Evaluation of antitumor activity of survivin short interfering RNA delivered by lipid nanoparticles in colon cancer *in vitro* and *in vivo*

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Received September 23, 2015; Accepted March 14, 2017

DOI: 10.3892/ol.2017.6404

Abstract. Survivin has been overexpressed in numerous types of cancer and is associated with a poor clinical outcome. A number of various approaches have been used to counteract survivin in order to inhibit tumor growth or promote cell apoptosis. The present study aimed to evaluate the efficiency and antitumor effect of a survivin-targeted short interfering RNA (siRNA) delivery system using lipid nanoparticles for the treatment of colon cancer. Survivin siRNA (si-survivin) nanoliposomes were prepared and transfected into LoVo cells. The mRNA expression level of survivin was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. Cell viability was evaluated by MTT assay. LoVo-bearing nude mice were treated with si-survivin intratumorally or intravenously. Tumor growth in LoVo-bearing mice was monitored and recorded, and tumor samples were obtained for evaluation of survivin expression levels using RT-qPCR, western blotting and immunohischemical staining. The expression level of survivin was significantly reduced by nanoliposomal si-survivin along with cell proliferation inhibition *in vitro*. Intravenous administration of si-survivin nanoliposomes may significantly inhibit tumor growth with less toxicity compared with doxorubicin hydrochloride treatment in LoVo-bearing mice. Nanoliposomal si-survivin may significantly reduce the expression level of survivin and inhibit cell proliferation of colon cancer cells *in vitro* and *in vivo*. si-survivin delivered by lipid nanoparticles may be a potential treatment approach for colon cancer.

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Abbreviations: siRNA, short interfering RNA; IAP, inhibitor of apoptosis; PLA, poylactic acid; PEI, polyethilenimine; MTT, methyl thiazolyl tetrazolium; IHC, immunohistochemistry; H&E, hematoxylin and eosin

Key words: survivin, siRNA, nanoliposome, colon cancer, xenograft

Introduction

Survivin, a member of the inhibitor of apoptosis (IAP) gene family, has previously been reported as overexpressed in numerous types of cancer and was associated with poor clinical outcome (1-3). Survivin contains a baculovirus IAP repeat, but does not contain a carboxyl-terminal RING finger. It is prominently expressed in various cancer cell lines and in numerous human types of cancer, including colon, lung and breast cancer (1). The potential of survivin as a biological target for anticancer therapies has been widely studied (4-7). The anticancer effects of survivin inhibition has been demonstrated in melanoma cells (8), human colon cancer cells (9) and K562 human leukemia cell line (10).

Short interfering RNA (siRNA)-mediated gene silencing is gradually becoming a powerful tool used to reduce abnormally high expression for target genes, which results in its potential applications in cancer therapy. Synthetic siRNA has been used for targeting oncogenes and genes involved in various stages of cancer cells, including proliferation, metastasis and apoptosis (11,12). However, the broad applications of siRNA in cancer therapy are based on a well-designed delivery system that is able to efficiently deliver siRNA molecules into tumors or target cells (13,14). Systemic therapeutic use of siRNA has major limitations, including rapid degradation by nucleases and renal clearance (15).

Nanocarriers are submicron size particles, ranging from 1 to 1,000 nm in diameter (16). They are able to overcome the majority of obstacles that limit the therapeutic use of siRNA (17,18). Nanoparticles are made of various biodegradable nanomaterials, including liposomes, poylactic acid and polyethilenimine (19). The nanoliposomal siRNA carrier has been demonstrated to efficiently carry and deliver siRNA in *in vivo* systems (20,21). The present study aimed to investigate the antitumor effect of survivin siRNA (si-survivin) delivered by lipid nanoparticles. The results revealed that nanoliposomal si-survivin may significantly reduce the expression level of survivin and inhibit cell growth *in vitro* and *in vivo*.

