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Original Article

Inhibitory effect of siRNA-Annexin A7 on growth, migration, and invasion in BGC823 cells and gastric cancer xenograftsin nude mice

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Abstract: Objective: To explore the inhibitory effect of siRNA-Annexin A7 on growth, migration, and invasion of transplanted gastric cancer in nude mice. Methods: The siRNA sequence targeting to human Annexin A7 gene was designed, and based on that a pair of complementary oligonucleotides were synthesized, annealed, and cloned into plasmid pGenesil-1.1 to construct recombinant plasmid siRNA-Annexin A7. Transplanted gastric cancer model was established by injecting s.c. nude mice with human gastric cancer BGC823 cells, and siRNA-Annexin A7 was injected into the tumors formed. The nude mice were observed for clinical manifestation relying on the size and weight of transplanted tumors. The tumor tissue and angiogenesis were examined by pathologic sections. Flow cytometry was used to detect the changes of cell cycle. Western blot and qRT-PCR were used to analyze the expression of PCNA, P27, MMP-2, and TIMP-2. Results: Both the size and weight of transplanted tumors of nude mice injected with siRNA-Annexin A7 were less than those of control groups ($P<0.05$). The examination of pathologic sections showed that, compared with in the control group, obvious necrosis of tumor cells was observed in siRNA-Annexin A7 group. The cells in stage S were fewer in siRNA-Annexin A7 group than those in the other two groups, while the cells in stage G0/G1 were much more in siRNA-Annexin A7 group. The results of western blot and qRT-PCR confirmed that the expression of PCNA and MMP-2 was down-regulated, whereas the expression of p27 was up-regulated. Conclusion: Gastric cancer xenografts were established in nude mice with human gastric cancer BGC823 cells. The volume and weight of tumor were decreased after inhibition of Annexin A7 expression in BGC823 cells. Tumor cells were arranged sparsely after inhibition of Annexin A7 expression in BGC823 cells. The siRNA-Annexin A7 inhibits Annexin A7 expression in transplanted gastric cancer of nude mice, and influences the growth, migration, and invasion of tumors by down-regulating the expression of PCNA and MMP-2, as well as up-regulating the expression of p27.

Keywords: Gastric cancer, Annexin A7, proliferation, migration, invasion

Introduction

Although the incidence of gastric cancer is decreasing in recent years, gastric cancer is still common. The latest epidemiologic data show that nearly one million cases of gastric cancer were added in 2012, and more than 700,000 deaths occurred [1-3]. Gastric cancer accounts for the fifth highest incidence of cancer in the world, second only to lung cancer, breast cancer, colon cancer and prostate cancer, accounting for the second place among cancer deaths, especially in East Asia [1]. The proliferation, invasion and metastasis characteristics of gastric cancer cells play an important role in the occurrence and development of tumors. In-depth study of the factors related to the occurrence, development, proliferation and invasion of gastric cancer may help us understand the mechanism of gastric cancer and provide a basis for early diagnosis and targeted therapy.

Annexin A7 is a member of the Ca^{2+}-dependent phospholipid binding protein multigene family. Previous studies have shown that abnormal heterozygous loss of Annexin A7 subcellular levels is associated with the occurrence, development, metastasis, and invasion of various tumors [4-7]. In this study, a nude mouse xenograft model was established based on the previous study. The inhibition of Annexin A7 gene

expression on human gastric cancer cell lines was observed by gross observation, routine pathologic staining, immunohistochemistry, western blot and qRT-PCR. The effects of proliferation, invasion, and metastasis of subcutaneous xenografts in nude mice were investigated.

Materials and methods

Animals and cells

Human gastric cancer cell line BGC823 was provided by the Research Center of the Fourth Hospital of Hebei Medical University. The cells were cultured in RPMI-1640 medium containing 10% inactivated fetal bovine serum, penicillin, and streptomycin at 37°C in an incubator containing 5% CO_2. A total of 15 male BALB/C nude mice, 4-25 weeks old, 20-25 g, were purchased from Beijing Huakang Biotechnology Co., Ltd. and were raised in the barrier environment of the Animal Center of the Fourth Hospital of Hebei Medical University.

Annexin A7 shRNA transfections

A shRNA that interferes with Annexin A7 RNA was constructed; a negative control plasmid (NS-shRNA) was designed and synthesized, and a pair of complementary oligo DNA sequences designed and synthesized according to the gene sequence of the target gene and a pair of negative control sequences were as follows (5'→3').

shRNA, S: CACCGGGACAGATGAGCAGGCAATTTCAAGAGAATTGCCTGCTCATCTGTCCCTTTTTTG; A: GATCCAAAAAAGGGACAGATGAGCAGGCAATTCTCTTGAAATTGCCTGCTCATCTGTCCC. NS shRNA, S: CACCGTTCTCCGAACGTGTCACGTCATTG; A: GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGACGTGACACGTTCGGAGAAC.

All of the above were designed and synthesized by Genepharma.

The cell lines were cultured in six-well plates for 24 h and washed with RPMI 1640 prior to transfection. We divided the cells into control, NS shRNA, and Annexin A7 shRNA groups. The control group was treated with Lipofectamine 2000 only. The cells were transfected with the shRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 24 h, the transfection efficiency was evaluated.

