Preparation of chitosan-silicon dioxide/BCSG1-siRNA nanoparticles to enhance therapeutic efficacy in breast cancer cells

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Abstract. Breast cancer is one of the most serious diseases, posing threats to women's physical and mental health. Gene therapy has been gradually regarded as an important part of tumor therapeutics. In the present study, the breast cancer-specific gene 1-small interference RNA (BCSG1-siRNA) plasmid was designed, then encapsulated by chitosan-silicon dioxide nanometer carriers. The results demonstrated a successful encapsulation of BCSG1-siRNA in chitosan-silicon dioxide nanoparticles (encapsulation efficiency exceeded 90%). BCSG1-siRNA was released slowly (the release rate was almost 30% after 24 h). The cytotoxic effect on MCF-7 cells was enhanced by increasing the concentration of nanoparticle (the proliferation rate was reduced to 13.4±5.3% and apoptosis rate was increased to 71.5±6.8%). Therefore, the materials presented in the current study acted as successful gene carriers and exhibited significant antitumor effects in breast cancer cells.

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Introduction

Breast cancer-specific gene 1 (BCSG1) is a group of specific expression genes in human breast cancers (1-5). The high expression of BCSG1 can promote the survival of tumor cells in adverse environment and reduce the effect of chemotherapeutics (6-9), thus making BCSG1 a potential target for breast cancer treatment (10-12). RNA interference (RNAi), as a gene-blocking technology widely used in the regulation of gene expression, has provided a new gene therapy for cancer and other serious diseases. However, RNAi is degradable *in vivo* and off-target distribution, which limits its application in cancer therapy. Therefore, the encapsulation and release technique for RNAi molecules has become one of the key issues in this field.

The recent decades have witnessed the emergence of some packaging materials (13-18), among which, chitosan, as a basic polysaccharide, is biocompatible, biodegradable, nontoxic, showing good tolerance in the human body (19-27). In addition, the positive charged chitosan nanoparticles are compatible with the RNAi molecules. Moreover, mesoporous silica nanoparticle has exhibited its high drug loading capability and low-toxicity degradation (28,29). Arginine-glycine-aspartate (RGD) peptide modification significantly increases the selectivity between cancer and normal cells, through receptor-mediated endocytosis (30,31). Taken together, RGD labeled chitosan-silicon dioxide nanoparticles are particularly prominent material for RNAi packing and releasing.

In this study, through comprehensive assessment on BCSG1 genes, the BCSG1-small interference RNA (siRNA) plasmid has been designed and synthesized for breast cancer

treatment. Moreover, chitosan-silicon dioxide is selected as targeted carrier to encapsulate BCSG1-siRNA. Finally, the feasibility of chitosan-silicon dioxide as gene carrier will be analyzed from the perspectives of its encapsulation and releasing efficiency.

Materials and methods

Materials. Chitosan (M_W: 100-150 kDa, DD, 85%; Amresco, Inc., Solon, OH, USA). Tetraethylorthosilicate (TEOS) and RGD was supplied by Shanghai Biological Technology Co. (Shanghai, China). MCF-7 cells were provided by Shanghai Cell Library (Shanghai, China). Fetal bovine serum, penicillin, streptomycin and trypsin were purchased from Chongqing Biological Pharmaceutical Co., Ltd. (Chongqing, China), pGPU6 carrier and RNAi turned dye reagents were obtained from Shanghai Zimmer Pharmaceutical Technology Co., Ltd. (Shanghai, China). PCR product recycling reagents box and BCSG1 probe were provided by Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China).

