Int J Clin Exp Pathol 2017;10(3):3588-3593
www.ijcep.com /ISSN:1936-2625/IJCEP0024159

Original Article

Effects of chitosan nanoparticle carrier-mediated small-interfering RNA silencing of SUMO-specific protease 1 on *in vitro* migration of breast cancer cells

Yuxi Xie[1*], Yonggui He[1*], Junwei Zhang[1], Aibin Cheng[1], Jinkun Xi[2]

[1]*Affiliated Hospital of North China University of Science and Technology, Tangshan 063000, China;* [2]*Heart Institute, Hebei United University, Tangshan 063000, China.* [*]*Equal contributors.*

Received January 16, 2016; Accepted March 26, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: Background: The aim of this study was to observe the effects of SENP1 silencing on the *in vitro* migration capacity of human breast cancer SK-BR-3 cells. Methods: They were transfected with chitosan nanoparticles (NPs) carrying SUMO-specific protease 1 small-interfering RNA (SENP1 siRNA). Chitosan-siRNA NPs were prepared by ionotropic gelation. Cells were transfected and divided into a chitosan-SENP1 siRNA NP (experimental) group, a Lipofectamine TM 2000 and SENP1 siRNA (positive control) group, a chitosan-siRNA NP (negative control) group and an empty chitosan NP (blank) group. The expressions of SENP1 in SK-BR-3 cells were detected by one-step reverse transcription-polymerase chain reaction and Western blot. The effects of SENP1 siRNA on in vitro migration capacity were evaluated by Transwell assay. One-way ANOVA and K-WH rank sum test were used for statistical analysis. Results: Spherical chitosan-siRNA NPs sized about 100 nm was successfully prepared. In the experimental group, SENP1 mRNA and protein expressions as well as *in vitro* cell migration were significantly inhibited compared with those in negative control and blank groups ($P<0.01$). Conclusion: Chitosan NP-mediated SENP1 siRNA effectively inhibited SENP1 expression in human breast cancer SK-BR-3 cells and their *in vitro* migration.

Keywords: Breast cancer, chitosan, nanoparticle, SENP1, RNA interference

Introduction

During treatment, invasion, metastasis and recurrence of breast cancer dominantly control clinical outcomes and survival rates. To control tumor invasion and metastasis, related genes have been used as RNA interference (RNAi)-based molecular targets to specifically block their expressions [1]. Novel nanoparticles (NPs), as valuable drug carriers, have attracted wide attention. RNAi genotherapy using NPs as carriers is a promising strategy for treating tumors.

Small ubiquitin-like modifier (SUMO), which functions for post-translational modification, can regulate protein activities and functions [2]. As an SUMO-specific protease, SUMO-specific protease 1 (SENP1) catalyzes deSUMOylation of numerous SUMO proteins. Hypoxia-inducible factor-1α (HIF-1α) plays important roles in the proliferation, angiogenesis, growth, invasion and metastasis of breast cancer cells [3]. In the hypoxia-HIF-1α signaling pathway, HIF-1α promotes generation of tumor tissues and blood vessels by activating the transcription of vascular endothelial growth factor (VEGF). Since SENP1 essentially regulates the maintenance of HIF-1α activity under hypoxic conditions [4], it directly or indirectly regulates formation of blood vessels as well as invasion and metastasis of tissues in tumors [4, 5]. The SENP1 positive rate of breast cancer tissues is higher than that in normal tissues, especially in the patients with lymphatic metastasis [6]. Therefore, efficient inhibition by using SENP1 as the target may be able to suppress the invasion and metastasis of breast cancer. In this study, human breast cancer SK-BR-3 cells were transfected with chitosan NPs carrying SENP1 small interfering RNA (siRNA) to observe the effects of SENP1 silencing on *in*

vitro migration capacity. The results provide experimental evidence for genotherapy of breast cancer.

Materials and methods

Reagents

Chitosan (87% degree of deacetylation) was purchased from Sigma-Aldrich. Sodium tripolyphosphate was bought from Fluka. Human breast cancer cell line SK-BR-3 was obtained from Stem Cell Bank, Chinese Academy of Sciences. α-MEM culture medium was purchased from Gibco. LipofectamineTM 2000 and TRIzol kits were bought from Life Technologies. Transwell chambers were obtained from Corning. Fibronectin (FN) was purchased from Chemicon. One-step reverse transcription-polymerase chain reaction (RT-PCR) kit was bought from Qiangen. Mouse anti-human β-actin and SENP1 monoclonal antibodies as well as electrochemiluminescence (ECL) kit were obtained from Santa Cruz. PCR primers were designed by our group and synthesized by BGI Biotechnology (Shenzhen) Co., Ltd.

