Effects of RNA interference-mediated knockdown of livin and survivin using monomethoxypolyethylene glycol-chitosan nanoparticles in MG-63 osteosarcoma cells

HUA-PENG GUAN[1*], JIAN-ZHONG SUN[1*], XIAO-LEI FENG[2], JIN-SHUI CHEN[1], FANG-JING CHEN[1], XIAO-FEI CHENG[1], XIN-WEI LIU[3] and BIN NI[1]

[1]Department of Spine Surgery, Changzheng Hospital, Second Military Medical University, Shanghai 200003; [2]Department of Orthopedics, People's Liberation Army 456 Hospital, Jinan, Shandong 250031; [3]Department of Orthopedics, General Hospital of Shenyang Military Area Command of Chinese People's Liberation Army, Rescue Center of Severe Wound and Trauma of Chinese People's Liberation Army, Shenyang, Liaoning 110840, P.R. China

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Abstract. MG-63 human osteosarcoma cells were transfected with short hairpin RNA (shRNA) against livin and survivin using monomethoxypolyethylene glycol-chitosan (mPEG-CS) nanoparticles (NPs) as carriers, with the aim of evaluating the effect on cell proliferation and apoptosis. mPEG-CS NPs sized ~100 nm were prepared by ionic crosslinking. mPEG-CS-livin shRNA, mPEG-CS-survivin shRNA and mPEG-CS-(livin shRNA + survivin shRNA) NPs were constructed by electrostatic adsorption at NP suspension/gene solution ratios of 3:1 to transfect MG-63 cells. The expression levels of livin and survivin mRNA and protein were measured by reverse transcription-polymerase chain reaction and western blotting, respectively. The inhibitory effects of downregulated livin and survivin expression on cell proliferation were measured using an MTT assay. The apoptosis-inducing effects of livin and surivin knockdown were investigated using a Hoechst staining kit. All shRNA groups resulted in reduced expression of livin and survivin mRNA and protein in MG-63 cells. The MTT assay and Hoechst staining indicated that simultaneous knockdown of livin and survivin genes inhibited the proliferation of MG-63 cells and promoted their apoptosis, to a greater extent than knocking down either gene individually. The simultaneous interference mediated by mPEG-CS NPs significantly reduced livin and survivin expression in MG-63 cells, suppressed proliferation and facilitated apoptosis, to a greater extent than knockdown of either livin or survivin alone were. Thus the results indicate a synergistic effect of livin and survivin.

Correspondence to: Dr Bin Ni, Department of Spine Surgery, Changzheng Hospital, Second Military Medical University, 415 Fengyang Road, Shanghai 200003, P.R. China
E-mail: nibindss@163.com; gg10536_2001@126.com

*Contributed equally

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Introduction

As the most common type of primary malignant bone tumor, osteosarcoma is typified by the formation of immature bone or bone-like tissues from proliferating tumor cells. Approximately 80% of osteosarcoma cases are observed in the long bones of limbs, with the majority at the metaphysis of knee joints, and the further 20% involving the axial skeleton and pelvis (1). The onset, progression, metastasis and prognosis of malignant tumors are closely associated with the expression of anti-apoptotic genes in tumor tissues (2). Under the protection of these genes, tumor cells survive, continuously proliferate and metastasize, leading to a poor prognosis. For example, the anti-apoptotic genes livin and survivin are highly expressed in human osteosarcoma tissues and serve important roles in the onset and progression of disease (3,4).

RNA interference is a valuable tool in the study of gene function, due to its high specificity and efficiency (5). Additionally, nanoparticles (NPs) that carry genes are able to effectively deliver exogenous DNA into cells and maintain high-level expression, thereby facilitating research into gene function and gene therapy. Thus, attention has been focused on developing gene-carrying NPs for cancer therapy.

Therefore, in the current study MG-63 osteosarcoma cells were transfected with monomethoxypolyethylene glycol-chitosan (mPEG-CS) NPs carrying livin and survivin short hairpin RNAs (shRNAs). The aim of the current study was to observe the effect of knocking down livin and survivin on apoptosis, and to investigate the association between the two genes and apoptosis.