Mouse xenograft of BGC823 cells

A total of 15 mice received subcutaneous injection into the right flanks with a 200-mL cell suspension containing 2×10^8 BGC823 cells. After 10 days, when established tumors of 0.5 cm in diameter were detected. The animals were randomly divided into three groups consisting of 5 animals each.

Annexin A7-siRNA group (n=5): on the first day, the recombinant plasmid Annexin A7-shRNA 20 μl (1 μg/μl) + in vivo transfection reagent 20 μl + 10 μl RPMI-1640 medium was injected, and then injected once every other day. A total of 7 injections were given.

NS-siRNA group (n=5): on the first day, the recombinant plasmid NS-shRNA 20 μl (1 μg/μl) + in vivo transfection reagent 20 μl + 10 μl RPMI-1640 medium was injected intratumorally, and then injected once every other day. A total of 7 injections were given.

Control group (n=5): on the first day, 20 μl of physiological saline + 20 μl of in vivo transfection reagent + 10 μl of RPMI-1640 medium was injected into the tumor, and then injected once every other day for 7 times.

One month after modeling, all nude mice were sacrificed by cervical dislocation, and the intact tumor was measured for tumor weight and tumor long dimension (a) and short dimension (b), and the tumor volume (V) was calculated using the Steel formula.

$V=a\times b^2/2$

Tumor specimen processing: each specimen was divided into 2 parts: ① one part was formalin-fixed for pathologic examination; ② one part was quickly frozen in liquid nitrogen, and stored in -80°C refrigerator for 4 hours, for extraction of total RNA and protein.

Hematoxylin-eosin staining

The gastric cancer specimens were immersed in 4% paraformaldehyde for 4 h, and transferred to 70% ethanol. Individual lobes of the gastric cancer specimen biopsy material were placed in processing cassettes, dehydrated

through a serial alcohol gradient, and embedded in paraffin wax blocks. Before immunostaining, 5-μm-thick lung tissue sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in PBS, and then stained with hematoxylin and eosin (H&E). After staining, sections were dehydrated through increasing concentrations of ethanol and xylene.

Immunohistochemistry

Tissue samples were fixed in formaldehyde (10%), embedded them paraffin, and serially sectioned into 10 separate 4 μm sections. After xylene dewaxing and rehydration using an ethanol gradient, we incubated the sections at room temperature in H_2O_2 (3%) for 15 min to remove any endogenous peroxidase activity. We then washed the sections with PBS and performed antigen retrieval using citrate buffer (0.01 M, pH 6.0), followed by heterogenetic antigen blocking with normal goat serum (5%) at room temperature for 40 min. We then added rabbit anti-human annexin 7 polyclonal antibody (Proteintech Group, Inc., 1:100 dilution), incubated the sections at 4°C overnight, and washed them with PBS. We then added biotin-conjugated, goat anti-rabbit IgG polyclonal antibody (Zhongshan Golden Bridge Inc.; 1:100 dilution), incubated the sections at 37°C for 30 min, and washed them again with PBS. Next, we added a horseradish peroxidase-conjugated streptavidin working solution, washed the sections with PBS, and applied DAB chromogen for color development. Finally, the sections were stained with hematoxylin, dehydrated, cleared, and mounted with neutral gum. PBS was substituted for the primary antibody as a negative control.

Two independent examiners determined the staining intensity and percentage of the total area with positive staining for each section. We scored the expression of Annexin A7 by multiplying the intensity scores by the percentage of the area of the sections that was positively stained. Briefly, we categorized the intensity scores into four groups: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. Similarly, we categorized the percentages of positively stained cells into four groups: 0, <5%; 1, 5-25%; 2, 25-50%; 3, 51-75%; 4, >75%. We then calculated a composite score by multiplying the intensity score and the percentage of staining. We grouped the composite scores into four grades: grade 1, score 0-1; grade 2, score 2-4; grade 3, score 5-8; grade 4, score 9-12.

Quantitative RT-PCR

We isolated total RNA from the cell lines using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. We synthesized cDNA from 2 μg total RNA using Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the SYBR Green RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μl containing 2 μl reverse-transcription product, 5 pmol each primer, 10 μl 2× SYBR Green mix (Applied Biosystems), and 0.4 μl 1,000× diluted reference dye (Applied Biosystems). The primers [11] used to detect Annexin A7 expression were 5'-GTATCCACAGCCACCTTCACAGTC-3' (forward) and 5'-TCCAAADAAACAGGAGAGAAAACAG-3' (reverse). As an internal control, we detected the expression of GAPDH mRNA using the following primers: 5'-CGCTGAGTACGTCGTGGAGTC-3' (forward) and 5'-GCTGATGATCTTGAGGCTGTTGTC-3' (reverse).

The PCR was performed using an Applied Biosystems Prism model 7900HT Sequence Detection System with the following settings: initial denaturation at 95°C for 5 min to ensure complete denaturation of the DNA and activation of the Taq polymerase, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. Fluorescence was detected after each cycle. All reactions were performed in triplicate. We normalized the relative expression levels to those of the GAPDH internal control and calculated the Annexin A7 expression levels using the $2^{-\Delta\Delta CT}$ method.