The design of BCSG1-siRNA. According to the BCSG1 cDNA sequences [provided by Ambion; Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and Takara Bio, Inc. (Otsu, Japan) website design and analysis software], four siRNA sequences and one control sequences of siRNA have been designed as follows: BCSG1-siRNA-1 sense strand, 5'-TGGTGAGCAGCG TCAACACTGT-3 and antisense strand, 5'-UUUGUGCAG CCAACCCUCCTT-3'; BCSGI-siRNA-2 sense strand, 5'-CCA AGGAGAATGTTGTACAGATT-3' and antisense strand, 5'-UUUGUGCAGCCAACCCUCCTT-3'; BCSG1-siRNA-3 sense strand, 5'-CAAGACCAAGGAGAATGTTGTTT-3' and antisense strand, 5'-UUUGUGCAGCCAACCCUCCTT-3'; BCSG1-siRNA-4 sense strand, 5'-GCCAAGACCAAGGAG AAIGTTTT-3' and antisense strand, 5'-UUUGUGCAGCCA ACCCUCCTT-3'; negative control siRNA sense strand, 5'-GTTCTCCGA ACGTGTCACGTTT-3' and antisense strand, 5'-ACGUGACACGUUCGGAGAATT-3'

Preparation of chitosan-silicon dioxide/BCSG1-siRNA nanoparticles. RGD-silica nanoparticles were prepared by sol-gel method. Briefly, cyclohexane (7.5 ml) was mixed with hexyl alcohol (1.6 ml) uniformly, then RGD peptide was added as core material under constant stirring for 30 min at room temperature. Then ammonia (100 μl) and TEOS (100 μl) were added drop wise to the mixture over a period of 24 h with stirring moderately. The products were collected by centrifugation, then washed by ethanol and water successively to remove the template cyclohexane.

To obtain chitosan-silicon dioxide/BCSG1-siRNA nanoparticles, 1% chitosan (100 μl) was added into the RGD peptide-silicon dioxide under stirring for 30 min at 180 r/min. Then preheated the equal volume of chitosan/silicon dioxide and BCSG1-siRNA plasmid at 55˚C, and mixed rapidly under stirring for 30 min at room temperature. Then a certain concentration of sodium tripolyphosphate solution (5 ml) was added into the mixture under stirring for 3.5 h at room temperature, and set over night. Finally, the nanoparticles were collected by centrifugation, then filtered by distilled water and lyophilized about 24 h.

Table I. The loop parameter of polymerase chain reaction.

Reaction conditions	Temperature (˚C)	Reaction time (min)
Initial denaturation	94	2
Denaturation	94	0.5
Annealing	56	0.5
Extension	72	0.5
Preservation	72	10

Characterization of chitosan-silicon dioxide/BCSG1-siRNA. The nanoparticles were characterized by laser particle size analyzer transmission electron microscope (TEM), scanning electron microscope (SEM), UV-visible spectroscopy and fluorescence spectroscopy.

The encapsulation efficiency of BCSG1-siRNA was measured in accordance with equation [1] (32-36):

$$\text{Encapsulation efficiency} = (C_0 - C_t)/C_0 \times 100\% \quad [1].$$

C_0 was the original amount of RNA before encapsulation, C_t was the remanent amount of RNA in supernatants after encapsulation.

The BCSG1-siRNA chitosan nanoparticles and phosphate-buffered saline (PBS) (pH 7.4) were put in cell incubators at 37˚C, respectively. Then the supernatant was taken to calculate the released BCSG1-siRNA concentration by using UV-spectrophotometer after 12, 24, 36, 48, 60 and 72 h. The release efficiency of BCSG1-siRNA was measured by equation [2]:

$$\text{Release rate} = C_2/C_1 \times 100\% \quad [2].$$

C_1 and C_2 were the quantity of BCSG1-siRNA in PBS before and after incubation, respectively.

BCSG1 protein expression by immunocytochemistry detection. According to SP immunohistochemistry promega, PBS was used as a negative control, and the positive signal of BCSG1 protein expression as a positive control.

Results showed the positive signal of BCSG1 is a brown granular substance, located within the cytoplasm. Ten microscopic fields were randomly selected under high magnification microscope (the amount of the cells are no less than 100). The results were caculated by the percentage of positive cells and the color intensity (37).

Semi-quantitative RT-PCR. DNase-treated RNA was extracted and used as a template, following the reverse transcription kit for cDNA synthesis.