Synthesis of siRNA fragment

siRNA sequences were synthesized by Shanghai GenePharma Co., Ltd. Sense strand for SENP1: 5'-AACTACATCTTCGTGTACCTC-3', anti-sense strand: 5'-CTAAACCATCTGAATTGGCTC-3'; sense strand for negative control: 5'-UUCUCCGAACGUGUCACGUTT-3', anti-sense strand: 5'-ACGUGACACGUUCGGAGAATT-3'.

Preparation of chitosan-siRNA NPs

By using ionotropic gelation, 3.5 μl of siRNA solution (50 μmol/L) was added into 1 ml of sodium tripolyphosphate solution (1 mg/ml), which was mixed under magnetic stirring, dropped into 3 ml solution of chitosan in acetic acid (2 mg/ml), magnetically stirred, and incubated at room temperature for 30 min, giving encapsulated chitosan-siRNA NPs. Meanwhile, siRNA-free chitosan NPs were prepared as blank control.

Morphological observation of chitosan-siRNA NPs

NP suspension (1 μl) was 1:5 diluted by 30 mmol/L Na_2SO_4, and dropped onto clean mica sheets and blown dry with nitrogen to observe the morphology of NPs under an atomic force microscope.

Cell culture

SK-BR-3 cells were cultured in α-MEM culture medium, incubated at 37°C in 5% CO_2 atmosphere after adding 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, digested with 0.25% trypsin and passaged. The cells in the logarithmic growth phase were selected for subsequent experiments.

Experimental grouping and cell transfection

One day before transfection, SK-BR-3 cells were inoculated onto 6-well plates and divided into four groups after 80% confluence. (1) Experimental group that was transfected with chitosan-SENP1 siRNA NPs; (2) Positive control group that was transfected with Lipofectamine TM 2000 and SENP1 siRNA; (3) Negative control group that was transfected with chitosan-siRNA NPs; (4) Blank group that was transfected with empty chitosan NPs. The final concentrations of siRNA in groups (1)-(3) were all 100 nmol/L.

Detection of SENP1 mRNA expression in SK-BR-3 cells by RT-PCR

The supernatant was discarded 24 h after transfection, into which was added 1 ml of TRIzol reagent to extract total RNA according to the kit's instructions. Upstream primer for SENP1: 5'-TTGGCCAGAGTGCAAATGG-3', downstream primer: 5'-TCGGCTGTTTCTTGATTTTTGTAA-3'; upstream primer for β-actin as internal reference: 5'-GACGTGGACATCCGCAAAGAC-3', downstream primer: 5'-TAGTTGCGTTACACCCTTCTTG-3'. RT-PCR reaction system was prepared according to the kit's instructions. Reaction parameters: Reverse transcription at 50°C for 30 min and reaction at 95°C for 15 min, after which cycling was initiated, including denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s, 30 cycles in total. Finally, the reaction was performed by extension at 72°C for 10 min. PCR products were subjected to agarose gel electrophoresis and photographed. The absorbances of DNA bands were analyzed, and the absorbance ratios of SENP1/β-actin were used as relation mRNA expression levels.

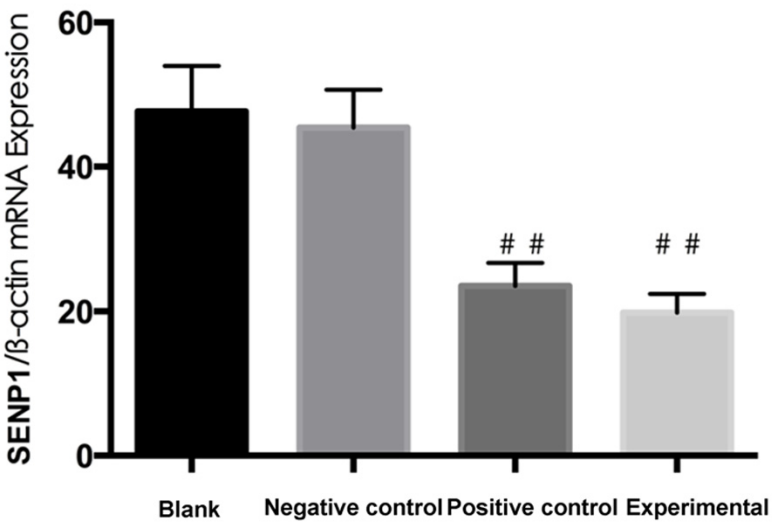

Figure 1. SENP1/β-actin mRNA expressions in SK-BR-3 cells detected by RT-PCR. Compared with blank and negative control groups, ##P<0.01.