Western blot

Cellular protein was extracted from the cell lines using lysis buffer and protease inhibitors (Beyotime, China). We separated 60 μg of protein from each sample by 12% SDS-PAGE and then electroblotted the gels onto PVDF membranes (Roche, Basel Switzerland). The membranes were then blocked in Tris-buffered

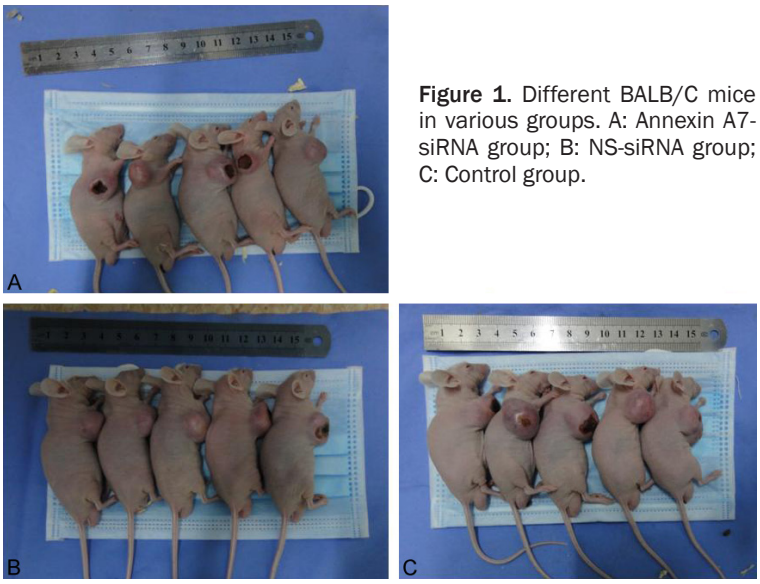

Figure 1. Different BALB/C mice in various groups. A: Annexin A7-siRNA group; B: NS-siRNA group; C: Control group.

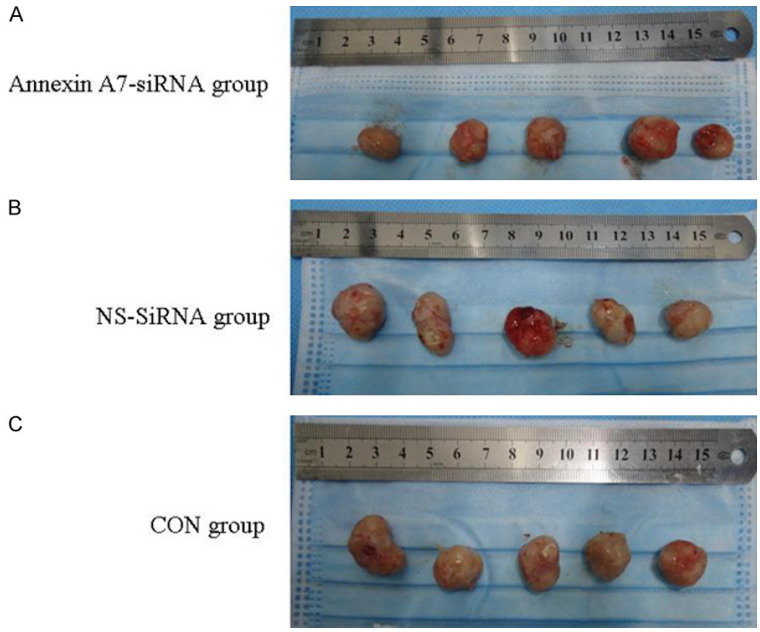

Figure 2. Different tumors in various groups. A: Annexin A7-siRNA group; B: NS-siRNA group; C: Control (CON) group.

saline with 0.1% Tween-20 (TBS-T) containing 5% non-fat milk for 1 h at room temperature, followed by incubation at 4°C overnight with primary antibodies for Annexin A7, proliferating cell nuclear antigen (PCNA), p16, p21, p27, cyclin D1, cyclin E1, cyclin A, tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2, ICAM-1, MMP-1, MMP-2, and MMP-9 (all antibodies were purchased from Santa Cruz, USA). After several washes with TBS-T, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. We used the GAPDH protein level as a control for equal protein loading.

The expression levels of PCNA, P27, MMP-2 and TIMP-2 in transplanted tumor tissues were detected by immunohistochemistry, RT-PCR and western blot.

Statistical analysis

Results are expressed as means ± standard deviation. Student's t test or one-way ANOVA were used to determine the significance of differences between groups. We used SPSS software version 22.0 for all statistical analyses, with $P<0.05$ as the threshold for significance.

Results

General conditions, tumor volume and weight changes of model animals

Nude mice in each group developed tumors, and the control group began to have decreased appetite, apathetic and weight loss in the control group 2 weeks after the modelling; while the appetite and body weight of the nude mice in the experimental group did not decrease significantly, and it was relatively active. Compared with the control group and the NS-siRNA group, the average volume and average weight of the nude mice in the Annexin A7-siRNA group were significantly decreased ($P<0.05$); there was no difference between the control group and the NS-siRNA group ($P>0.05$) (**Figures 1**, **2** and **Table 1**).