Materials and methods

Reagents. The reagents used were as follows: Sodium tripolyphosphate (TPP), Chengdu Kelong Chemical Co., Ltd.

(Chengdu, China); CS (degree of deacetylation, >90.0%), Shanghai Bo'ao Biological Technology Co., Ltd. (Shanghai, China); mPEG (5000), Sigma-Aldrich (St. Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM) culture medium, Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China); fetal bovine serum (FBS), Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China); TRIzol reagent, Thermo Fisher Scientific, Inc. (Waltham, MA, USA); reverse transcription-polymerase chain reaction kit, Takara Bio, Inc. (Otsu, Japan); trypsin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Roche Diagnostics (Basel, Switzerland); cell lysis buffer and Hoechst staining kit, Beyotime Institute of Biotechnology (Shanghai, China); Diaminobenzidine (DAB) Histochemistry kit (Thermo Fisher Scientific, Inc.); rabbit anti-human livin (cat. no. Ab5393) and survivin antibodies (cat. no. Ab76424) (1:1,000 dilution; Abcam, Cambridge, MA, USA), rabbit anti-human GAPDH polyclonal antibody (1:900 dilution; cat. no. Ab9485; Abcam) and horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G antibody (1:900 dilution; cat. no. Ab97051; Abcam).

Cell line. The MG-63 human osteosarcoma cell line was purchased from the College of Life Sciences, Wuhan University (Wuhan, China).

Synthesis of livin and survivin shRNA plasmids. According to the gene sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), the IDs for livin and survivin were NM-022161 and NM-001168, respectively. Livin and survivin shRNA plasmids were designed, synthesized and sequenced by Obio Technology (Shanghai) Co., Ltd. (Shanghai, China).

Preparation of mPEG-CS NPs. mPEG-CS NPs were prepared by ionic crosslinking as described previously (6). In brief, 50 mg CS was dissolved in 100 ml of 1% (V/V) acetic acid and stirred for 30 min until completely dissolved, following which 1 ml of 1% (V/V) mPEG solution was added and stirred for a further 2 h to form the mPEG-CS solution. Subsequently, 1 mg/ml TPP solution was slowly added whilst the solution was stirred until a visible opalescence was observed, following which the solution was stirred overnight at room temperature to produce an NP suspension. The suspension was centrifuged at 17,860 x g for 10 min, washed twice with sterile double-distilled water (ddH_2O), dispersed ultrasonically in fresh ddH_2O with the same volume, and stored at 4˚C prior to use.

Preparation of gene-carrying NPs. Gene-carrying NPs were prepared by electrostatic adsorption as described previously (7). Solutions (0.35 mg/ml, 2.5 μl each) of recombinant livin and survivin shRNA plasmids were mixed with 15 μl mPEG-CS NP solution, incubated in a 5˚C water bath for 20 min and vortexed for 30 min, forming an mPEG-CS-(livin shRNA + survivin shRNA) NP suspension. Subsequently, mPEG-CS-livin shRNA and mPEG-CS-survivin shRNA alone NP suspensions were obtained via the same method.

Cell culture. MG-63 cells were cultured in DMEM containing 10% FBS, into which streptomycin-penicillin double antibiotic solutions (Sigma-Aldrich) were added to a final concentration of 100 U/ml. The cells were then incubated in a 5% CO_2 atmosphere at 37˚C, digested with 0.25% trypsin and passaged, and cells in the logarithmic growth phase were selected for investigation.

Grouping and cell transfection. One day prior to transfection, MG-63 cells in the logarithmic growth phase were seeded into 6-well plates at a density of $2x10^5$ cells/well, and cultured in DMEM without double antibiotic solution or serum in a 5% CO_2 atmosphere at 37˚C. When 80% confluent, the cells were divided into four groups and transfected as follows: Livin + survivin interference group, transfected with mPEG-CS-(livin shRNA + survivin shRNA) NPs; livin interference group, transfected with mPEG-CS-livin shRNA NPs; survivin interference group, transfected with mPEG-CS-survivin shRNA NPs; and negative control group, transfected with mPEG-CS-blank plasmid NPs. Cells were transfected according to the instructions of the Lipofectamine 2000 kit (Thermo Fisher Scientific, Inc.) at 40-60% confluence. Following 4-6 h of transfection, the cells were cultured in complete DMEM.

