Original Paper

SUMO-1 Gene Silencing Inhibits Proliferation and Promotes Apoptosis of Human Gastric Cancer SGC-7901 Cells

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Key Words

Gastric cancer • SUMO-1 • Gene silencing • shRNA • Cell proliferation • Cell cycle • Cell apoptosis

Abstract

Background: It has been reported that blocking small ubiquitin-like modifier (SUMO) conjugation by silencing SUMO gene remarkably decreased tumor growth *in vivo*. However, few studies have examined the relationship between SUMO gene silencing and gastric cancer (GC). The study aims to explore the effects of SUMO-1 gene silencing on GC cell proliferation and apoptosis. **Methods:** GC cells were cultured and divided into 5 groups: the blank group (without any transfection or treatment), the empty vector group (transfected with empty vector), the shRNA-SUMO-1-1 group (transfected with shRNA-SUMO-1-1 plasmid), the shRNA-SUMO-1-2 group (transfected with shRNA-SUMO-1-2 plasmid), and the shRNA-SUMO-1-3 group (transfected with shRNA-SUMO-1-3 plasmid). Cell Counting Kit-8 (CCK-8) assay was performed to examine cell proliferation. Annexin V/PI staining combined with flow cytometry were used to detect cell apoptosis. Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting were employed to measure the mRNA and protein expressions of SUMO-1, P53, Bcl-2 and c-myc, respectively. **Results:** SUMO-1 mRNA and protein expressions were decreased after transfecting with shRNA-SUMO-1. Compared with the blank group, the shRNA-SUMO-1-1 group presented a remarkable decreased proliferation of SGC-7901 cells. Significant increase in cell apoptosis rate was observed. Bcl-2, c-myc and P53 expressions were declined after transfecting with shRNA-SUMO plasmid. **Conclusion:** Our study provided evidence that SUMO-1 gene silencing could decrease proliferation and promote apoptosis in GC cells.

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Introduction

According to the data released by the International Agency for Research on Cancer, gastric cancer (GC) ranks the fourth most frequently occurring cancer worldwide after lung,

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breast and colorectal cancers and also accounts for the second most common cancer-related cause of death after lung cancer [1]. With a geographical variation in incidence of the cancer, its mortality in China tends to increase with a 5-year survival rate at only 30~40% and a poorer prognosis for advanced tumors, which severely pose a threat to the health and life of people [2, 3]. It has been suggested that approximately 66~75% of GC risk could be reduced with high intake of fruit and vegetables and low consumption of salted foods [4]. Currently, GC relies on surgery combined with chemotherapy and adjuvant chemoradiotherapy for its clinical treatment, which is somewhat effective in prolonging patients' overall survival and relapse-free survival but failed to inhibit GC recurrence and metastasis [5, 6]. Furthermore, in spite of the advances in early diagnosis and treatment modalities, the side effects of chemotherapy and frequent recurrence remain tricky problems in its treatment, thus fueling a great need for identification of new and effective anti-cancer agents for reducing the mortality [7]. It has been revealed that certain genes such as vitamin E succinate (VES) can block cell cycle, arrest DNA synthesis and induce apoptosis in human GC SGC-7901 cells, therefore inhibiting cell growth [8]. The mechanism of the apoptosis of SGC-7901 cells, however, still remains unclear [9].

Small ubiquitin-like modifier (SUMO) is known as a group of proteins which conjugate to lysine residues of target proteins, and further modifying their activity, stability, and subcellular localization, a large number of whose substrates are transcription factors or other nuclear proteins involved in gene expression [10, 11]. SUMO conjugation, or SUMOylation, plays crucial roles in chromosomal organization and function, genome stability, quality control of newly synthesized proteins, proteasomal degradation of proteins and DNA damage repair, which makes obvious increase in levels of SUMO-conjugated proteins likely to exert a major impact on the fate of cells [12, 13]. SUMOylation is known to affect cellular processes in a wide range of diseases, including human astrocytic brain tumors, glioblastoma and breast cancer [12, 14]. It is reported that blocking SUMO conjugation by silencing *SUMO* expression remarkably modifies gene expression and reduces cancer tumorigenesis, making silencing *SUMO* a promising method to treat cancers [15]. However, few studies have been reported so far to inquire into the relationship between *SUMO* gene silencing and GC. Thus, the present study initiated to probe into what an effect SUMO-1, one isoform of SUMO family, has on the proliferation and apoptosis of SGC-7901 cells in GC.