Pathological results of each group of tumors

H&E staining showed that the growth of cells of the NS-siRNA group and the control group were densely packed, while the cells of the Annexin A7-siRNA group showed sparse cells (**Figure 3**).

Table 1. Mean sizes and weights of tumors in various groups (n=5)

	Size (mm^2)	Weight (g)
Annexin A7-siRNA group	654.99±311.6*	1.30±0.67*
NS-siRNA group	1198.46±362.42	2.27±0.60
Control group	1228.29±47.50	1.97±0.32

Note: *: *P*<0.05 compared to the other two groups.

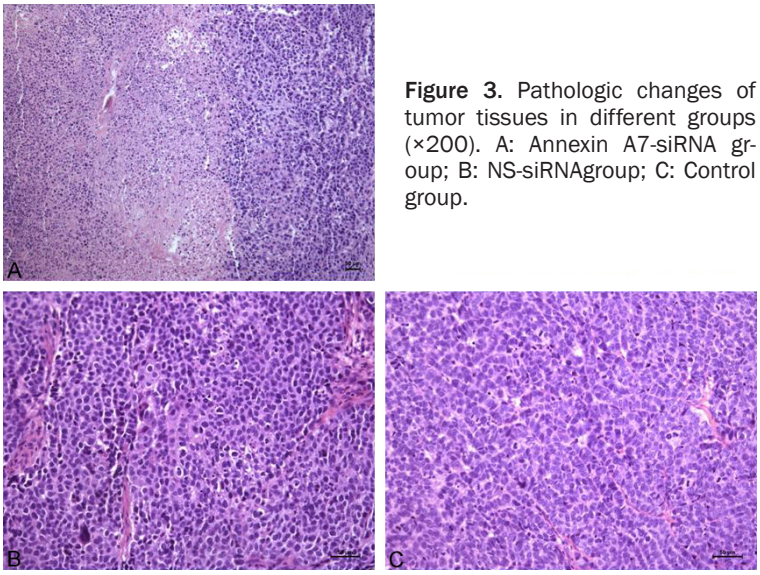

Figure 3. Pathologic changes of tumor tissues in different groups (×200). A: Annexin A7-siRNA group; B: NS-siRNAgroup; C: Control group.

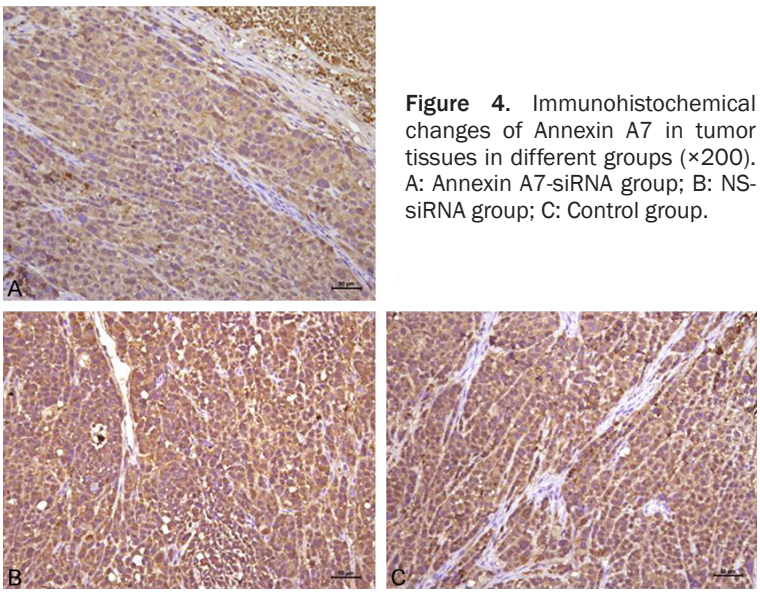

Figure 4. Immunohistochemical changes of Annexin A7 in tumor tissues in different groups (×200). A: Annexin A7-siRNA group; B: NS-siRNA group; C: Control group.

Annexin A7 immunohistochemical staining results in each group of tumor

Immunohistochemical results showed that there was no significant difference in staining of tumor cells between NS-siRNA group and control group, while tumor cells stained with Annexin A7-siRNA group were lighter (**Figure 4**).

Western blot of Annexin A7 in each group of tumor

Western blot analysis showed that there was no significant difference in the expression of Annexin A7 protein between the NS-siRNA group and the control group, while the expression of Annexin A7 protein in the tumor tissue of Annexin A7-siRNA group was significantly down-regulated (**Figure 5**).

Annexin A7 mRNA expression in each group of tumors

The results of qRT-PCR showed that there was no significant difference in mRNA expression of Annexin A7 in NS-siRNA group and control group, while mRNA expression of Annexin A7 in tumor tissue of Annexin A7-siRNA group was significantly downregulated (*P*<0.05) (**Figure 6**).