Materials and methods

Cell culture. LoVo, a human colon cancer cell line, was obtained from Biomics Biotechnologies Co., Ltd. (Nantong, China) and maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare, Logan, UT, USA). The medium was supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). The LoVo cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Nanoliposomal siRNA construction. The siRNA oligonucleotides targeting human survivin (si-survivin) were designed and synthesized by Biomics Biotechnologies Co., Ltd. The sequence of si-survivin was as follows: Sense, 5'-GCA UCU CUA CAU UCA AGAA-3' and anti-sense, 5'-UUC UUG AAU GUA GAG AUGC-3'. A scrambled sequence was used as the negative control (si-NC) with the sequences as follows: Sense, 5'-UUC UCC GAA CGU GUC ACGU-3' and anti-sense, 5'-ACG UGA CAC GUU CGG AGAA-3'. The siRNAs were then encapsulated into disaturated phosphatidylcholine (DSPC; Avanti Polar Lipids, Alablaster, AL, USA); Avanti Polar Lipids, Alablaster, AL, USA), cholesterol, dioctadecyldimethylammonium chloride (DODAC) and N-palmitoyl-sphingosine-1-succinyl (PEG-CerC16; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 100% ethanol at a 25/45/25/2.5 molar ratio. The average diameter of nanoliposomal si-survivin and si-NC was 70.7±29.077 and 64.9±26.128 nm, respectively.

In vitro transfection. Cultured LoVo cells were seeded at a density of $1x10^5$ cells per well on a 24-well plate. Survivin and control siRNA-liposomes were mixed with Opti-MEM (10 μl liposomes in a total volume of 255 μl Opti-MEM; Life Technologies; Thermo Fisher Scientific, Inc.) and left to stand for 5 min at room temperature. Subsequently, nanoliposomes loaded with si-survivin or control siRNA were added to each well at a concentration of 100 nmol/l at 37˚C for 4-6 h. The mRNA level of survivin following transfection was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Total RNA isolation and RT-qRCR. Total RNA was isolated using RISO™ RNA reagent (Biomics Biotechnologies Co., Ltd.) and DNase (2.5 μl stock solution diluted to a final volume of 100 μl; Biomics Biotechnologies Co., Ltd.) was used for DNA digestion during the extraction procedure. cDNA was synthesized using PrimeScript reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. The mRNA level of survivin was determined by qPCR using SensiMix™ One-Step kit (Quantace, Taunton, MA, USA). RT-qPCR was performed on the ABI PRISM Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using 2X One-Step qPCR mix (12.5 μl), 50X SYBR-Green I (0.5 μl), primers (0.5 μl), and cDNA template (100 ng in a total volume of 4 μl; all from Quantace; Bioline USA, Inc., Taunton, MA, USA). The conditions for RT-qPCR were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 20 sec, 58˚C for 30 sec and 72˚C for 30 sec. Specific primers were used to detect survivin: Forward, 5'-ACG ACC CCA TAG AGG AAC AT-3' and reverse, 5'-TCC GCA GTT TCC TCA AAT TC-3'. The housekeeping gene GAPDH was amplified as the internal control using specific primers: Forward, 5'-GAA GGT GAA GGT CGG AGT C-3' and reverse, 5'-GAA GAT GGT GAT GGG ATT TC-3'. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (22). All analyses were performed in triplicate.

Western blot analysis. To determine the protein expression level of survivin following LoVo cellular uptake of the si-survivin complex, total protein was extracted using lysis buffer supplemented with 25 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l EGTA, 5 mmol/l EDTA, 10 mmol/l NaF, 1 mmol/l phenylmethyl sulfonylfluoride, 1% TritonX-100, 0.5% Nonidet P40, 10 mg/l aprotinin and 10 mg/l leupeptin, as previously described (10). Proteins were quantified using a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol, and equal amounts of protein were subjected to 8% SDS-PAGE. The primary antibodies used for western blotting were rabbit polyclonal anti-survivin antibody (dilution, 1:500; cat. no. ab469; Abcam, Cambridge, UK) and mouse anti-β-actin monoclonal antibody (dilution, 1:400; cat. no. BM0005; Wuhan Boster Biological Technology, Ltd., Wuhan, China) overnight at 4˚C. The secondary antibodies used were relative horseradish peroxidase-conjugated secondary IgG antibodies (dilution, 1:500, cat. nos. BA1054 and BA1051; Wuhan Boster Biological Technology, Ltd.) at room temperature for 2 h. An enhanced chemiluminescence system (Sino-American Biotechnology Co., Ltd., Luoyang, China) was used to detect the expression levels of proteins. Band intensity quantification was performed using Image J software version 1.441 (National Institutes of Health, Bethesda, MA, USA).