Materials and Methods

Ethics statement

The study was approved by clinical experiment ethical committee of China-Japan Union Hospital of Jilin University and informed consents were obtained from all study subjects.

Specimens collection

Specimens were extracted from GC tissues and adjacent normal tissues (5 cm beyond cancer lesions) of 35 GC patients who were diagnosed by pathological examination in GC surgical department of China-Japan Union Hospital of Jilin University. These 35 patients included 23 male and 12 female patients, and they were 28 years old to 82 years old with an average age of 55.89 ± 15.12 years old. The inclusion criteria of patients were: complete medical records, no other elementary diseases or chronic diseases or combined other body malignant tumor. And patients with incomplete medical records, or had radiotherapy or chemotherapy before operation were excluded. Specimens were washed by normal saline many times, and then connective tissues and necrotic tissues were cut off. Two or three pieces of $0.5 \times 0.5 \times 0.3$ cm³ GC tissues were extracted. All specimens were disintegrated by lysis solution (7 mol/L urea, 2 mol/L thiourea and 4% CHAPS), added with proteinase inhibitor (Cocktail), after that, supernatant extracted by centrifugation was quantitated by butyrylthiocholine (BTC) method, and stored at -80°C.

Immunohistochemistry (IHC)

GC tissues were immobilized by 40 g/L formaldehyde at once separated, then paraffin-embedded, and finally were cut into slices. After that, they were routinely dewaxed and hydrated. A total of 0.01 mol/L citrate buffer were applied for antigen retrieval at high temperature and high pressure. The horse

serum sealing fluid was dropwise added into specimens and extra fluid was throwing away after 20 min conservation at indoor temperature. The specimens were added with 50 μ L SUMO-1 (ab32058, 1: 500 attenuation, Abcam company) as primary antibody, and stored one night at 4°C. Then they were reheated for 45 min at 37°C, washed by phosphate buffer saline (PBS) 3 times with 5 min each time. A total of 40 ~ 50 μ L second antibody were added into them, and stored for 1 h at 37°C. Then they were washed by PBS 3 times with 5 min each time. The coloration was made by 3,3'-diaminobenzidine (DAB) for 5~10 min, and then they were washed by PBS for 10 min. Specimens were counterstained by hematoxylin for 2 min, and then experienced dehydration, transparent disposal, sealing sheet and microscopy. Under optical microscope, positive expression was indicated by uneven thickness of dyed cell nucleus with color of yellowish to claybank. As a negative control, PBS was used instead of the primary antibody.

Cell culture

GC primary cells were collected by digesting GC tissues with collagenase digestion method, and then to be observed after trypan blue staining. Primary cells were purified and sub-cultured to be stable continuous cell lines. Their cellular morphology was observed under optical microscope before and after hematoxylin-eosin (HE) staining when they were immobilized by 95% ethyl alcohol. Purification: after the fusion degree of primary cells which grew with adherence came to be 80%, 0.25% trypsin were applied to digest the specimens; when cancer cells turned round and floated under microscope and fibroblasts started to fall off, 2 mL 10% FBS were added to stop digestion; then falling fibroblasts and cancer cells with low activity were removed; specimens culture continued till fibroblasts adhered to wall while cancer cells did not adhere to wall, where these cancer cells were cultured in a new culture plate. Subculture: purified cancer cells were inoculated to 24-well plate; when fusion degree came to be 80% ~ 90% area of whole culture dish, specimens were digested by pancreatin, and were inoculated to 12-well plate, 6-well plate and 10 cm plate in turn; when fusion degree came to be 80% of 10 cm plate, they were digested and sub-cultured. The specimens were cultured and sub-cultured by RPMI-1640 medium (HyClone company) which contained 10% fetal calf serum (FCS) (Gibco Company, USA), at 37°C and 5% CO₂ constant temperature incubator.