Expression of PCNA, P27, MMP-2 and TIMP-2 in transplanted tumor tissues

Immunohistochemistry showed that there was no significant difference in the staining of PCNA, P27, MMP-2, and TIMP-2 between the NS-siRNA group and the control group, while the PCNA and MMP-2 staining of the tumor cells in the Annexin A7-siRNA group was lighter than that of the P27 staining. There was no significant difference in TIMP-2 (**Figure 7**).

The results of western blot showed that the protein expression of PCNA was significantly downregulated in the Annexin A7-siRNA group compared with the NS-siRNA group and the control group, while the protein expression of P27 was significantly increased (*P*<0.01). The expres-

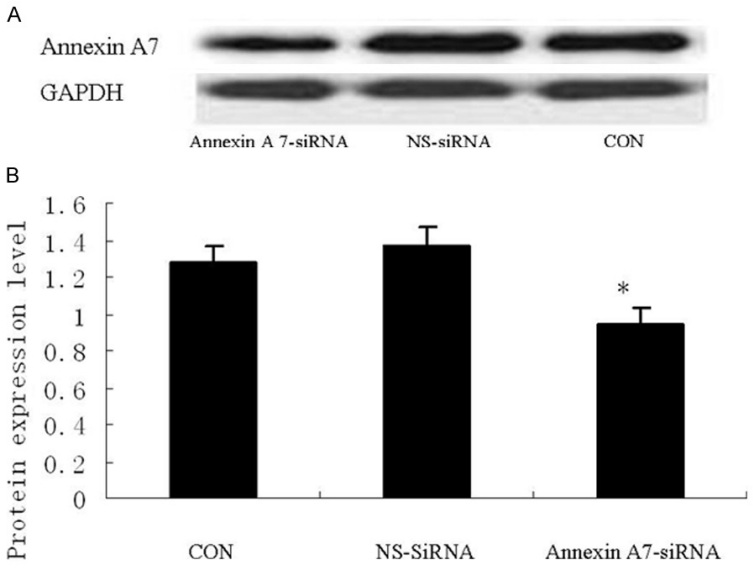

Figure 5. Protein expression of Annexin A7 in tumors tissues of different groups. A: Protein expression of Annexin A7; B: Protein expression level of Annexin A7.

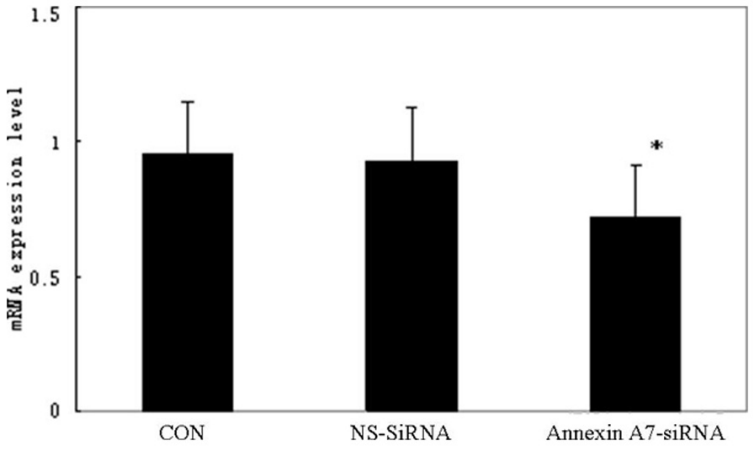

Figure 6. The expression of Annexin A7 in different groups. mRNA expression levels detected by qRT-PCR. GAPDH was used as an endogenous reference to standardize mRNA expression levels. *P<0.05 compared with control group.

sion of MMP-2 protein was significantly down-regulated, while the expression of TIMP-2 protein was not significantly changed. There was no difference between the blank plasmid group and the NS-control group (**Figure 8**).

The results of qRT-PCR showed that the mRNA expression of PCNA was significantly down-regulated in shRNA-Annexin A7 group compared with NS-siRNA group and control group, while the mRNA expression of P27 was significantly increased (P<0.01). The mRNA expression of MMP-2 was down-regulated, and the expression of TIMP-2 protein was not significantly changed. There was no difference between the NS-siRNA group and the control group (**Figure 9**).

Discussion

Gastric cancer is one of the most common malignant tumors of the digestive system. Its prevalence includes high incidence, frequent early invasion and metastasis, and poor prognosis [8, 9]. Although surgical resection techniques and assisted radiotherapy and chemotherapy techniques have made some progress in recent years, the survival rate of gastric cancer patients has not increased significantly. This is an important challenge facing the current treatment of gastric cancer. Since the occurrence and development of gastric cancer involve complex biologic processes, the characteristics of gastric cancer proliferation, invasion, and metastasis, as well as the molecular biologic effects of related genes in this process, will explain the biological characteristics of gastric cancer. It provides an important reference for early diagnosis, risk assessment and targeted therapy of gastric cancer. The previous study confirmed from the tissue, cell and molecular biology levels that Annexin A7 plays an important role in the proliferation, invasion, and metastasis of gastric cancer cells, and can be used as a predictor of lymph node metastasis and long-term survival in patients with gastric cancer. The main purpose of this study was to investigate the effect of shRNA inhibition of Annexin A7 on proliferation, invasion, and metastasis of human gastric cancer cell lines transplanted subcutaneously in nude mice on the basis of previous studies.