Measurement of livin and survivin mRNA expression in MG-63 cells by reverse transcription-polymerase chain reaction (RT-PCR). Following 48 h of transfection, the culture medium was discarded, and 1 ml TRIzol reagent was added to extract total DNA according to the manufacturer's instructions. cDNA synthesis from total RNA (1 μg) was performed in a reaction volume of 20 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate mix, 200 units SuperScript™III reverse transcriptase (all from Thermo Fisher Scientific) and 1 μl random primers (hexanucleotide mix, 10X; Roche Applied Science, Mannheim, Germany). Initially, RNA was denatured at 65˚C for 5 min, followed by addition of the reaction mixture and reverse transcription at 50˚C for 50 min. The reaction was stopped by denaturing the enzyme at 70˚C for 15 min. The primers used for PCR amplification and the length of the amplified fragments were as follows: Livin forward, 5'-TAAAGA CAGTCCAAGTGCCT-3' and reverse, 5'-TGATGGCCTGTG TGGAAGAA-3', length of amplified fragment was 348 base pairs (bp); survivin forward, 5'-ACCACAGTCCATGCCATC AC-3' and reverse, 5'-GTTCTTGGCTTTCTCTGTCC-3', length of amplified fragment was 300 bp; and GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TGCTGT AGCCAAATTCGTTG-3', length of amplified fragment was 450 bp. The primers were supplied by Thermo Fisher Scientific. RT-PCR was conducted as follows: Reverse transcription at 50˚C for 30 min, cycling at 95˚C for 15 min, denaturation at 94˚C for 30 sec, annealing at 57˚C for 30 sec, extension at 72˚C for 30 sec (30 cycles in total), and extension at 72˚C for 10 min. PCR products were subjected to agarose gel electrophoresis.

Measurement of livin and survivin protein expression in MG-63 cells by western blotting. Following 48 h of transfection, the culture medium was discarded, and cells were washed twice with 4˚C phosphate-buffered saline (PBS), 150 μl pre-cooled cell lysis buffer was added and incubated on ice for 30 min, prior to centrifugation at 4˚C and

13,980 x g for 15 min. Subsequently, the supernatant was collected as the protein sample and quantified using a BCA kit (Sigma-Aldrich). Proteins were denatured, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Thermo Fisher Scientific), electronically transferred onto a nitrocellulose membrane and then blocked for 3 h at 37˚C using blocking buffer (Sigma-Aldrich). Membranes were incubated with primary antibodies overnight at 4˚C, washed three times with PBS and then incubated with secondary antibodies at room temperature for 2 h. After several washes with PBS, the membranes were incubated with Luminata Forte Western HRP Substrate (EMD-Millipore, Billerica, MA, USA) for 3 min or Western Bright (Advansta, Inc., Menlo Park, CA, USA) diluted 1:1 with water for 30 sec. Images were captured under red safe light on X-ray Film (Fujifilm, Tokyo, Japan) using an X-ray developing unit (Agfa, Köln, Germany) for 10 min. The DAB kit was used for color development. Relative protein expression levels were calculated from the ratios of livin/β-actin and survivin/β-actin.

Measurement of the effect of RNA interference on the inhibition of MG-63 cell proliferation using an MTT assay. Following transfection, the cells were cultured for 24, 48 and 72 h. MTT solution (10 μl, 5 mg/ml) was added to each well and cells were incubated at 37˚C for 4 h. Subsequently, the supernatant was removed, 10 μl dimethyl sulfoxide was added to each well and the cells were agitated for 10 min at 37˚C. Absorbance (A) of each well was measured with a Standard Filter-Microplate Photometer (wavelength at 490 nm; Applied Biosciences, Thermo Fisher Scientific). The inhibitory rate was calculated as follows: (IR%) = (1 - $A_{experiment\ group}/A_{blank\ control\ group}$) x 100. The values of each group are presented as the mean ± standard deviation (SD).