Detection of SENP1 protein expression in SK-BR-3 cells by Western blot

The supernatant was discarded 48 h after transfection, into which was added pre-cooled lysate [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfonate (SDS), 1% octylphenoxypolyethoxyethanol (NP-40), 0.5% deoxysodium cholate, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml (aprotinin)]. The mixture was then reacted in ice bath for 20 min and centrifuged at 4°C and 12000×g for 15 min. The supernatant was then subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE), electronically transferred to a nitrocellulose (NC) membrane, blocked in blocking solution at room temperature for 2 h, incubated overnight with β-actin and SENP1 antibodies that had been diluted by blocking solution at room temperature, incubated with horseradish peroxidase-labeled secondary antibodies at room temperature for 2 h, color-developed by using ECL kit, exposed by X-ray, developed and fixed. After absorbance scan and image analysis, the absorbance ratios of SENP1/β-actin were used as relative protein expression levels.

Effects of SENP 1 siRNA on in vitro migration capacity of SK-BR-3 cells

Cells were transfected in 6-well plates, collected after 48 h, counted and adjusted to the density of 1×10^6/ml. Outer surface of filter membrane in Transwell chamber was coated with 10 μg FN and air-dried. α-MEM culture medium containing 0.1% bovine serum albumin was added in 24-well plates, into which were added the Transwell chambers. Cell suspension (100 μl) was then added into each chamber, followed by 6 h of culture at 37°C in 5% CO_2. Afterwards, the chambers were taken out, and the filter membrane was fixed in methanol for 1 min and subjected to HE staining. After unpenetrated cells on the inner surface of the filter membrane were wiped off, the membrane was sealed for microscopic observation. The penetrating cells were counted under a ×400 light microscope. Five visual fields were randomly selected for each membrane, and four samples were set for each group.

Statistical analysis

All data were analyzed by SPSS11.0. SENP1/β-actin mRNA expressions between groups were compared by one-way ANOVA. Pairwise comparisons were performed by using LSD test. K-WH rank sum test was employed to compare SENP1/β-actin protein expressions between groups and effects of SENP1 siRNA on cell migration. Pairwise comparisons were conducted by the Bonferroni method. P<0.01 was considered statistically significant.

Results

Inhibitory effects of SENP1 siRNA on SENP1 mRNA expression in SK-BR-3 cells

Total RNA was extracted from each group and subjected to RT-PCR. The SENP1 mRNA expression levels of the negative control and blank groups were similar 24 h after transfection (P>0.05), which were significantly higher than those of the positive control and experimental groups (P<0.01) (**Figure 1**).

Inhibitory effects of SENP1 siRNA on SENP1 protein expression in SK-BR-3 cells

Western blot showed that 48 h after transfection, SENP1 protein expression levels de-