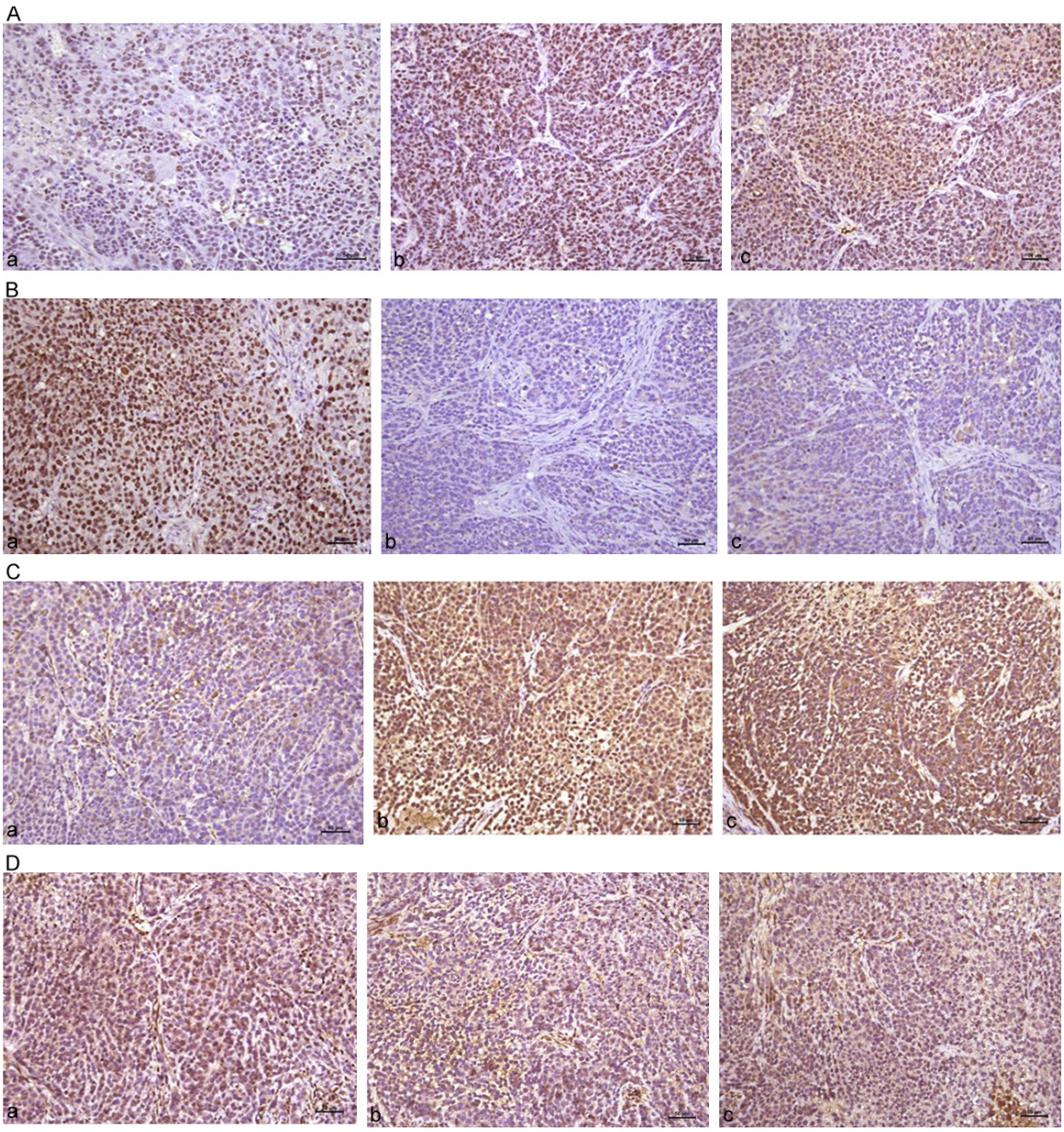

Figure 7. Immunohistochemical measurement of PCNA, P 27, MMP-2, and TIMP-2. A: PCNA in different groups; B: P27 in different groups; C: MMP-2 in different groups; D: TIMP-2 in different groups. a: Annexin A7-siRNA group; b: NS-siRNA group; c: Control group.