Model construction

The pSilencer™ 3.1-H1 neo plasmid was used to build RNA interference (RNAi) system that was effective to interfere SUMO-1. In accordance with mRNA sequence of SUMO-1 (NM003352), following the classic siRNA design principles, the siRNA software with online program of Promega company was applied to seek target sequence complied with design feature, from the downstream of SUMO-1 initiation codon. The basic local alignment search tool (BLAST) Search was made to confirm that these target sequence had no homology with the known sequences. BamH 1 and Hind III were selected as restriction sites. The target sequences sites and their base sequences showed as Table 1. According to the base sequences of SUMO-1 target sequences in Table 1, the oligonucleotide template expressing shRNA was transferred by online program of Ambion company, and synthesized by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. The synthesized oligonucleotide were mixed according to equal molar ratio, and then they were added into annealing buffer and slowly annealed to indoor temperature after heated in 95°C water bath for 5 min. The pSilencer™ 3.1-H1 neo plasmid was linearized by dual-enzyme digestion of BamH 1 and Hind III, and next it was fixed with three annealing and linked template DNAs in the above table overnight. The pSilencer™ 3.1-H1 neo plasmid was connected with oligonucleotide inserted in three target sequences of SUMO-1 in Table 1, of which recombinant plasmids were separately named as shRNA-SUMO-1-1, shRNA-SUMO-1-2, and shRNA-SUMO 1-3. These plasmids were processed overnight and stored at -20°C. These three plasmids had restriction enzyme digestion by BamH 1 and Hind III, of which enzyme-digested products were analyzed and identified by agarose gel electrophoresis.

Table 1. SUMO-1 target sequence sites and their base sequences. Note: SUMO-1, small ubiquitin-like modifier-1

Target sequence sites	Base sequences
001 (267-285)	5'-TGACAACACATCTCAAGAA-3'
002 (309-327)	5'-GACAGGGTGTTCATGAA-3'
003 (342-361)	5'-TCTTTGAGGGTCAGAGAAT-3'

Cell transfection

The cells were transfected by Lipofectamine2000 liposome transfection reagent kit of Invitrogen company. One day before transfection, 0.25% pancreatin were used to digest cells and count as well. And then these cells were added into 6-well plate of RPMI 1640 complete medium with 5×10^5 cells/well. These plasmids were respectively attenuated by 100 μ L serum-free RPMI 1640 medium. And a total of 40 μ L Lipofectamine2000 were attenuated by 1000 μ L serum-free RPMI 1640 medium. These attenuated plasmids and Lipofectamine2000 solution were mixed together in 10 min, and then stored at indoor temperature for 20 min. A total of 250 μ L were extracted and added into 6-well plate, blending lightly. The subjects were divided into 5 groups, including the blank group (without any transfection or treatment), the empty vector group (transfected with empty vector), the shRNA-SUMO-1-1 group (transfected with shRNA-SUMO-1-1 plasmid), the shRNA-SUMO-1-2 group (transfected with shRNA-SUMO-1-2 plasmid), the shRNA-SUMO-1-3 group (transfected with shRNA-SUMO-1-3 plasmid). After transfection, they were cultured for 6 h at 37°C and 5% CO₂, and finally continued to culture after changing media with serum RPMI-1640 complete medium.

Cell counting kit-8 (CCK-8)

GC SGC-7901 cells in logarithmic phase were extracted and digested by 0.25% pancreatin, and to be counted in its single-cell suspension. Then 1×10^4 cells/mL cell suspension was prepared, where cells were inoculated into 96-well plate by 150 μ L and 1500 cells per well, 3 complex holes per group. When fusion degree of cells in each group came to 80%, the complete medium was removed, and cells were continued to be cultured for 4 ~ 6 h with serum-free medium, which synchronized cell cycle of all groups. Cell plate were taken out at 0 h, 24 h, 48 h and 72 h respectively and then continue to be cultured for 2 h after 10 μ L CCK-8 (making no bubble, otherwise influence optical density (OD) value reading) were added into them. The OD value were read at the location of 450 nm on microplate reader, and cell growth control rates were calculated by the following formula: control rate (%) = (A value of control well - A value of experimental well)/A value of control well $\times$ 100%. Each experiment repeated five times.