MTT assay. To detect the effect of si-survivin on the viability of LoVo cells, MTT assay was performed using MTT reagent (Sigma-Aldrich; Merck KGaA), according to the manufacture's protocol. Briefly, $5x10^3$ cells were seeded into 96-well plates following overnight growth and were incubated with si-survivin complex (100 nmol/l) or nanoliposomes with si-NC. MTT was added 48 h after uptake of siRNA and the cells were incubated at 37˚C for an additional 4 h. Absorbances were determined 4 h after the addition of MTT and the optical density value at a wavelength of 570 nm was determined.

In vivo tumorigenicity assay. Male athymic BALB/c nude mice (20-24 g, 7-9 weeks old), were obtained from Shanghai Laboratory Animal Center (Shanghai, China). Mice were maintained in specific pathogen-free conditions with free access to food and water, under a constant temperature of 22±2˚C and a 12 h light/12 h dark cycle (7:00 a.m. to 7:00 p.m.). All animal experiments were approved by the Ethics Committee of the Capital Institute of Pediatrics (Beijing, China). Cells were trypsinized, washed and re-suspended with sterile PBS. A total of 200 μl cell suspension ($6x10^6$ cells) was injected subcutaneously into the forelimb area of male BALB/c athymic nude mice 4-6 weeks of age. When the xenograft tumors grew to ~1 cm in diameter, the mice were sacrificed and the tumors were obtained. The xenograft tumors were

than sectioned into tissue blocks (1x1x1 mm) and implanted into the right flank of forelimb area of healthy male BALB/c athymic nude mice by subcutaneous injection. The mice were then randomly divided into four groups (6 mice/group). Mice treated with nanolipsomal-si-NC complex at a concentration of 3 mg/kg (twice a week for 5 weeks) were in the NC group. Mice from the doxorubicin hydrochloride (DOX; Shenzhen Wanle Pharmaceutical Co., Ltd., Wuhan, China). group were treated with an intraperitoneal injection of 2.5 mg/kg (once a week for 5 weeks) DOX as the positive control. Mice treated with nanolipsomal-si-survivin intravenously (3 mg/kg, twice a week for 5 weeks) or intratumorally (50 μg per mouse, twice a week for 5 weeks) were the SU-IV or SU-IT groups, respectively. DOX was administrated once a week, whereas siRNA-nanoliposomes were administrated twice a week. All experimental procedures were performed according to the guidelines of the Beijing Children's Hospital, Capital Medicine University (Beijing, China). The present study was approved by the Ethics Committee of The Beijing Children's Hospital.

Tumor size was evaluated every 5 days using a caliper and tumor volume was determined using the formula: Volume = length x width x width/2. The length was the largest perpendicular diameter and the width was the smallest. Following 33 days of treatment, mice were sacrificed. Tumors were obtained, weighed and stored at -80˚C for further analysis. The relative tumor proliferation rate, which was using to evaluate the effect of the treatment, was calculated by the following formula:

$$\text{Inhibition rate (\%)} = \left[1 - \frac{\text{Tumor weight (treatment)}}{\text{Tumor weight (control)}}\right] \text{ x } 100$$

Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining. Tumor tissues were fixed in 4% paraformaldehyde at 4˚C for at least 24 h and embedded in paraffin blocks to obtain longitudinal and transverse sections. The sliced sections were then used to perform IHC and H&E staining. IHC staining of samples was performed as previously described (23) and the primary antibody used was the rabbit polyclonal anti-survivin antibody (1:500, cat. no. ab76424, Abcam). The sliced sections were stained with hematoxylin for 10 min followed by staining with eosin for 1-3 min at room temperature, as described previously (24). Representative areas of digital photomicrographs from each group were selected at a fixed magnification of x100 using a Nikon 50i light microscope (Nikon Corporation, Tokyo, Japan).