The loop parameter of PCR was shown in Table I, it was about 35 cycles. The agarose electrophoresis of PCR product: PCR product (3-5 μl) was mixed with loading buffer (1 μl), then the point sample was added to 2% agarose gel. And voltage was adjusted to 5 V/cm about 30 min in the 0.5 X TBE electrophoresis fluids. Finally, PCR products electrophoresis strips were observed by gel imaging system. The melting

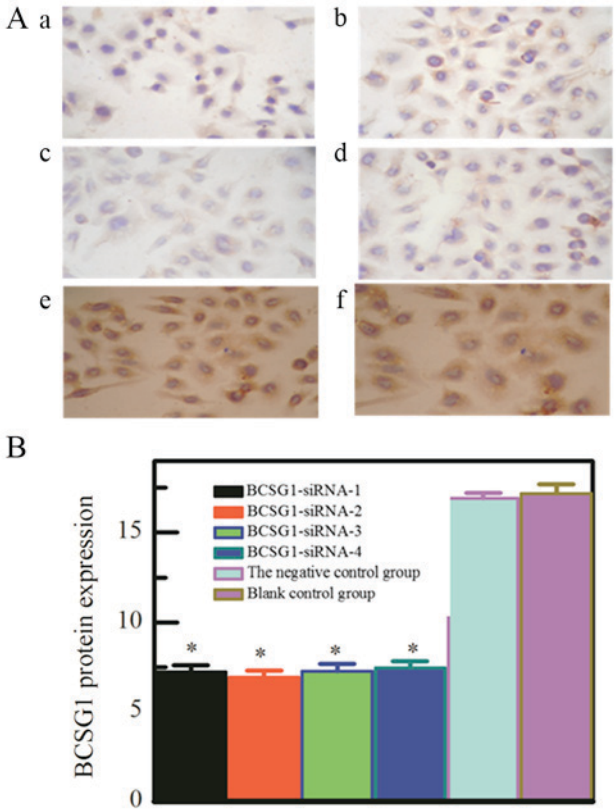

Figure 1. The protein expression of BCSG1 of MCF-7 cells in breast cancer. (A) The microscopic photos of immunostaining of BCSG1 protein (a, BCSG1-siRNA-1; b, BCSG1-siRNA-2; c, BCSG1-siRNA-3; d, BCSG1-siRNA-4; e, negative control group; f, blank control group). (B) The relative protein expression levels were determined (*P<0.05 vs. control). BCSG1, breast cancer-specific gene 1; siRNA, small interference RNA.

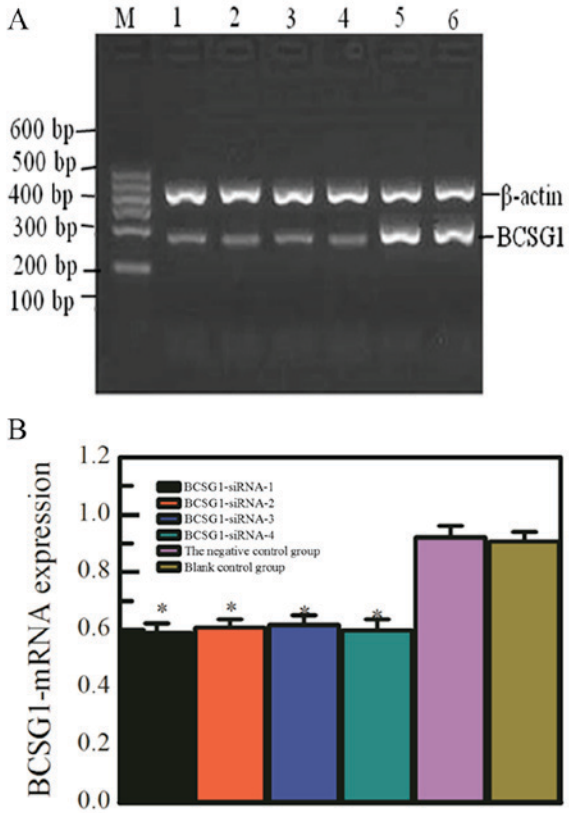

Figure 2. BCSG1-mRNA expression of MCF-7 cells in breast cancer (*P<0.05 vs. control). (A) Representative western blot images. (B) The relative BCSG1-mRNA expression levels were determined. β-actin was used as a loading control. BCSG1, breast cancer-specific gene 1; siRNA, small interference RNA.

curve was obtained by quantitative analysis software of PCR instrument (38-40).