Annexin family is a family of Ca^{2+}-dependent phospholipid-binding proteins widely distributed in various tissues and cells of animals and plants, and has the ability to reversibly bind to phospholipid membranes and bind to calcium ions. Annexin A7 belongs to the Annexin A family, also known as synthesin, and was subsequently found to contain such proteins in a wide variety of genus cells. The Annexin A7 gene is located on the human chromosome 10q21. In the past, it was considered that this region is the region where the tumor suppressor gene is located. We know that Annexin A7 is involved in the development and progression of tumors, as follows: 1) Annexin A7 may be a tumor suppressor gene in glioblastoma, glioblastoma multiforme, melanoma and prostate cancer; 2) Annexin A7 is an oncogene in liver cancer, gastric cancer, nasopharyngeal carcinoma, colorectal cancer and breast cancer; 3) Annexin

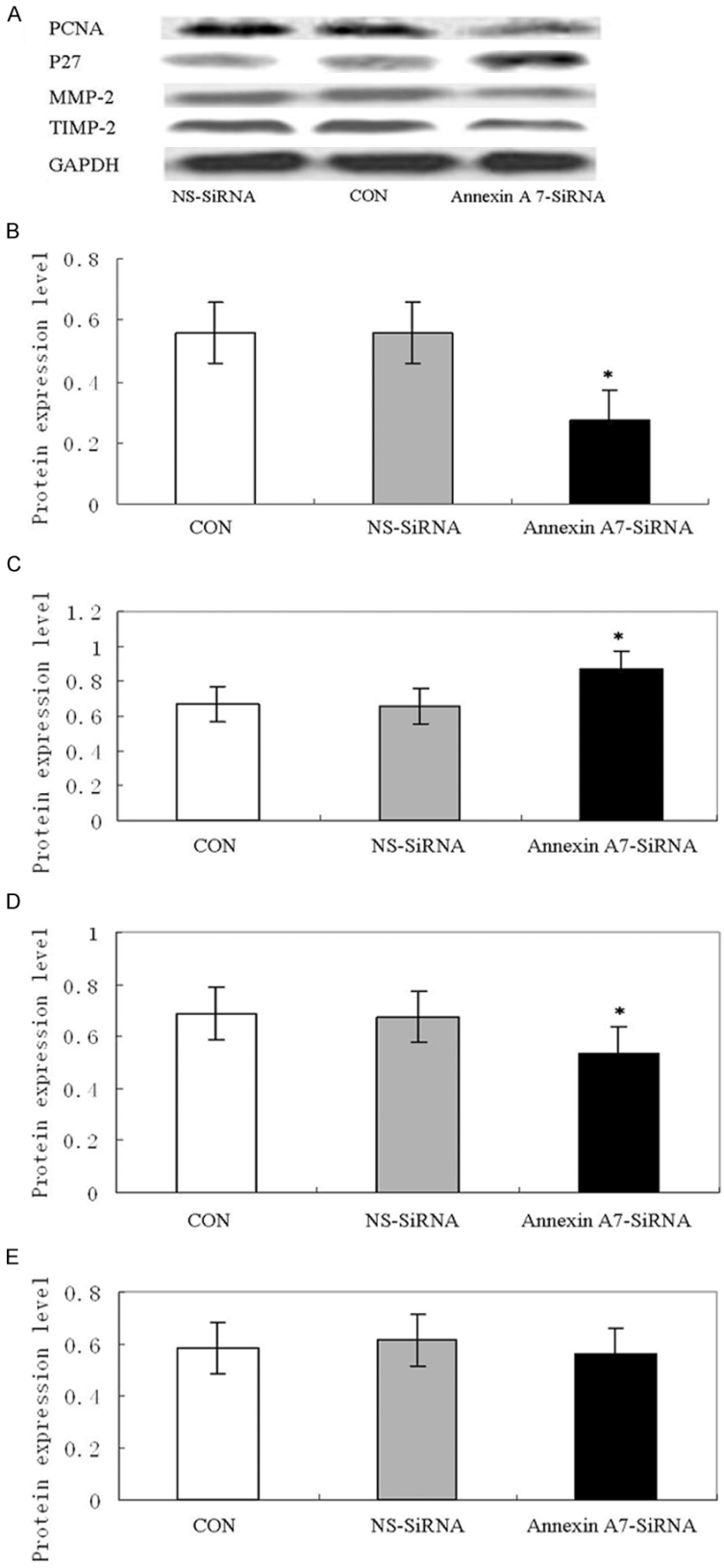

Figure 8. Protein levels of PCNA, P27, MMP-2, and TIMP-2 among different groups by western blot. A: Protein expression bands; B: Protein expression of PCNA; C: Protein expression of P27; D: Protein expression of MMP-2; E: Protein expression of TIMP-2. *P<0.01 compared with control (CON) group.

A7 subcellular localization and down-regulation, heterozygous deletion and tumor occurrence, development, invasion and metastasis with sexual relevance [10].

RNA interference (RNAi) technology is a method developed in recent years to specifically inhibit gene expression. It is a highly conserved, double-stranded RNA (dsRNA) induced by evolution. The phenomenon of efficient and specific degradation of homologous mRNA is characterized by high specificity, high efficiency, transitivity and time-dose dependence [11, 12]. Previous studies have used RNAi technology to study and explore some gene functions related to the occurrence, development, and metastasis of gastric cancer. Recently, shRNA (short hairpin RNA, shRNA) has been successfully transfected in mammals.

