C. Altieri, A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma, Nat. Med. 3 (1997) 917–921.
[18] F. Li, G. Ambrosini, E.Y. Chu, J. Plescia, S. Tognin, P.C. Marchisio, D.C. Altieri, Control of apoptosis and mitotic spindle checkpoint by survivin, Nature 396 (1998) 580–584.
[19] L.A. Emens, G. Middleton, The interplay of immunotherapy and chemotherapy: harnessing potential synergies, Cancer Immunol. Res. 3 (2015) 436–443.
[20] F. Milano, T. Jorritsma, A.M. Rygiel, J.J. Bergman, C. Sondermeijer, A.T. Brinke, S.M. VanHam, K.K. Krishnadath, Expression pattern of immune suppressive cytokines and growth factors in oesophageal adenocarcinoma reveal a tumour immune escape-promoting microenvironment, Scand. J. Immunol. 68 (2010) 616–623.
[21] M.H. Qu, R.F. Zeng, S. Fang, Q.S. Dai, H.P. Li, J.T. Long, Liposome-based co-delivery of siRNA and docetaxel for the synergistic treatment of lung cancer, Int. J. Pharm. 474 (2014) 112–122.
[22] X.R. Bao, H. Liao, J. Qu, Y. Sun, X. Guo, E.X. Wang, Y.H. Zhen, Synthesis, characterization and cytotoxicity of alkylated quercetin derivatives, Iran. J. Pharm. Res. 15 (2016) 329–335.
[23] G.Q. Hou, L.X. Xue, Z.M. Lu, T.L. Fan, F. Tian, Y.L. Xue, An activated mTOR/p70S6K signaling pathway in esophageal squamous cell carcinoma cell lines and inhibition of the pathway by rapamycin and siRNA against mTOR, Cancer Lett. 253 (2007) 236–248.
[24] Y. Tang, F. Wang, C. Jin, H. Liang, X.H. Zhong, Y.J. Yang, Mitochondrial injury induced by nanosized titanium dioxide in A549 cells and rats, Environ. Toxicol. Phar. 36 (2013) 66–72.
[25] K. Jha, M. Shukla, M. Pandey, Survivin expression and targeting in breast cancer, Surg. Oncol. 21 (2012) 125–131.
[26] I. Tamm, Y. Wang, E. Sausville, D.A. Scudiero, N. Vigna, T. Oltersdorf, J.C. Reed, IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs, Cancer Res. 58 (1998) 5315–5320.
[27] G. Himani, S. Prerna, J.C. Gupta, G.P. Talwar, D. Shweta, Survivin: a unique target for tumor therapy, Cancer Cell Int. 16 (2016) 1–14.
[28] H. Caldas, M.P. Holloway, B.M. Hall, S.J. Qualman, R.A. Altura, Survivin-directed RNA interference cocktail is a potent suppressor of tumour growth in vivo, J. Med. Genet. 43 (2006) 119–128.
[29] H. Uchida, T. Tanaka, K. Sasaki, K. Kato, H. Dehari, Y. Ito, M. Kobune, M. Miyagishi, K. Taira, H. Tahara, H. Hamada, Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth in vitro and in vivo, Mol. Ther. 10 (2004) 162–171.
[30] M. Creixell, N.A. Peppas, Co-delivery of siRNA and therapeutic agents using nanocarriers to overcome cancer resistance, Nano Today 7 (2012) 367–379.
[31] L.P. Li, N.C. Liang, C.Q. Luo, Construction of survivin siRNA expression vector and its regulation on cell cycle and proliferation in MCF-7 cells, Chin. J. Cancer 23 (2004) 742–748.
[32] S. Sharma, M. Stolina, Y. Lin, B. Gardner, P.W. Miller, M. Kronenberg, S.M. Dubinett, T cell-derived IL-10 promotes lung cancer growth by suppressing both T cell and APC function, J. Immunol. 163 (1999) 5020–5028.
[33] L. Zeng, C. O'Connor, J. Zhang, A.M. Kaplan, D.A. Cohen, IL-10 promotes resistance to apoptosis and metastatic potential in lung tumor cell lines, Cytokine 49 (2010) 294–302.
[34] P. Pradhan, H. Qin, J. Leleux, D. Gwak, I. Sakamaki, L.W. Kwak, K. Roy, The effect of combined IL10 siRNA and CpG ODN as pathogen-mimicking microparticles on Th1/Th2 cytokine balance in dendritic cells and protective immunity against B cell lymphoma, Biomaterials 35 (2014) 5491–5504.
[35] W. Dummer, B.C. Bastian, N. Ernst, C. Schänzle, A. Schwaaf, E.B. Bröcker, Interleukin-10 production in malignant melanoma: preferential detection of IL-10-secreting tumor cells in metastatic lesions, Int. J. Cancer 66 (1996) 607–710.
[36] A.M. Wang, A.A. Creasey, M.B. Ladner, L.S. Lin, J. Strickler, J.N. Van Arsdell, R. Yamamoto, D.F. Mark, Molecular cloning of the complementary DNA for human tumor necrosis factor, Science 228 (1985) 149–154.
[37] K.W. Moore, R.D.W. Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.
[38] D.I. Shin, U. Banning, Y.M. Kim, J. Verheyen, M. Hannen, H. Bnig, D. Krholz, Interleukin 10 inhibits TNF-alpha production in human monocytes independently of interleukin 12 and interleukin 1 beta, Immunol. Investig. 28 (1999) 165–175.
[39] D.O. Moon, M.O. Kim, J.D. Lee, Y.H. Choi, G.Y. Kim, Rosmarinic acid sensitizes cell death through suppression of TNF-alpha-induced NF-kappaB activation and ROS generation in human leukemia U937 cells, Cancer Lett. 288 (2010) 183–191.
[40] M. Jamaluddin, S. Wang, I. Boldogh, B. Tian, A.R. Brasier, TNF-α-induced NF-κB/RelA Ser^{276}, phosphorylation and enhanceosome formation is mediated by an ROS-dependent PKAc pathway, Cell. Signal. 19 (2007) 1419–1433.
[41] H.L. Dong, L.Y. Yao, W.L. Bi, F.S. Wang, W. Song, Y.G. Lv, Combination of survivin siRNA with neoadjuvant chemotherapy enhances apoptosis and reverses drug resistance in breast cancer MCF-7 cells, J. Cancer Res. Ther. 11 (2015) 717–723.
[42] R.A. Lake, B.W. Robinson, Immunotherapy and chemotherapy–a practical partnership, Nat. Rev. Cancer 5 (2005) 397–405.
[43] V. Joguparthi, T.X. Xiang, B.D. Anderson, Liposome transport of hydrophobic drugs: gel phase lipid bilayer permeability and partitioning of the lactone form of a hydrophobic camptothecin, DB-67, J. Pharm. Sci. 97 (2010) 400–420.
[44] C. Main, L. Bojke, S. Griffin, G. Norman, M. Barbieri, L. Mather, D. Stark, S. Palmer, R. Riemsma, Topotecan, pegylated liposomal doxorubicin hydrochloride and paclitaxel for second-line or subsequent treatment of advanced ovarian cancer: a systematic review and economic evaluation, Health Technol. Asses. 10 (2006) 1–132.