Flow cytometry

Cell cycle of cells each group were detected by propidium iodide (PI) staining and flow cytometry. SGC-7901 cells of each group were separately inoculated into five 10 cm cell culture plates by 1×10^6 cell/plate. They were cultured for 24 h at 37°C 5% CO₂ incubator. After that, cells were collected and washed twice with pre-cooled PBS. Then they were immobilized by 75% frozen ethyl alcohol at -20°C, followed by which they were washed with pre-cooled PBS. The next stage was that cells were re-suspended by 200~500 μ L pre-cooled PBS, 30 min in 37°C water bath after added with 20 μ L Rnase A solution. And then 400-mesh sieve were used to filter cells, followed by incubated for 30 min to 1 h at 4°C away from light after blending with 400 μ L PI. Finally, specimens were filtered by yellow nylon mesh. Cell cycle of cells in each group was detected by Becton Dickinson flow cytometer (BD, USA) (cells collection and washing should avoid mechanical damage which might cause loss of cells DNA to influence the results accuracy).

Cell apoptosis detection used Annexin V/PI double-staining. SGC-7901 cells of each group were separately inoculated into five 10 cm cell culture plates by 1×10^6 cell/plate. They were cultured for 24 h at 37°C 5% CO₂ incubator. After that, cells were collected and prepared into single-cell suspension. Then cells were blending with 500 μ L Annexin V Binding Buffer, 5 μ L Annexin V-FITC and 5 μ L Propidium Iodide in order, 10 min at indoor temperature away from light. Finally, specimens were filtered by yellow nylon mesh. Cell apoptosis also were detected by BD flow cytometer (BD, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of cells in each group were extracted by Trizol, while reverse transcription was processed by invitrogen kit, and at last PCR amplified reaction were conducted. The first stage was pre-denaturation at 94°C for 5 min. The second stage included denaturation at 94°C for 35 s, annealing at 51°C for 35 s and extension at 72°C for 35 s, with totally 30 circulations. The third stage was extension at 72°C for 7 min. The reaction system was 25 μ L in total, including 1 μ L cDNA, 1 μ L upstream primer and 1 μ L downstream primer of SUMO-1, 12.5 μ L Master Mix (2 $\times$ Taq PCR), and the rest deionized water completed the 25 μ L. The agarose gel electrophoresis (containing 5 μ g/mL ethidium bromide) were conducted with 6 μ L PCR products, where observation and analysis were done. With target gene = 2^{- $\Delta\Delta C_t$} , the relative expression of

Table 2. Upstream and downstream primer sequences of *SUMO-1* and β -actin. Note: SUMO-1, small ubiquitin-like modifier-1

Gene		Primer sequence
<i>SUMO-1</i>	Sense	5'-AGGAGGCAAAACCTTCAACT -3'
	Anti-sense	5'-TTCTTCCTCCATTTCCAGTT-3'
β -actin	Sense	5'-ACACTGTGCCCATCTACGAGG -3
	Anti-sense	5'-AGGGGCCGGACTCGTCATACT-3'

target gene was calculated. $\Delta\Delta Ct = (\text{target gene Ct value of experimental group} - \text{reference gene Ct value of experimental group}) - (\text{target gene Ct value of control group} - \text{reference gene Ct value of control group})$. Ct value was the recurring number when fluorescence signal in each reaction tube reached the given field value. The quantitative primer in the experiments showed as Table 2.