Statistical analysis. Data is presented as the mean ± standard deviation. Statistical significance was assessed using SPSS version 17.0 (IBM SPSS, Armonk, NY, USA). Significance among groups was analyzed using one-way analysis of variance, followed by Bonferroni's post-hoc test to determine the differences between groups and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Nanoliposomal si-survivin inhibits cell proliferation in vitro. The expression levels of survivin in LoVo cells were detected by RT-qPCR. As presented in Fig. 1A, the mRNA expression level of survivin was significantly inhibited in the group treated with nanoliposomal si-survivin compared with the normal and NC groups. In order to investigate the effect of nanoliposomal si-survivin on the proliferation of colon cancer, an MTT assay was performed. The results demonstrated that the proliferation of LoVo cells was significantly inhibited by the treatment of nanoliposomal si-survivin (Fig. 1B). No significant difference was revealed in the nanoliposomal NC siRNA nanoliposome-transfected cells and normal groups.

Nanoliposomal si-survivin inhibits proliferation of colon cancer cells in vivo. The proliferation inhibition effect of nanoliposomal si-survivin was further investigated *in vivo*. Tumor growth was significantly reduced in the si-survivin nanoliposomes treatment group compared with the NC group ($P=0.031$, SU-IV group vs. NC group). The antitumor effect of DOX was greater compared with the effect of nanoliposomal si-survivin. However, 3 mice in the DOX group succumbed prior to the end of the experiment and the average body weight in the DOX group was significantly lower compared with in other groups, suggesting that DOX treatment may have increased toxicity (Table I). Mice treated with si-survivin nanoliposomes intratumorally or intravenously, had similar body weights compared with mice in the NC group (Table I). The tumor inhibition rate of each treatment group is presented in Table I. The results revealed that the inhibition rates of DOX treatment and intravenous administration of nanoliposomal si-survivin were 68.9 and 31.1%, respectively; whereas, intratumoral administration of si-survivin nanoliposomes did not exhibit any obvious antitumor effects. Furthermore, tumor growth was significantly inhibited with the treatment of nanoliposomal si-survivin (Table II).

The mRNA and protein expression levels of survivin in tumor tissues were detected by RT-qPCR and western blot analysis, respectively. It was demonstrated that the mRNA and protein expression levels of survivin were significantly reduced following si-survivin treatment, intratumorally and intravenously, compared with the NC group, which was treated with nanolipsomal-si-NC complex. The expression levels of survivin were decreased following DOX treatment but this was not significant (Fig. 2). The results of IHC indicated that survivin was mainly located in the cytoplasm and that the expression level of survivin was reduced following si-survivin treatment (Fig. 3). Tumor histological examination was detected by H&E staining and the results are presented in Fig. 4. The expression level of survivin was lower in the DOX group than in the NC group (Fig. 4). These results suggested that intravenous injection of nanoliposomal si-survivin may significantly inhibit tumor growth in mice, and may be less toxic compared with DOX treatment.

Discussion

Survivin serves an important role in cell apoptosis and acts as a suppressor of apoptosis. It has been reported as strongly expressed in numerous types of common human neoplasms, and was associated with prognostic relevance, ionizing radiation and cell resistance to antitumor agents (25). These findings suggest that survivin may be a promising target for novel antitumor therapies. In recent years, a number of various approaches have used to counteract survivin to inhibit tumor

Table I. Effect of antitumor activity of survivin-targeted short interfering RNA nanoliposomes.