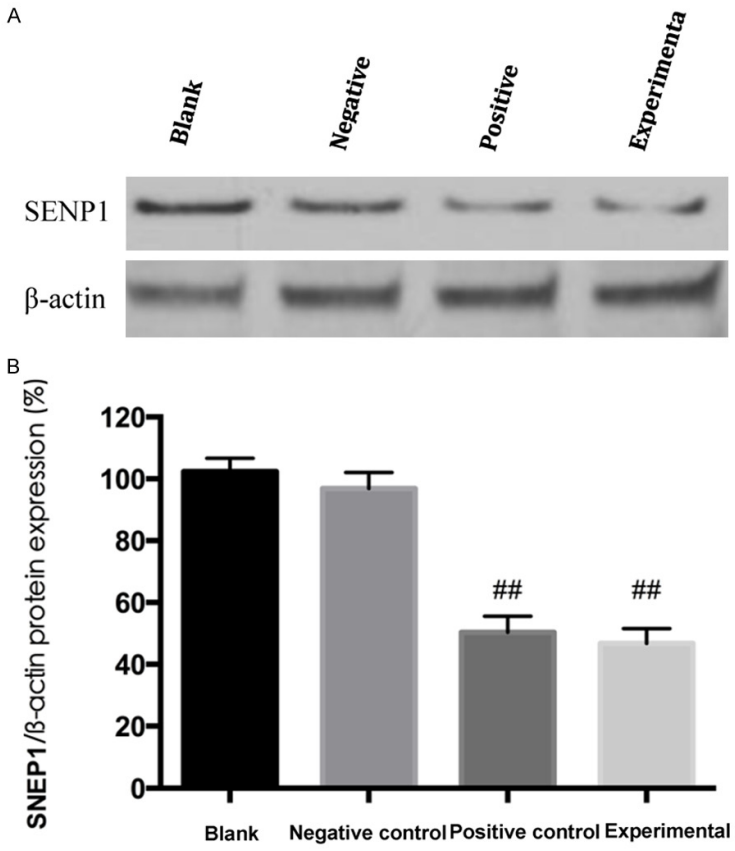

Figure 2. SENP1 protein expressions in SK-BR-3 cells detected by Western blot. A: Western blot results; B: relative grayscales. Compared with blank and negative control groups, ##P<0.01.

creased in the experimental and positive control groups (**Figure 2A**). Absorbance integration analysis showed that the inhibition rates of SENP1 protein expression in the two groups were 47.9% and 51.2% respectively, which were significantly different from those of the blank and negative control groups (P<0.01) (**Figure 2B**).

Effects of SENP1 siRNA on in vitro migration capacity of SK-BR-3 cells

Under the ×400 light microscope (**Figure 3**), the cells counts of the experimental and positive control groups differed significantly from those of the negative control and blank groups (P<0.01) (**Table 1**), suggesting that SENP1 siRNA obviously inhibited the migration of SK-BR-3 cells.

Discussion

SUMOylation mainly regulates the interactions between modified proteins, protein transport between cell nucleus and cytoplasm, protein location and transcriptional activity, as well as antagonizes ubiquitination [7]. SUMO-modified reverse reaction, also known as deSUMOylation, is completed by a group of SENPs [8]. SENPs in mammalian cells included six members, i.e. SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7. SENP1 can modify a wide range of substrates and catalyze the deSUMOylation of numerous SUMO proteins as a nuclear protease [9].

Hypoxia often plays key roles in regulating tumor angiogenesis, survival and spread, mainly coordinated by HIF-1α. SENP1 is a critical regulatory factor for maintenance of HIF-1α activity under hypoxic condition [4]. In the hypoxia-HIF-1α signaling pathway, HIF-1α further activates the transcription of VEGF and thus facilitates the formation of blood vessels in tumors [10]. It has previously been reported that high HIF-1α and VEGF expressions are important to the onset, progression, invasion and metastasis of breast cancer, predicting poor prognosis as well [11]. The SENP1 positive rate of breast cancer tissues remarkably surpasses that in normal tissues, particularly in the patients with lymphatic metastasis [6]. Hypoxia-induced HIF-1α expression is evidently reduced in the cells of mice depleted of SENP1, with significantly shorter half-life of HIF-1α protein than that of wild-type mice. In the meantime, hypoxia-induced VEGF expression is also dramatically decreased in the embryonic fibroblasts of these mice, indicating that SENP1 predominantly regulates tumor angiogenesis [4]. Increase of SENP1 protein allows deSUMOylation of bound SUMO proteins. As a result, the inhibitory effects of SUMOylation are relieved, which enhances the transcriptional activity of HIF-1α protein and significantly increases induced expression of VEGF. Therefore, SENP1 is indeed involved in angiogenesis of tumors [3]. Since tumor invasion and metastasis are closely related with angiogenesis,