In vitro cellular uptake assays. The BCSG1-siRNA chitosan nanoparticles were diluted to 10, 5, 1 and 0.5 μg/ml. MCF-7 breast cancer cells were divided into 6 groups and treated with the indicated concentrations of BCSG1-siRNA chitosan nanoparticles, further incubated for a week. At 2 days interval, culture medium and BCSG1-siRNA chitosan nanoparticles were replenished. Afterward, the amount of MCF-7 cells in the five groups was measured by inverted microscope. Cell suspension (20 μl) was dropped into the counting slide and observed by the 10X objective lens. The experiments were carried out in triplicate and were independently repeated at least 3 times. Then the proliferation rate and apoptosis rate (equations 3 and 4) were determined to evaluate the effect of BCSG1-siRNA chitosan nanoparticles to MCF-7 cells.

$$\text{Proliferation rate} = (N_0 - N_1)/N_0 \times 100\% \quad [3].$$

N_1 and was N_0 were the number of cells in experimental group and control group.

$$\text{Apoptosis rate} = N_2/(N_2 + N) \times 100\% \quad [4].$$

N_2 and N were the number of apoptotic cells apoptosis and normal cells.

Statistical analysis. All the quantitative data have been presented as the mean ± standard deviation. Analysis of variance with Tukey post hoc test was used to evaluate statistical significance among different groups using SPSS software version 20 (SPSS, Inc., Chicago, IL, USA). Significance was considered when P<0.05.

Results

The immunocytochemistry analysis. The immunocytochemistry result (Fig. 1) has shown that the BCSG1 protein expression of the four specific siRNA transfection groups, the negative control group and blank control group was 7.22, 6.93, 7.26, 7.46, 16.89 and 17.17. The differences among the four specific siRNA transfection groups were not significant (P>0.05), while the differences between the former four groups and control groups had statistical significance (P<0.05).

Compared with negative control group and blank control group, the protein expression of BCSG1 in the four specific siRNA transfection groups is significantly lower. The results showed that the designed siRNA transfection groups could specifically downregulated the protein expression of BCSG1.

The PCR analysis of mRNA expression. RNA for reverse transcription, PCR amplification and electrophoresis in gel image system photo were experimented after transfection (Fig. 2),

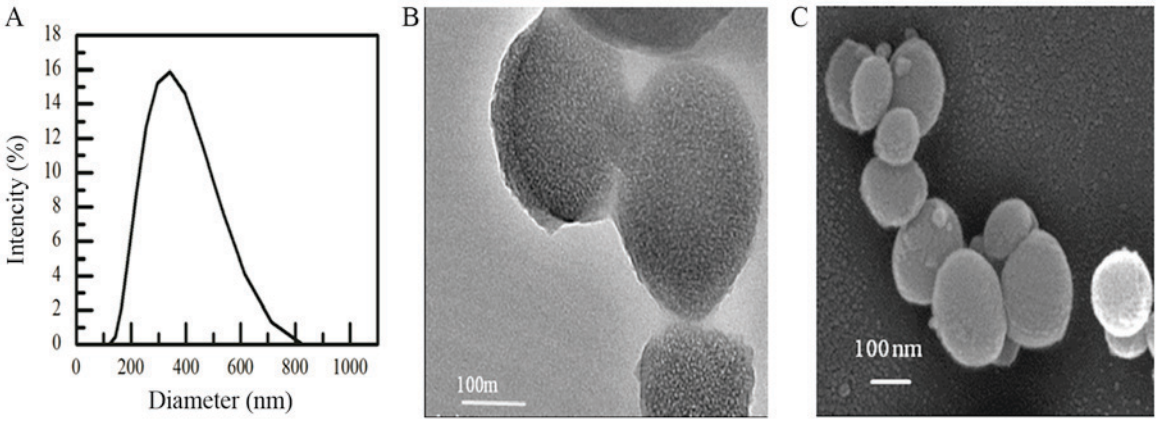

Figure 3. Structural characterization of BCSG1-siRNA/chitosan-silicon dioxide nanoparticle. (A) Size distribution of BCSG1-siRNA/chitosan-silicon dioxide nanoparticle. (B) TEM images of BCSG1-siRNA/chitosan-silicon dioxide nanoparticle. (C) SEM images of BCSG1-siRNA/chitosan-silicon dioxide nanoparticle. BCSG1, breast cancer-specific gene 1; siRNA, small interference RNA; TEM, transmission electron microscope; SEM, scanning electron microscope.