Group	Mice, n		Body weight, g		Tumor weight, g	Inhibition rate, %
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment		
NC-IV	6	6	18.80±0.76	20.30±1.37	1.64±0.45	/
DOX	6	3	19.40±1.10	15.70±2.30	0.51±0.21	68.90
SU-IT	6	6	19.10±0.38	21.70±1.51	1.53±0.12	6.71
SU-IV	6	6	18.80±1.60	19.80±4.24	1.13±0.46	31.10

NC-IV, intravenous injection of negative control short interfering RNA; DOX, intraperitoneal injection of doxorubicin hydrochloride; SU-IV, intravenous injection of survivin short interfering RNA nanoliposome; SU-IT, intratumoral injection of survivin short interfering RNA nanoliposome.

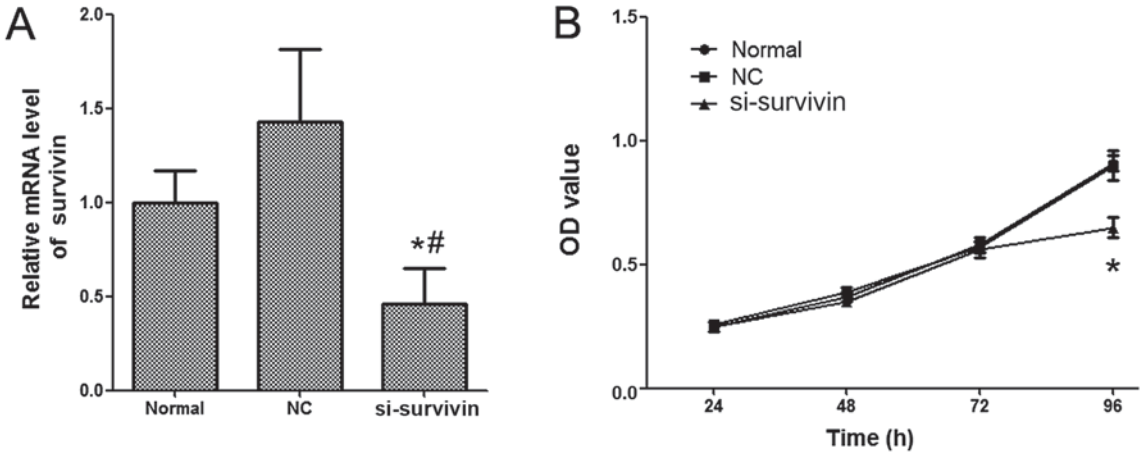

Figure 1. Nanoliposomal si-survivin inhibited cell proliferation *in vitro*. (A) The expression levels of survivin in LoVo cells were detected by reverse transcription-quantitative polymerase chain reaction. (B) MTT assay to detect the effect of nanoliposomal si-survivin on the proliferation of colon cancer cells. OD, optical density; NC, negative control; si-survivin, survivin short interfering RNA. *P<0.05 vs. normal group; #P<0.05 vs. NC group.

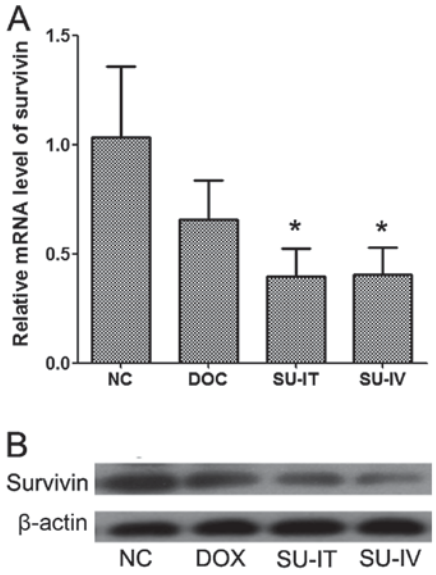

Figure 2. The mRNA and protein expression levels of survivin in tumor tissues *in vivo*. The expression levels of suvivin in tumor tissues were detected by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis. *P<0.05 vs. NC group. NC, negative control; DOX, intraperitoneal injection of doxorubicin hydrochloride; SU-IV, intravenous injection of survivin short interfering RNA nanoliposomes; SU-IT, intratumoral injection of survivin short interfering RNA nanoliposomes.

growth or promote cell apoptosis (26-28). It has been reported that survivin anti-sense oligonucleotides may specifically inhibit the expression levels of survivin mRNA and protein and reduce cell proliferation in cell lines originated from various tumors, including lung, head, neck and bladder cancer (29-31).