Proliferating cell nuclear antigen (PCNA) protein is an acidic protein expressed only in proliferating cells. The substances necessary for DNA replication, a variety of proteins involved in cell replication and repair, bind to the replication step, so PCNA reflects a very sensitive and accurate indicator of cell proliferation [13]. Increased cell proliferation activity makes cancer cells easily detach from the primary tumor, which is conducive to cancer invasion and metastasis [14]. A number of studies at home and abroad have shown that PCNA has important implications for the prognosis of patients with malignant tumors [15]. Gastric cancer occurs mostly in proliferating cells, PCNA is overexpressed; and PCNA high expression indicates that the cells are in a proliferative state,

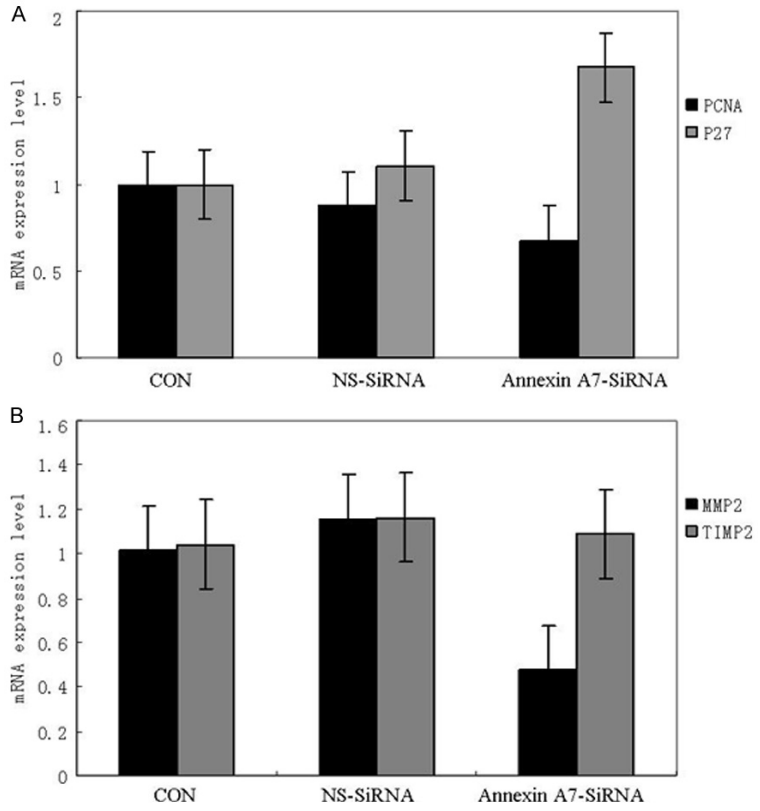

Figure 9. mRNA expression levels of PCNA, P27, MMP-2 and TIMP-2 from different groups. **P*<0.01 compared with control (CON) group.

with a large chance of tumor invasion and metastasis, and poor prognosis. The p27 protein belongs to the CKI family, and p27 overexpression inhibits cell progression from the G1 phase to the S phase through the cyclin E-CDK2 pathway, thereby restricting cell cycle progression [16]. MMP-2 can degrade type IV collagen (the main component in the basement membrane) and may therefore be involved in tumor invasion and metastasis. Studies have shown that the expression of MMP-2 is closely related to gastric cancer, and is related to the depth of tumor invasion, lymph node metastasis, and differentiation [17, 18]. Tissue inhibitors of metalloproteinase (TIMPs) are natural inhibitors of MMPs. TIMP-2 is mainly secreted by tumor cells and interstitial cells. It has a broad spectrum of MMPs inhibition, is the inhibitor of most MMPs except MMP-9, and has certain selectivity. TIMP-2 inhibits MMP-2 enzyme by non-covalent bond in a ratio of 1:1. The original and active form, thereby protecting the integrity of the extracellular matrix (ECM) to combat tumor invasion and metastasis. It can also initiate or inhibit the activation of MMP-2 in a concentration-dependent manner [19].

In this study, the above indicators were used as markers to analyze the role of Annexin A7 in the proliferation, invasion, and metastasis of gastric cancer. The Annexin A7 gene interference plasmid Annexin A7-siRNA was injected into the subcutaneously transplanted tumor of nude mice and found to inhibit the expression of Annexin A7 in nude mice. It was further analyzed that the mechanism of Annexin A7 inhibiting cell proliferation may be due to its down-regulation of PCNA. MMP-2 expression, while up-regulating P27 expression, affects the cell cycle progression, and slows the process of tumor cell proliferation, invasion and metastasis, thereby exerting anti-tumor effects. This result is consistent with previous studies, thus confirming that Annexin A7 may be one of the important oncogenes in the development and progression of gastric cancer, and may be an important marker for predicting lymph node metastasis and prognosis in gastric cancer patients and it has become an important target for molecular targeted therapy.

In conclusion, a transplanted gastric cancer model was successfully established by injecting s.c. nude mice with human gastric cancer BGC823 cells. The volume and weight of tumor were decreased after inhibition of Annexin A7 expression in BGC823 cells. Tumor cells arranged sparsely after inhibition of Annexin A7 expression in BGC823 cells. The siRNA-Annexin A7 inhibited the Annexin A7 expression in transplanted gastric cancer of nude mice, and influenced the growth, migration and invasion of tumors by down-regulating the expressions of PCNA and MMP-2, as well as up-regulating the expression of P27.

Disclosure of conflict of interest

None.

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