Effect of RNA interference on MG-63 cell apoptosis using Hoechst staining. Following 48 h of transfection, the cells were fixed according to the instructions of the Hoechst staining kit. Following the removal of the fixing solution, cells were stained whilst agitated at room temperature for 5 min. Subsequently, the staining solution was removed and the microscope slide was covered with a coverslip and sealed with antifade solution. Cell morphology was observed using an inverted fluorescence microscope (FV1200; Olympus, Tokyo, Japan) and images were captured. Five visual fields were randomly selected in each well and the cells were counted. The rate of apoptosis was calculated as follows: Apoptotic index (%) = Number of apoptotic cells/(number of normal cells + number of apoptotic cells) x 100. The data were presented as the mean ±SD.

Statistical analysis. All data were analyzed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). Inter-group differences in livin and survivin mRNA expression levels were compared by univariate analysis of variance, and pairwise comparison was performed with Fisher's least significant difference test. Inter-group differences in livin and survivin protein expression levels and the effect of RNA interference on cell survival and apoptosis were compared using the Kruskal-Wallis test, and pairwise comparison was conducted with the Bonferroni correction. $P<0.05$ was considered to indicate a statistically significant difference.

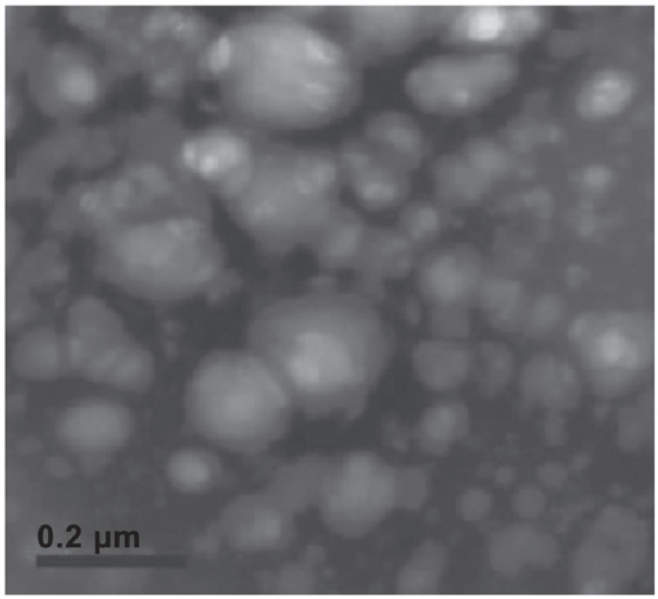

Figure 1. Transmission electron micrograph of monomethoxypolyethylene glycol-chitosan-livin and monomethoxypolyethylene glycol-chitosan-survivin short hairpin RNA nanoparticles.

Results

Morphologies of mPEG-CS, mPEG-CS-livin shRNA and mPEG-CS-survivin shRNA NPs. Transmission electron micrographs of mPEG-CS-livin shRNA and mPEG-CS-survivin shRNA NPs exhibit uniform approximate spheres sized 100-200 nm that are distributed evenly, without obvious aggregation (Fig. 1).

Inhibition of mRNA expression by RNA interference measured using RT-PCR. RT-PCR products were resolved by 2% agarose gel electrophoresis to analyze the effects of the different interference groups. Fig. 2 indicates that compared with the negative control group, the livin and livin + survivin interference groups significantly reduced the expression levels of livin mRNA ($P<0.05$). Fig. 3 indicates that compared with the negative control group, the survivin and livin + survivin interference groups significantly reduced the expression levels of survivin mRNA ($P<0.05$).

Inhibition of protein expression by RNA interference measured using western blotting. The effect of the different interference groups on livin and survivin protein expression levels were measured by western blotting. β-actin was used as the internal reference, and the relative expression levels of livin protein in the negative control, livin interference and livin + survivin interference groups were 93.31±2.47, 50.22±3.42 and 6.43±2.16%, respectively (Fig. 4). The expression levels of livin in the livin and livin + survivin interference groups were significantly downregulated compared with the negative control group ($P<0.05$).