Western blotting

SGC-7901 cells of each group were separately inoculated into five 10 cm cell culture plates by 1×10^6 cell/plate. They were cultured for 24 h at 37°C 5% CO₂ incubator. After that, Real-time Instant Presence with Advertisement (RIPA) lysis buffer was used to disintegrate them, where cell proteins extracted. After collecting supernatant by centrifugation, cell concentration of each group was measured by BCA Protein Assay Kit (Beyotime Biotechnology). Loading buffer added into proteins, they were boiled for 10 min at 95°C. With 30 μ g loading amount in each well, proteins were isolated by 10% polyacrylamide gel electrophoresis, where the voltage of electrophoresis was 80 v (spacer gel) to 120 v (separation gel). After electrophoresis, membrane was transferred to Polyvinylidene Fluoride (PVDF) membrane in 60 ~ 90 min by wet transfer method, where current was 250 mA. Then they were closed for 2 h with 5% defatted milk at room temperature. The primary antibody SUMO-1 (ab32058, 1: 1000 attenuation), P53 (ab1101, 1: 1000 attenuation), bcl-2 (ab32124, 1: 1000 attenuation), and c-myc (ab32072, 1: 1000 attenuation) all bought from Abcam company (Boston United States), were added into specimens to shake overnight at 4°C. After that, they were washed by Phosphate Buffer Solution with Tween-20 (PBST) 3 times with 10 min per time. The next stage was incubating for 1 h on shaker at indoor temperature after adding matched second-antibody (1: 5000 attenuation), followed by which washed the membrane 3 times with 10 min per time. The results were observed by motored molecular imaging system. The protein expression of SUMO-1 was represented by SUMO-1/ β -actin value.

Statistical analysis

All statistics were analyzed by SPSS 21.0 statistical software (SPSS, Chicago, IL, USA), and the data were expressed by mean value $\pm$ standard deviation (mean $\pm$ SD). Comparison between two groups was detected by *t*-test, while comparison among multiple groups was detected by one-way analysis of variance (One-Way ANOVA). Difference was considered as statistical significant if $P < 0.05$.

Results

Comparison of SUMO-1 expression between GC tissues and adjacent normal tissues

SUMO-1 locates in cell nucleus and its expressions were widely distributed in various tissues and cells. Under optical microscope, positive expression was indicated by uneven thickness of dyed cell nucleus with color of yellowish to claybank. SUMO-1 expressions in GC tissues presented to be positive while the majority of that in adjacent normal tissues presented to be negative expression (Fig. 1). The color of poor-undifferentiated tumor showed to be deeper than well-moderate differentiated tumor.

Morphological differences of GC primary cells and passage cells under light microscope

The collagenase digestion extracted large quantity of GC cells, and caused low cell damage. After 60 minutes of collagenase digestion, cells were mostly single cell. The trypan blue staining detected rate of live cells by calculating stain-resistance rate, where live cells appeared to be transparent globule with stain-resistance rate over 91%, while dead cells were blue (Fig. 2A). With short cell culture cycle, adherent cells reached 43% after 48 h, and cellular morphology appeared to be fusiform and stoning to grow adhering to the wall. At the

Fig. 1. SUMO-1 protein expression in different differentiated GC tissues and adjacent normal tissues ($\times 400$). Notes: GC, gastric cancer; A, adjacent normal tissue; B, gastric cancer tissue; C, well-moderate differentiated tumor; D, poor-undifferentiated tumor; E, negative control.

Fig. 2. Morphological differences of GC primary cells and passage cells extracted by collagenase digestion ($200\times$). Notes: A, trypan blue staining of SGC-7901 cells; B, morphological changes of SGC-7901 cells under light microscopy; C, HE staining of SGC-7901 cells.

Fig. 3. The electrophoretogram of enzyme digestion analysis. Notes: M: Marker; 1: Empty vector; 2: shRNA-SUMO1-1; 3: shRNA-SUMO-1-2; 4: shRNA-SUMO-1-3.

third day, cells proliferated rapidly. One week later, primary cells grew to over 74% (Fig. 2B). Observed under light microscopic, major cellular morphology appeared to be fusiform and oval, a few appeared to be polygonal with tapering at two ends and clear nucleus, even some of them were polynuclear with clear nucleolus, which indicated to be big and deep color by HE staining, complying with features of SGC-7901 cells (Fig. 2C).

Enzymic digestion analysis and sequence analysis of recombinant plasmids

The study built three recombinant plasmids including shRNA-SUMO1-1, shRNA-SUMO1-2 and shRNA-SUMO1-3. They were double-enzyme digested by BamH 1 and Hind III, where 66 bp insert fragment and 4.2 kb linear plasmid pSilencerTM 3.1-H1 neo. The empty vector group was only transfected with empty vector plasmid. The results of recombinant plasmids identified by enzyme digestion analysis were correct as Figure 3.