With the identification of RNA interference using synthetic 21-23 nucleotide RNA duplexes, si-survivins have been used for various types of cancer treatments (32,33). Carvalho *et al* (34) reported that si-survivins may specifically decrease the expression level of survivin in HeLa cells and inhibited cell growth. This study also demonstrated that si-survivins had a short half-life time and were not detected 60 h following transfection (34). Paduano *et al* (23) revealed that si-survivins markedly reduced the expression level of survivin and produced supra-additive growth suppression in human androgen-independent prostate cancer cells. Numerous previous studies have directly added siRNA mimics into cell cultures (35-37). However, the major limitations of direct addition of siRNA mimics to cells are the instability and short half-life time. It has been reported that the half-life of siRNA in serum was only ~15 min (15).

In the present study, instead of using survivin antisense oligonucleotide treatment or direct si-survivin treatment, an alternative therapeutic approach for RNA interference was

Table II. *In vivo* antitumor effects of nanonanoliposomal survivin-targeted short interfering RNA on Balb/c nude mice bearing LoVo tumor cells.

	NC-IV			DOX			SU-IT			SU-IV		
Day	Tumor volume, mm^3	RTV	T/C	Tumor volume, mm^3	RTV	T/C, %	Tumor volume, mm^3	RTV, mm^3	T/C	Tumor volume, mm^3	RTV	T/C
0	27.65±8.38	/	/	18.60±6.36	/	/	24.01±5.26	/	/	28.57±4.56	/	/
1	69.40±22.48	2.53±0.39	/	48.68±22.94	2.58±0.57	102.03	57.48±4.02	2.46±0.40	97.29	73.86±13.63	2.58±0.25	102.13
5	132.35±48.63	4.30±1.56	/	98.45±59.13	4.09±2.89	95.27	94.11±18.01	3.98±0.71	92.73	105.26±28.28	3.69±0.85	85.82
8	198.39±70.56	6.30±2.95	/	131.38±72.69	5.51±3.42	87.48	148.22±28.50	6.38±1.74	101.31	187.36±79.46	6.39±1.95	101.47
12	311.80±129.29	9.97±5.57	/	185.71±96.64	7.87±4.53	78.90	232.60±57.12	9.80±2.28	98.27	269.46±115.52	9.24±3.04	92.68
15	478.16±182.52	13.83±6.35	/	165.61±107.26	8.34±6.43	60.29	372.07±59.58	15.94±3.84	115.25	380.30±123.96	13.19±3.18	95.34
19	674.53±269.54	21.49±13.10	/	271.88±175.04	13.82±10.73	64.32	524.02±109.30	22.41±5.92	104.29	401.46±175.41	14.03±4.43	65.31
22	789.29±114.64	25.76±15.80	/	290.86±201.10	15.07±12.55	58.49	641.16±169.76	27.08±6.33	105.12	498.24±184.16	17.43±4.97	67.69
26	1,009.91±154.20	37.65±15.40	/	454.00±250.80	23.49±17.20	62.40	764.20±120.19	32.79±7.53	87.10	655.62±189.66	22.94±8.31	60.10
29	1,133.79±283.40	42.40±16.90	/	380.59±59.83	27.33±21.73	64.45	941.79±107.78	40.66±9.86	95.89	784.17±158.16	27.45±7.14	64.73
33	1,452.67±314.40	54.20±24.90	/	645.41±225.20	34.96±23.83	64.51	1,121.84±102.95	48.43±10.91	89.36	889.65±197.29	31.13±7.06	57.45

T/C, relative tumor proliferation rate; RTV, relative tumor volume compared to the tumor volume at day 0; NC-IV, intravenous injection of negative control short interfering RNA; DOX, intraperitoneal injection of doxorubicin hydrochloride; SU-IV, intravenous injection of survivin short interfering RNA nanoliposome; SU-IT, intratumoral injection of survivin short interfering RNA nanoliposome.