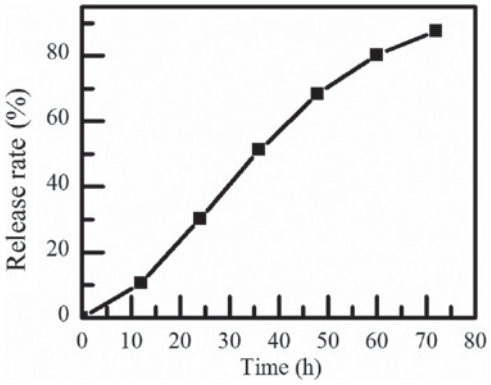

Figure 4. The release curve of BCSG1-siRNA from chitosan-silicon dioxide nanoparticles. BCSG1, breast cancer-specific gene 1; siRNA, small interference RNA.

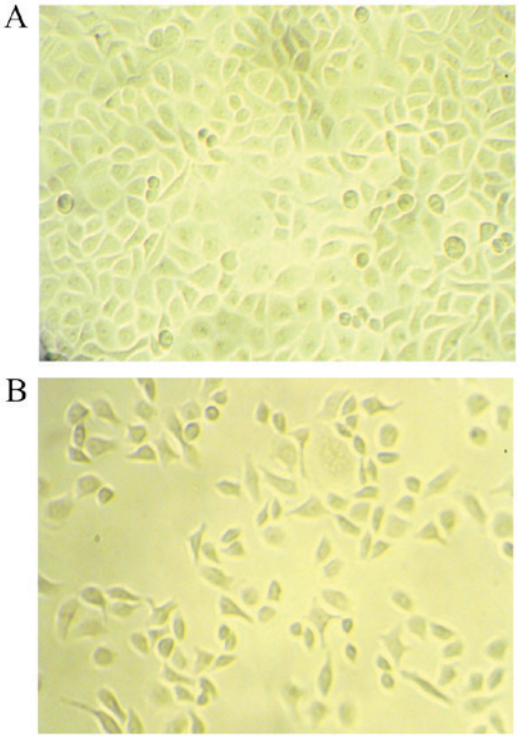

Figure 5. The change of shape in breast cancer MCF-7 cells transfected with BCSGI-siRNA. (A) The control group, (B) the transfected group. BCSG1, breast cancer-specific gene 1; siRNA, small interference RNA.

including the four specific siRNA transfection groups, the negative control group and blank control group.

The first four groups were significantly darkened strip, showed the lower expression of BCSG1-mRNA. Compared with the gray value of β-actin mRNA, the PCR result indicated that the relative transcript level of mRNA in each group was 0.589±0.033, 0.608±0.029, 0.617±0.033, 0.598±0.037, 0.922±0.040, 0.908±0.031, it can be seen that there were no significant differences among the former four groups ($P>0.05$), but the difference between the former four groups and control groups had statistical significance ($P<0.05$).

Compared with negative control group and blank control group, the mRNA expression of BCSG1 in the four specific siRNA transfection groups is significantly lower. The results showed that the designed siRNA transfection groups could specifically downregulated the mRNA expression of BCSG1.

The characteristic of chitosan nanoparticle. According to the laser particle size Analyzer test (Fig. 3A), results showed that the particle sizes was between 200-600 nm, average out at 303±51 nm, the polydispersity index was 0.18. However, the shapes and sizes of nanoparticles after freeze drying were irregular sphere, whose size was slightly smaller than 200 nm from the TEM and SEM (Fig. 3B and C).