Effect of shRNA transfection on SUMO gene silencing in the shRNA-SUMO-1-1, shRNA-SUMO-1-2 and shRNA-SUMO-1-3 groups

The target sequences of SUMO-1 mRNA were separately encoded to shRNA-SUMO-1-1, shRNA-SUMO-1-2 and shRNA-SUMO-1-3. Agarose gel electrophoresis results indicated shRNA-SUMO-1-1 group without SUMO-1 band while shRNA-SUMO-1-2 group and shRNA-SUMO-1-3 group with visible SUMO-1 band, which suggested the latter two had poor gene

Fig. 4. Effects of shRNA transfection on *SUMO-1* gene silencing. Notes: A, the electrophoretogram of SUMO-1 mRNA expressions in each group; B, the cartogram of SUMO-1 mRNA expressions in each group; C, the electrophoretogram of SUMO-1 mRNA expressions at different time in the shRNA-SUMO-1-1 group; D, the changing trend diagram of SUMO-1 mRNA expressions at different time in the shRNA-SUMO-1-1 group; E, the Western blotting image of SUMO-1 protein expressions at different time in the shRNA-SUMO-1-1 group; ** represented $P < 0.01$ compared with the blank group; * represented $P < 0.05$ compared with the blank group.

Fig. 5. Comparison of SGC-7901 cell proliferation in each group. Notes: CCK-8, cell counting kit-8; * represented $P < 0.05$ when compared with the blank group.

silence effect. There was no significant influence on *SUMO-1* gene expression in the empty vector group (Fig. 4A). qRT-PCR results indicated *SUMO-1* mRNA levels were significantly up-regulated in the shRNA-SUMO-1-1, shRNA-SUMO-1-2 and shRNA-SUMO-1-3 groups (all $P < 0.05$), which were significantly different from both the blank group and the empty vector group (both $P < 0.05$), as Figure 4B. By Image J software analysis of gray value, silence efficiency of shRNA-SUMO-1-1 at 24 h, 48 h, and 72 h were respectively 12.47%, 75.84 % and 49.36% (Fig. 4C~D). By Western blot, the change of SUMO-1 expression was consistent with the change rule of qRT-PCR (Fig. 4E). All these results indicated that shRNA-SUMO-1-1 had the best silence effect on SUMO-1 than other two recombinant plasmids, where the best silence time was 48 h.

Comparison of SGC-7901 cell proliferation in each group

CCK-8 method was applied to detect SGC-7901 cell proliferation after SUMO-1 interfered by shRNA. It's indicated that cell proliferation ability was significantly reduced by 24 h, 48 h and 72 h interference in the shRNA-SUMO-1-1 group when compared with the blank group

Table 3. The proportions of specific cell cycle in each group by flow cytometry detection (n = 3, %, mean $\pm$ standard deviation). Note: * represented $P < 0.05$ when compared with the blank group

Groups	G0/G1	S	G2/M
Blank	47.22 $\pm$ 1.59	20.00 $\pm$ 1.49	32.78 $\pm$ 2.22
Empty vector	48.35 $\pm$ 1.31	19.22 $\pm$ 1.35	32.43 $\pm$ 2.62
shRNA-SUMO-1-1	56.70 $\pm$ 2.36*	3.37 $\pm$ 1.00*	39.92 $\pm$ 1.95*
shRNA-SUMO-1-2	47.04 $\pm$ 1.29	20.19 $\pm$ 1.24	32.77 $\pm$ 1.10
shRNA-SUMO-1-3	48.46 $\pm$ 1.28	19.32 $\pm$ 2.24	32.22 $\pm$ 2.35

Fig. 6. Comparison of SGC-7901 cell apoptosis in each group. Notes: A, cell apoptosis detection by Annexin V/PI double staining and flow cytometry; B, statistical results of cell apoptosis in each group; C: expressions of bcl-2, c-myc and P53 mRNA in each group detected by agarose gel electrophoresis; D, expressions of bcl-2, c-myc and P53 protein detected by Western blotting; * represented $P < 0.05$ when compared with the blank group.