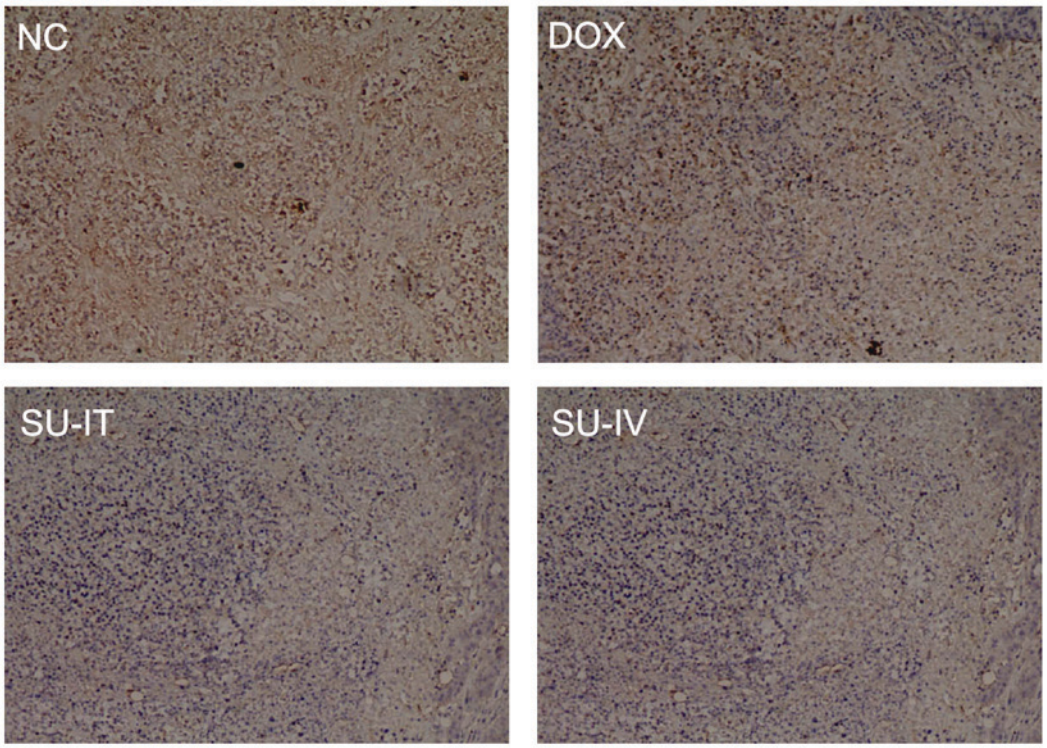

Figure 3. Tumor histological examination was detected by hematoxylin and eosin staining (magnification, all x100). NC, negative control; DOX, intraperitoneal injection of doxorubicin hydrochloride; SU-IV, intravenous injection of survivin short interfering RNA nanoliposomes; SU-IT, intratumoral injection of survivin short interfering RNA nanoliposomes.