According to the UV-Spectrophotometric analysis, the encapsulation rate of chitosan nanoparticles was 94.23±0.43%, showing that most of the DNA are encapsulated in chitosan nanoparticles, the encapsulation effect was good. Similarly, the release efficiency of BCSG1-siRNA was measured and drawn into the release curve, shown in Fig. 4. The release rate was almost 30% after 24 h, and the releasing effect of BCSG1-siRNA nanoparticles was sustained. It has a good application in drug controlled release.

The proliferation and apoptosis rate assay. As shown in Fig. 5, under the inverted microscope, cells of the control group were in good condition and grew with attaching on the plate;

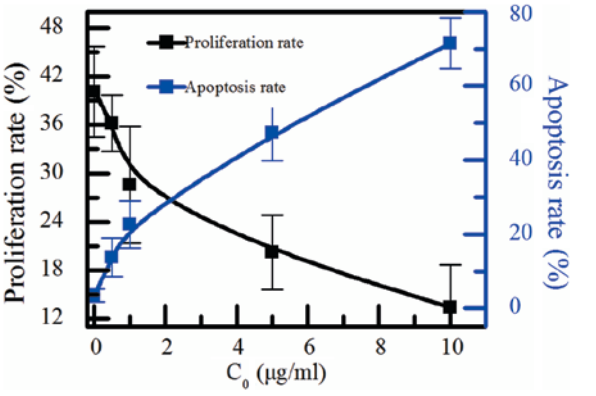

Figure 6. BCSG1-siRNA effect of chitosan-silicon dioxide nanoparticle. BCSG1, breast cancer-specific gene 1; siRNA, small interference RNA.

interestingly, cells of the trancfected group exhibited smaller volume, narrow shape and loose junctions, more cells floated on the plate.

The proliferation rate and the apoptosis rate was shown in Fig. 6. When the concentration of the BCSG1-siRNA chitosan nanoparticles up to 10 μg/ml, the proliferation rate reduced from 40.1±5.6 to 13.4±5.3%, and the apoptosis rate increased from 3.5±1.8 to 71.5±6.8%. When the concentration of BCSG1-siRNA chitosan nanoparticles increases, the proliferation effect of MCF-7 cell will decrease, the apoptosis rate will increase.

The results showed that the designed BCSG1-siRNA/chitosan-silicon dioxide nanoparticle could significantly inhibit tumor growth. The BCSG1-siRNA was encapsulated by chitosan-silicon dioxide, then delivered to the tumor, avoiding the degradation in the tissue. As improving the concentration of BCSG1-siRNA, the BCSG1 expression would be well downregulated, then the tumor growth would be inhibited.

Discussion

In this study, the BCSG1-siRNA plasmid has been designed to downregulate the BCSG1 gene expression. Chitosan-silicon dioxide nanoparticle containing BCSG1-siRNA has been successfully synthesized, its feasibility and effect as drug carriers has been analyzed.

From the immunocytochemistry experiment and the PCR results, the designed BCSG1-siRNA plasmid could significantly downregulate the BCSG1 gene expression. The encapsulation efficiency of nanoparticle was almost 90% (compared with the traditional materials, the encapsulation efficiency was 65-75% in the purified chitosan nanometer carrier, 65-70% in liposomes or other loading material).

Chitosan-silicon dioxide nanoparticle has a good application in drug controlled release, because of the positive charged chitosan and mesoporous silica nanoparticle. The release efficiency was enhanced, as increasing the concentration of gene drug. And the releasing effect of BCSG1-siRNA nanoparticles was sustained.

As improving the concentration of BCSG1-siRNA, the BCSG1 expression would be well downregulated, then the tumor growth would be inhibited. Results reveal the significant selectivity of BCSG1-siRNA nanoparticles.

Chitosan-silicon dioxide carrier can not only improve the encapsulation efficiency, but also enhance the release rate of gene drugs. As increasing the concentration of BCSG1-siRNA chitosan nanoparticle, its effect for breast cancer cells was enhanced, while the damage on normal cell is relatively small. Therefore, it is a better targeted gene delivery system, the chitosan-silicon dioxide as a targeted carrier for gene therapy is feasible.

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