(all $P < 0.05$). With the culture time increased, the inhibition of cell proliferation by shRNA-SUMO-1-1 showed a time-dependent effect. Compared with the blank group, after 24 h and 48 h interference in the shRNA-SUMO-1-2 group, cell proliferation was not reduced much, but after 72 h interference it was reduced significantly ($P < 0.05$). However, in the shRNA-SUMO-1-3 group, these three time points showed no obvious reduction of cell proliferation. And cell proliferation had no great changes in the empty vector group in comparison with the blank group (both $P > 0.05$) (Fig. 5).

Comparison of cell cycle of SGC-7901 cells in each group

The cell cycle detection showed that cells transfection at G0/G1 stage in the shRNA-SUMO-1-1 group took up 56.70 ± 2.36 proportion of the whole cell cycle, which were significantly higher than that in the blank group ($G0/G1 = 47.22 \pm 1.59$) and that in the empty vector group (48.35 ± 1.31) (both $P < 0.05$). The proportion of S stage cells in the blank group was 20.00 ± 1.49 while that in the shRNA-SUMO-1-1 group was 3.37 ± 1.00 , much lower than that in the blank group ($P < 0.05$). The proportion of G2/M stage cells in the shRNA-SUMO-1-1 group was 39.92 ± 1.95 , which was much higher than that in the blank group ($G2/M = 32.78 \pm 2.22$) ($P < 0.05$). Compared with the blank group, the proportions of G0/G1, S and G2/M stage in the empty vector group, the shRNA-SUMO-1-2 group and the shRNA-SUMO-1-3 group had no much changes (all $P > 0.05$). The proportions of specific cell cycle in each group were showed as Table 3.

Comparison of SGC-7901 cell apoptosis in each group

Cell apoptosis was detected by Annexin V/PI double staining and flow cytometry. After 48 h of transfection, cell apoptosis rate of the blank group was 1.87 ± 0.22 , while the proportion of cell apoptosis in the shRNA-SUMO-1-1 group was 5.56 ± 0.88 , which showed an increase of apoptosis rate in comparison with the blank group ($P < 0.05$). But the cell apoptosis rate of the shRNA-SUMO-1-2 group (1.92 ± 0.30) and that of the shRNA-SUMO-1-3 group (2.02 ± 0.77) were not significantly changed ($P > 0.05$). The cell apoptosis rate of the empty vector group was 1.80 ± 0.12 , which is not different with that in the blank group ($P > 0.05$) (Fig. 6A~B). The apoptosis relevant mRNA expressions before and after transfection were detected by PCR method. The mRNA expressions of bcl-2 and c-myc in the shRNA-SUMO-1-1 group were declined significantly in comparison with blank group ($P < 0.05$). The plasmids shRNA-SUMO-1-1, shRNA-SUMO-1-2 and shRNA-SUMO-1-3 all had an inhibitory effect on the mRNA expressions of P53 (all $P < 0.05$), among which shRNA-SUMO-1-1 had the greatest inhibitory effect, and this indicated shRNA-SUMO-1-1 can inhibit P53 endogenous expression of SGC-7901 cells (Fig. 6C). The apoptosis relevant expressions before and after transfection were detected by Western blot. Compared with the blank group, expression levels of bcl-2, c-myc and P53 proteins in the shRNA-SUMO-1-1 group were much reduced (all $P < 0.05$), while expression levels of bcl-2 and c-myc in the shRNA-SUMO-1-2 group and the shRNA-SUMO-1-3 group were changed no so much (all $P > 0.05$), and P53 in these two groups were decreased ($P < 0.05$). However, these three protein expressions in the empty vector group had no obvious change by comparison with the blank group (all $P > 0.05$) (Fig. 6D).

Discussion

GC is one of the leading causes of cancer mortality worldwide and its management has evolved relatively little especially in advanced stages [16]. In recent years, significant progress has been made in the survival rate of GC patients, however, to reach a successful management of GC patients, biomarkers with high sensitivity and specificity in the prediction of the disease severity of GC are still in urgent needs [17]. It is reported that some genes are expressed in human malignancies abnormally, making their biological importance more apparent than before [18]. Hence, a better understanding of the molecular events of GC can lead to new preventive, diagnostic and therapeutic strategies to the disease [19].