The relative expression levels of survivin protein in the negative control, survivin interference and livin + survivin interference groups were 86.29±4.24, 57.52±4.21 and 35.73±3.14%, respectively (Fig. 5). Compared with the negative control group, survivin protein expression levels in the survivin and

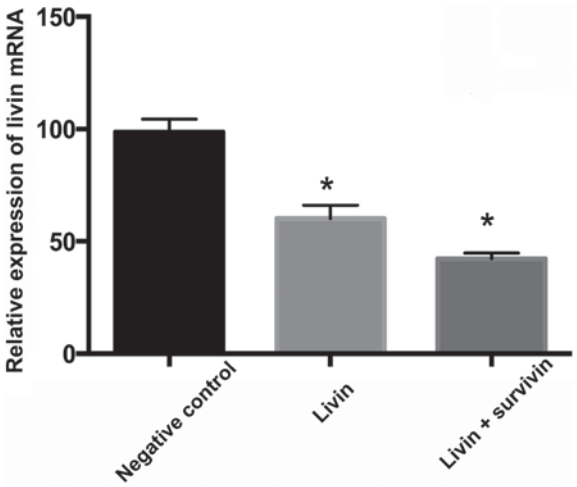

Figure 2. Effect of livin and survivin RNA interference on livin mRNA expression levels in MG-63 cells. *P<0.05 vs. the negative control group.

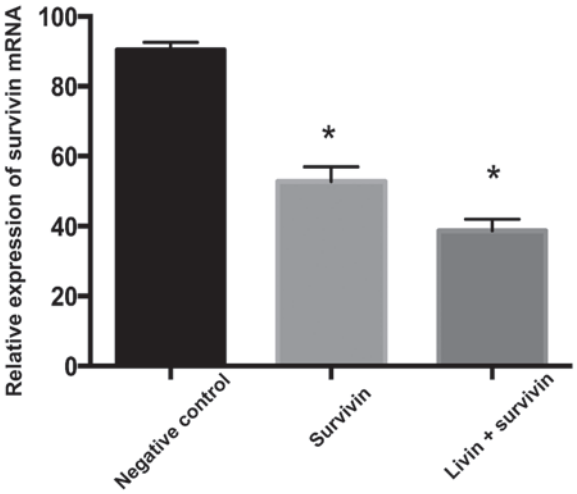

Figure 3. Effect of livin and survivin RNA interference on survivin mRNA expression levels in MG-63 cells *P<0.05 vs. the negative control group.

livin + survivin interference groups were significantly down-regulated (P<0.05).

Inhibition of MG-63 cell proliferation by RNA interference measured using an MTT assay. The inhibitory effects of RNA interference on MG-63 cell proliferation were assessed using an MTT assay. The inhibitory effects of livin, survivin and livin + survivin RNA interference compared with the proliferation in the negative control group, were significant (P<0.05) and, in the livin and livin + survivin groups, increased in a time-dependent manner. In addition, livin + survivin interference had a significantly greater effect compared with livin or survivin interference alone (P<0.05; Table I).

Morphological analysis of apoptosis. Morphological alterations associated with apoptosis were observed in MG-63 cells using a fluorescence microscope following Hoechst staining. The nuclei of the normal cells were blue, while those of apoptotic cells were fragmented, densely stained and white. The apoptotic indices of the negative control, livin, survivin