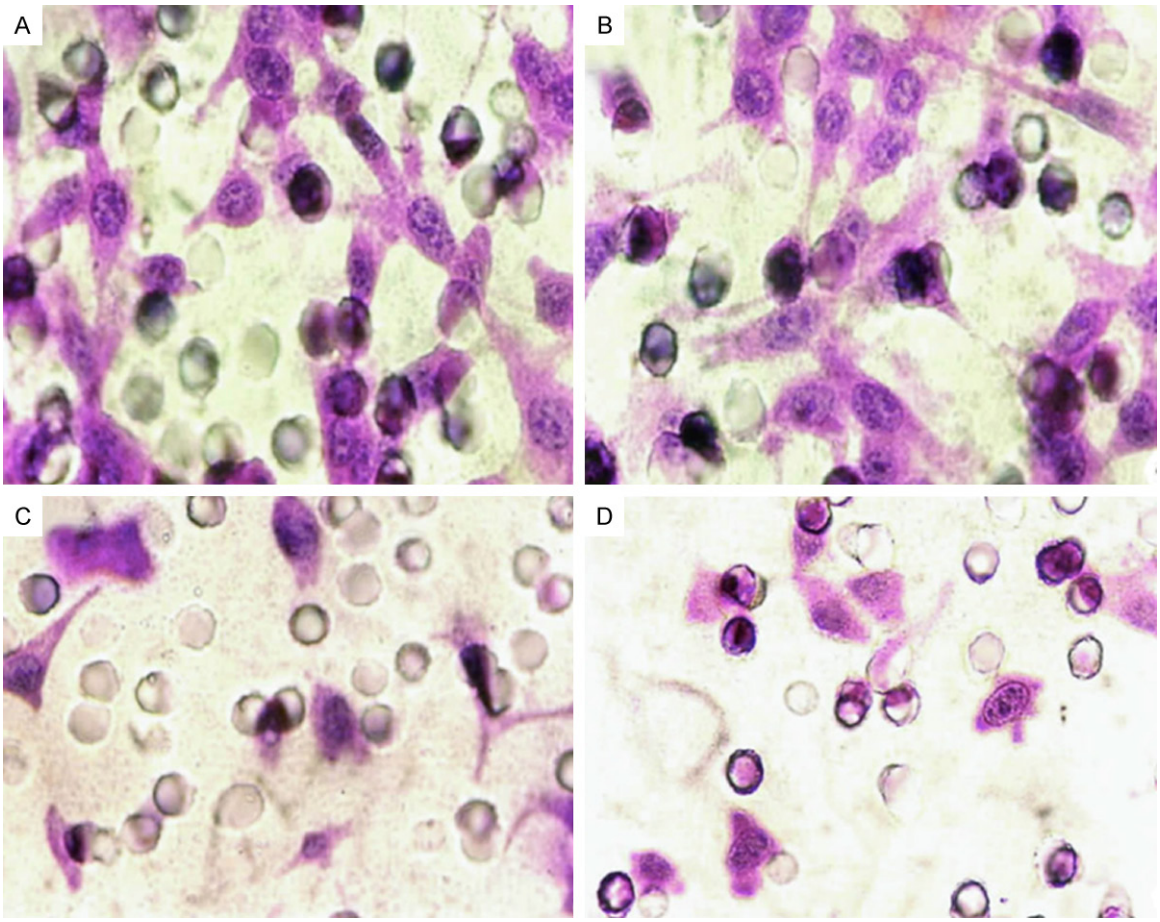

Figure 3. Effects of SENP1 siRNA on migration of SK-BR-3 cells (light microscope, HE staining, ×400). A: Blank group; B: Negative control group; C: Positive control group; D: Experimental group.

Table 1. Effects of SENP1 siRNA on migration of SK-BR-3 cells

Group	Sample (n)	Cell count in each visual field [M (QR)]	Z value	*P* value
Blank group	4	110.00 (96.50-111.75)	11.712	0.007
Negative control group[a]	4	98.50 (94.50-108.25)		
Positive control group[b]	4	38.50 (35.50-42.50)		
Experimental group[c]	4	36.50 (32.75-38.00)		

The data not conforming to the normal distribution were compared by K-WH rank sum test. Pairwise comparisons were conducted by the Bonferroni method. a: Compared with blank group, P>0.05; b: compared with negative control and blank groups, P<0.01; c: compared with negative control and blank groups, P<0.01, compared with positive control group, P>0.05. M (QR): Median (quartile range).

SENP1-targeted therapy may be applicable to inhibition of breast cancer migration. In this study, breast cancer cells transfected with SENP1 siRNA significantly inhibited SENP1 mRNA and protein expressions as well as the resulting strong tendency to *in vitro* migration. In contrast, the cells transfected with negative control siRNA were free from inhibition, which can be attributed to dependence of SENP1 siRNA on the specificity of target sequence.