In the present study, higher expression of SUMO-1 protein was found in GC tissues than in adjacent normal tissues. SUMO-1, a small protein significant for various biological processes, is conjugated to many intracellular proteins covalently, particularly to regulatory components of the transcriptional machinery, such as transcription and histones factors [20]. SUMO-1 is contained in SUMO family, and SUMO could maintain alternative lengthening of telomeres [21, 22], and tumor cells always escape from the proliferation limitation that normal telomere shortening imposes by activating a telomere maintenance mechanism, either alternative lengthening of telomeres or telomerase [23]. Besides, SUMO was found to play a role in the signaling regulation of nuclear factor- κ B, a tumor promoter in inflammation-associated cancer [24, 25]. Therefore, it can be concluded that SUMO plays an important role in the tumor development. In the present study, higher expression of SUMO-1 in GC tissues could further confirm the previous related studies. Similarly, over-expression of SUMO-1 appears in many mammalian cancer cells, such as squamous cell carcinoma of the lip, liver cancer and hepatocellular carcinoma [26-28].

Next, in the study, after transfection of shRNA-SUMO-1, GC SGC-7901 cells have decreased proliferation ability; in terms of cell cycle, proportion of cells at G0/G1 phase increased while that of cells at G2/M phase decreased. SUMOylation is a vital post-translational modification characterized by reversibly and covalently binding SUMO proteins to target proteins [29]. Niu et al. also pointed out that SUMOylation plays an important regulatory role in various physiological process, including modulation of transcription, cell cycle and apoptosis

[6]. Derksen et al. reported that stimulation of Wnt/ β -catenin signaling can increase cell proliferation, while blocking Wnt/ β -catenin signaling interferes with cancer cell growth [30]. Expression and activity of Wnt/ β -catenin target genes can be down-regulated after interference with SUMOylation through *SUMO-1* silence, and SUMOylation inhibition can down-regulate β -catenin via promotion of ubiquitin-proteasomal mediated degradation at protein level [31]. Therefore, in the study, shRNA-SUMO-1 inhibits SUMOylation, blocks Wnt/ β -catenin signaling, and thus decreases cells proliferation. β -catenin may regulate the expression of CyclinD1 to promote cell invasion and proliferation [32], and Cyclin D1 is an important regulatory protein at the G1/S check point [33]. Therefore, with β -catenin down-regulated by shRNA-SUMO-1, Cyclin D1 decreased, causing cells arrest at G0/G1 phase. Besides, it has been reported that apoptosis is closely associated with the G0/G1 arrest of cell cycle [34], therefore, higher apoptosis rate is observed in the study. To confirm the result, in Hao et al. study, inhibition of SUMO1 conjugation causes G1 arrest and abolishes the tumorigenic property and self-renewal of glioblastoma initiating of stem cells [35].

Finally, after transfection of shRNA-SUMO-1, expression level of Bcl-2, c-myc and p53 were declined. Bcl-2 family proteins, which have either anti- or pro-apoptotic activities, have always been studied for the past years owing to their significance in the regulation of tumorigenesis, apoptosis and cellular responses to anti-cancer therapy [34], and Bcl-2 is an anti-apoptotic protein [36]. A study has shown that β -Catenin up-regulate Bcl-2 [37], therefore in the study, shRNA-SUMO-1 inhibits β -catenin, further down-regulating Bcl-2. Besides, β -catenin can also regulate c-myc, whose transcription factor occurs frequently in human cancers and is related to tumor aggression and poor clinical prognosis [38]. Hence, in the same manner, shRNA-SUMO-1 inhibits β -catenin, further down-regulating c-myc. As for the correlation between SUMO-1 and p53, many human tumors has been found to carry missense mutations in the *TP53* gene [39], and cancer-associated p53 mutations also endow the mutant protein with new activities that can actively contribute to increased resistance to anticancer treatments and to various tumor progression stages [40]. Based on the result, it is believed that *SUMO-1* gene promotes the expression of p53 mutations; therefore, with shRNA-SUMO-1, the p53 expression is down-regulated.

In summary, *SUMO-1* gene silencing can decrease GCSGC-7901 cells proliferation, change cell cycle, promote cell apoptosis and down-regulate the expressions of Bcl-2, c-myc and P53 mutation, which provides a theoretical foundation for gene therapy against GC clinically.

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Disclosure Statement

The authors declare no conflicts of interest.

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