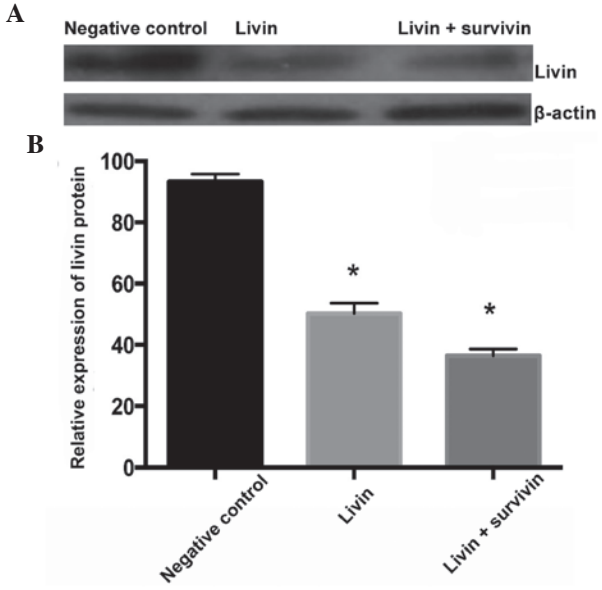

Figure 4. Inhibition of livin protein expression by RNA interference measured using western blotting. (A) Western blot image. (B) Quantification of relative expression. *P<0.05 vs. the negative control group.

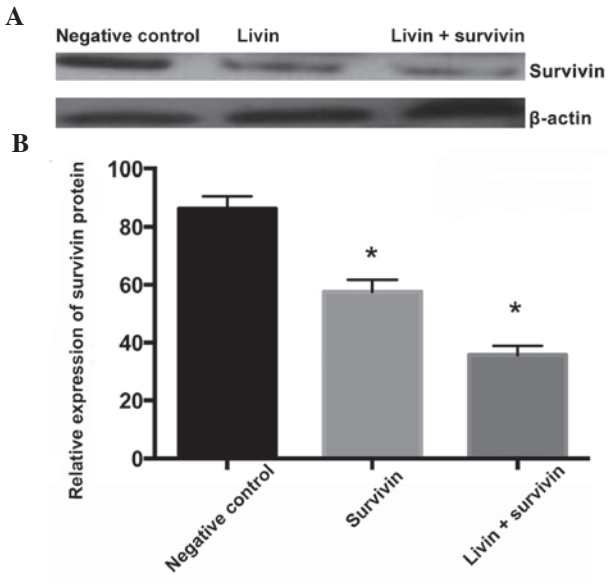

Figure 5. Inhibition of survivin protein expression by RNA interference measured using western blotting. (A) Western blot image. (B) Quantification of relative expression. *P<0.05 vs. negative control group.

and livin + survivin interference groups were 6.88±0.63, 31.76±2.45, 29.92±3.23 and 51.28±1.14%, respectively. Compared with the negative control group, the apoptotic indices of the three interference groups were significantly greater (P<0.05). In addition, livin + survivin interference induced significantly greater levels of apoptosis compared with the livin and survivin interference groups alone (P<0.05; Fig. 6).

Discussion

Originating from bone mesenchymal cells, osteosarcoma is a primary malignant bone cancer characterized by the

Table I. Rate of proliferation inhibition (%) in the livin and survivin RNA interference groups.

Group	Rate of proliferation inhibition over time (%)		
	24 h	48 h	72 h
Livin interference	31.12±1.02[a]	38.72±0.68[a]	41.29±1.51[a]
Survivin interference	34.23±0.97[a]	39.92±1.67[a]	44.71±0.96[a]
Livin + survivin interference	50.27±1.34[a,b]	54.74±0.87[a,b]	59.22±2.12[a,b]
Negative control	2.36±1.54	3.05±1.14	5.54±1.44

Values are expressed as the mean ± standard deviation. [a]P<0.05 vs. negative control group, [b]P<0.05 vs. livin and survivin interference alone groups.