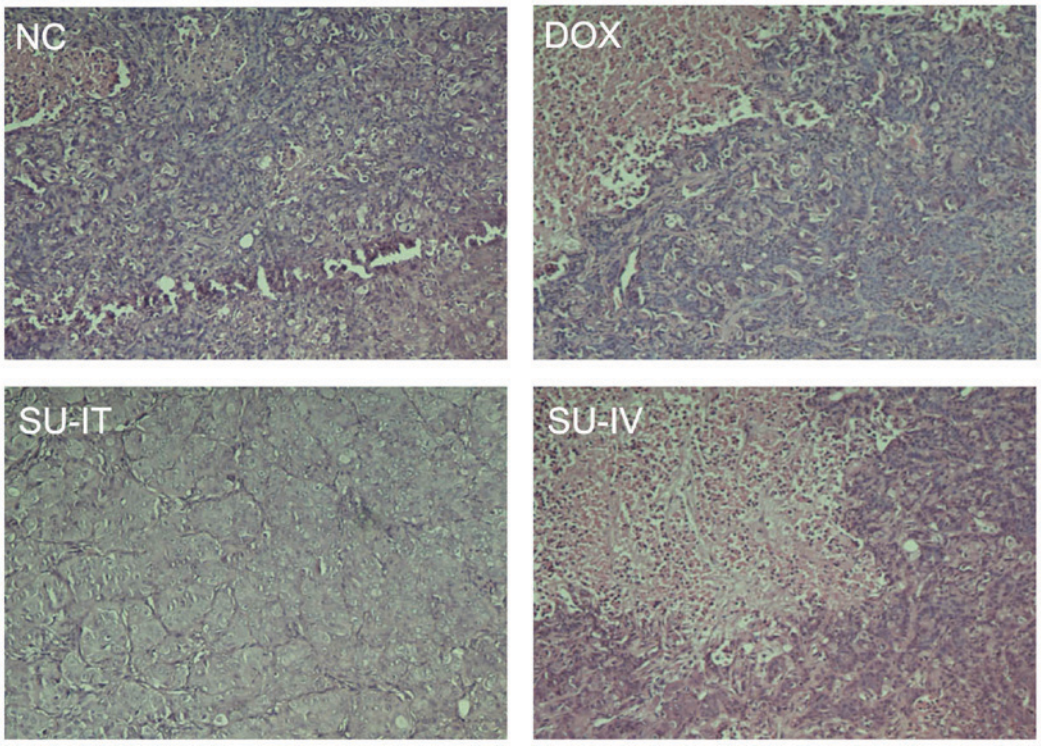

Figure 4. Survivin expression in tumor tissues was detected by immunohistochemistry assay (magnification, all x100). NC, negative control; DOX, intraperitoneal injection of doxorubicin hydrochloride; SU-IV, intravenous injection of survivin short interfering RNA nanoliposomes; SU-IT, intratumoral injection of survivin short interfering RNA nanoliposomes.

used. si-survivins were encapsulated in the nanoliposomes and then transfected into LoVo colon cancer cells. Nanocarriers have been reported to be able to effectively deliver siRNAs and may also prolong the half-lift time (17,18). Lipid nanoparticles, which have been recognized as one of the most efficient delivery systems for siRNAs, have been used extensively (38,39). In

the present study, lipid nanoparticles were synthesized using DSPC, cholesterol, DODAC and PEG-CerC16 at a 25/45/25/2.5 molar ratio. The particle diameter was ~70 nm following encapsulation with siRNAs. The nanoliposomal siRNAs effectively delivered siRNAs into target cells. The results of the present study demonstrated that the expression level of survivin was significantly reduced and cell growth was significantly inhibited following transfection with nanoliposomal si-survivin *in vitro*. Furthermore, tumor growth was significantly inhibited following systematic administration of nanoliposomal si-survivin by intravenous injection into nude mice with LoVo cell xenografts. Of note, the present study revealed that the average body weight of mice following DOX treatment was lower compared with other groups, whereas no significant changes of body weight were observed in the group treated with si-survivin nanoliposomes. A total of three mice succumbed prior to the end of the experiment in the DOX treatment group. These results suggested that lipid nanoparticles encapsulated with specific siRNAs may effectively inhibit tumor growth with less toxicity compared with traditional anticancer drugs.

In the present study, an efficient siRNA delivery system using lipid nanoparticles was utilized to investigate the potential treatment effect of si-survivin. The results demonstrated that nanoliposomal si-survivin significantly reduced the expression levels of survivin and inhibited cell growth *in vitro*. Furthermore, si-survivin nanoliposomes significantly inhibited tumor growth in nude mice bearing LoVo cell tumors with less toxicity compared with DOX. The results of the present study suggested that si-survivin delivered by nanoliposomes may be a potential therapy for colon cancer treatment.

Acknowledgements

The present study was supported by the Chinese National 863 Project (grant no. 2012AA020804).

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