As one of the crucial mechanisms for post-transcriptional silencing, RNAi cleaves double-stranded RNA into 19~25 nt fragments through nucleases and forms siRNA silencing complexes that recognize and degrade mRNA sharing homologous sequences, specifically silencing gene expressions eventually [12]. Compared to other gene silencing technologies such as antisense gene silencing, RNAi can exert effects with gene substances at low concentrations. In this study, the cells transfected with SENP1 RNA expressed significantly less

SENP1 mRNA and protein than the blank group did. On the other hand, non-viral gene carriers have attracted global attention due to safety concern. Therefore, an eligible gene nanocarrier, chitosan, was selected herein to perform RNAi, to ensure effective cellular absorption of siRNA and the carrier and to prevent degradation. As the sole natural biodegradable, cationic group-containing polysaccharide, chitosan has biocompatibility, non-cytotoxicity and non-immunogenicity, being capable of protecting nucleic acid complexes from degradation in the blood [13]. Given that particle size and shape play key roles in gene transfection, NPs are preferred to others owing to higher cellular absorption rates [14]. By controlling reaction parameters, we herein prepared chitosan NPs uniformly sized approximately 100 nm, which were suitable for carrying gene. Compared with the positive control group that utilized cationic liposome, the experimental group using chitosan NPs expressed similar levels of SENP1 mRNA and protein, i.e. these NPs were comparatively capable of gene transfer.

In conclusion, we managed to silence SENP1 gene in human breast cancer SK-BR-3 cells through mediation of chitosan NPs carrying synthesized siRNA, thereby reducing the *in vitro* migration capacity of these cells.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jinkun Xi, Heart Institute, Hebei United University, Tangshan 063-000, China. Tel: +86 315 3726279; Fax: +86 315 3725495; E-mail: xijkhi@126.com

References

[1] Elbashir SM, Harborth J, Weber K, Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 2002; 26: 199-213.

[2] Melchior F. SUMO–nonclassical ubiquitin. Annu Rev Cell Dev Biol 2000; 16: 591-626.

[3] Mukhopadhyay D, Dasso M. Modification in reverse: the SUMO proteases. Trends Biochem Sci 2007; 32: 286-295.

[4] Cheng J, Kang X, Zhang S, Yeh ET. SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. Cell 2007; 131: 584-595.

[5] Li QF, Dai AG. Hypoxia-inducible factor-1 alpha regulates the role of vascular endothelial growth factor on pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. Chin Med J 2004; 117: 1023-1028.

[6] Chen J, Wang J, Gu HY, Li QJ, Cheng ZG. [The clinical significance of expression of SENP1 and CerBb-2 in breast carcinoma]. Journal of Diagnostics Concepts & Practice 2009; 8: 175-179.

[7] Howe K, Williamson J, Boddy N, Sheer D, Freemont P, Solomon E. The ubiquitin-homology gene PIC1: characterization of mouse (Pic1) and human (UBL1) genes and pseudogenes. Genomics 1998; 47: 92-100.

[8] Yeh ET, Gong L, Kamitani T. Ubiquitin-like proteins: new wines in new bottles. Gene 2000; 248: 1-14.

[9] Hay RT. SUMO-specific proteases: a twist in the tail. Trends Cell Biol 2007; 17: 370-376.

[10] Yamakawa M, Liu LX, Date T, Belanger AJ, Vincent KA, Akita GY, Kuriyama T, Cheng SH, Gregory RJ, Jiang C. Hypoxia-inducible factor-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors. Circ Res 2003; 93: 664-673.

[11] Hao LS, Zhu X, Qian K, Zhang QH, Luo T, Li XJ, Wang G, Wu XT. [Expression of Hypoxia Inducing Factor 1 Alpha and Its Relationships with Proliferating Cell Nuclear Antigen and Microvessel Density in Human Breast Cancer]. Chinese Journal of Bases and Clinics in General Surgery 2007; 14: 203-207.

[12] Hu XX. [RNA interference (RNAi) as novel approach for gene silencing--review]. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2005; 13: 1141-1144.

[13] Tan ML, Choong PF, Dass CR. Cancer, chitosan nanoparticles and catalytic nucleic acids. J Pharm Pharmacol 2009; 61: 3-12.

[14] Schmitz T, Bravo-Osuna I, Vauthier C, Ponchel G, Loretz B, Bernkop-Schnurch A. Development and in vitro evaluation of a thiomer-based nanoparticulate gene delivery system. Biomaterials 2007; 28: 524-531.