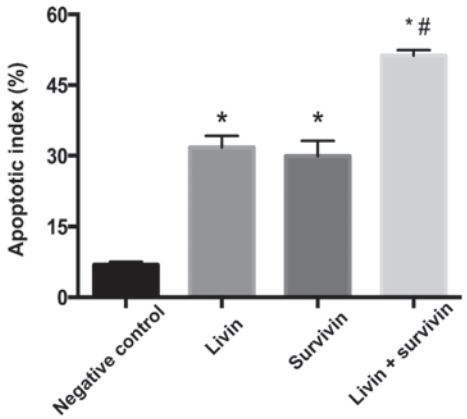

Figure 6. Effects of livin and survivin RNA interference on MG-63 cell apoptosis. *P<0.05 vs. the negative control group, #P<0.05 vs. the livin and survivin interference alone groups.

transformation of proliferating tumor cells into immature bone or bone-like tissues. Upon diagnosis, 80% of patients with osteosarcoma have suffered from micrometastases, with on average 8 months from surgical treatment to pulmonary metastasis (8). In addition, the 5-year survival rate is low, with a high rate of cancer-associated mortality within one year (9). In recent years, amputation has been replaced with limb-salvage therapy due to the development of chemotherapy, improvements in surgical techniques, bone reconstruction, genotherapy, immunotherapy and targeted molecular therapy (8).

The pathogenesis of osteosarcoma has been attributed to a complicated process involving the activation of numerous anti-apoptotic genes and the deactivation of pro-apoptotic genes. Alterations in apoptosis serve an essential role in tumor onset, with the inhibition of the expression of pro-apoptotic genes and excess upregulation of anti-apoptotic genes contributing to tumor onset, progression and metastasis (10).

Livin and survivin, as important members of the inhibitor of apoptosis family (11), are overexpressed in numerous types of malignant tumor tissues (12,13) and are novel targets for genotherapy, due to their effect on invasion and metastasis in addition to prognosis. Through various pathways, livin is able to suppress apoptosis by blocking apoptotic receptors and by inhibiting the mitochondria-based intrinsic apoptotic pathways (11). Additionally, downregulated livin expression is able to promote apoptosis and inhibit tumor growth (14). Livin expression is upregulated in human osteosarcoma tissue, with a rate of positivity for livin expression of 58.7%, which is significantly correlated with microvessel density (15). Survivin has a similar molecular structure to that of livin. Besides suppressing apoptosis, survivin also facilitates cell transformation by participating in angiogenesis of tumor tissues and generation of drug resistance (16). Survivin is expressed in malignant tumors, however, not in normal tissue (17); thus, it is possible that tumor cells may be killed selectively by targeted survivin immunotherapy and genotherapy. Therefore, survivin is a tumor antigen that is widely applicable to the clinical genotherapy of osteosarcoma.

Compared with traditional carriers such as plasmids, NPs are superior due to their large surface area, high adsorption capacity, facile surface modification and passive targeting (18). In the current study CS, a marine organism-derived polysaccharide, was selected due to its biocompatibility, safety, non-toxicity and degradability (18). Carrying a positive charge, CS is able to react with negatively charged DNA to form a polyelectrolyte complex that effectively protects DNA from degradation by DNA enzymes (19). However, ordinary CS is insoluble in water or common organic solvents, so it alone is not applicable in the biomedical field (20). Therefore, the current study altered the characteristics of CS through chemical modification. mPEG-CS NPs had low cytotoxicity, a suitable size for gene delivery, protective effects on genes and long circulating ability, in addition to high drug loading and encapsulation efficiency. Together, this indicates that this derivative is promising in gene transfection and genotherapy.

To verify the biological effects of livin and survivin on osteosarcoma cells and the applicability of CS NPs in tumor treatment, the current study generated mPEG-CS, mPEG-CS-livin shRNA, mPEG-CS-survivin shRNA and mPEG-CS-(livin shRNA + survivin shRNA) NPs to transfect MG-63 cells, and to reduce livin and survivin mRNA and protein expression levels. The present study indicates that mPEG-CS NPs are suitable carrier surrogates for traditional plasmids to mediate transfection.

Furthermore, the current study demonstrated that downregulated expression of livin and survivin in MG-63 cells significantly suppressed proliferation and enhanced apoptosis in MG-63 cells. Notably, simultaneous livin and survivin RNA interference exerted greater inhibitory effects on the cells compared with livin or survivin interference alone.

In conclusion, the anti-apoptotic genes livin and survivin participate in the onset and progression of osteosarcoma, and simultaneous inhibition is potentially eligible for future genotherapy.

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