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Original Paper

Downregulation of NGAL is Required for the Inhibition of Proliferation and the Promotion of Apoptosis of Human Gastric Cancer MGC-803 Cells

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Key Words
Neutrophil gelatinase-associated lipocalin • Gene silencing • Gastric cancer • Proliferation • Apoptosis

Abstract
Background/Aims: Gastric cancer is considered as a common malignancy with a poor prognosis as well as unsatisfactory treatment. Neutrophil gelatinase-associated lipocalin (NGAL) has been reported to affect multiple aspects of human tumor, including gastric cancer. This study aims to explore the effects of NGAL gene silencing on the proliferation as well as apoptosis of human gastric cancer MGC-803 cells. ***Methods:*** This study included 87 patients with gastric cancer. MGC-803 cells were collected and mainly treated with siRNA against NGAL and recombinant NGAL plasmid. The expression of NGAL mRNA and the expressions of NGAL protein and apoptosis-related proteins were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, respectively. Cell cycle and apoptosis were tested by flow cytometry, and cell proliferation was detected by water soluble tetrazolium-1 (WST-1) assay. The effect of NGAL gene silencing on tumorigenicity of MGC-803 cells *in vivo* was detected through establishment of xenograft in nude mice. ***Results:*** NGAL was highly expressed in gastric cancer tissues. The protein and mRNA expressions of NGAL gene in MGC-803 cells treated with NGAL-siRNA were obviously reduced, and the amount of cells in G0/G1 phase was increased. Moreover, MGC-803 cells treated with NGAL-siRNA exhibited inhibited proliferation, enhanced apoptosis, decreased expressions of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) as well as B-cell lymphoma-2 (Bcl-2) and increased expressions of cysteine-aspartic acid specific protease-9 (caspase-9) and Bcl2-associated X (Bax), as well as repressed tumorigenicity *in vivo*. ***Conclusion:*** NGAL gene silencing inhibits proliferation and promotes apoptosis of MGC-803 cells, which can provide a novel theory for treatment of gastric cancer.

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Introduction

Gastric cancer is a common malignancy with a high mortality rate, accounting for nearly 70, 000 new cases and 650, 000 deaths every year [1, 2]. It is the fourth most common cancer diagnosed in men globally (the fifth in women), and the third leading cause of cancer-associated deaths in men (the fifth in women) [1]. The pathogenesis of gastric cancer lies in the accumulation of epigenetic and genetic alterations in tumor suppressor genes and oncogenes, which result in the dysregulation of many signaling pathways, thereby leading to the disturbance of cell cycle as well as the balance between cell proliferation and death [3]. Various factors, including helicobacter pylori infection, host genetic polymorphisms, epigenetic and environmental factors, and lifestyle, contribute to the occurrence of gastric cancer [4].

At present, gastric cancer is primarily treated with surgery, radiotherapy and chemotherapy [5]. In spite of improvements in strategies for early detection, many patients are still diagnosed in advanced stage and don't receive effective curative surgery [2]. Furthermore, prognosis is still extremely poor for most patients with gastric cancer, especially for those with tumor recurrence and metastasis [1, 6]. It was reported that poor overall survival in human gastric cancer was related to the excessive expression of epithelial-to-mesenchymal transition (EMT) biomarkers [7]. Increasing evidences indicate that neutrophil gelatinase-associated lipocalin (NGAL) expression is remarkably reduced after induction of EMT by epidermal growth factor [8-10]. Therefore, novel therapy targeting NGAL might be a new treatment strategy.

NGAL, also known as Lipocalin-2 or Lcn-2, is a kind of 25kDa protein involved in the host defense against some gram-negative bacterium through binding with iron loaded bacterial siderophores, which limits the availability of this essential nutrient to bacteria, leading to the inhibition of their growth and pathogenicity [11]. It was frequently related to the size, stage, and invasiveness of tumor [12]. Recent evidence has also indicated that abnormal expression of NGAL gene might happen in lots of pathologic conditions including tumor [12]. Therefore, we suspect that the NGAL gene can contribute to the treatment of human gastric cancer. To achieve this, we studied the impact of silenced NGAL gene on the biological behavior of the MGC-803 cells to provide an experimental basis for targeted NGAL gene therapy against human gastric cancer.

Materials and Methods

Ethics statement

Experimental procedures were performed in line with the guidelines for using laboratory animals established by Henan Provincial People' Hospital. This study was conducted in strict accordance with the Helsinki declaration and approved by the Institutional Review Board of Henan Provincial People' Hospital (ethical number: 201712002). Participants provided written informed consent to participate in this study.

Study subjects

This study included 87 patients with gastric cancer in Henan Provincial People' Hospital between September 2016 to October 2017. The patients who had a Karnofsky performance score (KPS) score of more than 70 and patients pathologically diagnosed with gastric cancer after operation were included [13]. Exclusion criteria concluded [14]: The patients with primary tumors in other parts, patients who had an infectious disease or an acute inflammatory response, patients who had a history of immune system disease or a chronic disease, and patients who recently used drugs, antibiotics, or chemotherapeutic drugs that affected white blood cells were excluded. All gastric cancer tissues and adjacent tissues were cut into small pieces, quickly stored in liquid nitrogen in a cryopreservation tube and then moved to a -80°C refrigerator.

Immunohistochemistry

The positive expression rate of NGAL protein in gastric cancer tissues and adjacent normal tissues was detected by immunohistochemistry. Gastric cancer tissues and adjacent normal tissues were collected, fixed in 4% paraformaldehyde, embedded, and sliced into 4 μm sections. After 60 h of baking, the sections were dewaxed with xylene, dehydrated with gradient alcohol, soaked in 3% H_2O_2 for 10 min and washed by distilled water. High pressure antigen repair was performed for 90 s, and the temperature was cooled at room temperature. Next, the sections were blocked with 5% bovine serum albumin (BSA) and incubated for 30 min at 37°C. The diluted 100 μL NGAL anti-rabbit primary antibody (1:1000, ab63929, Abcam Inc., Cambridge, MA, USA) was incubated at 4°C overnight. Next, diluted biotin-labeled goat anti-rabbit (1: 100; HY90046, Shanghai Hengyuan Biotechnology Co., Ltd., Shanghai, China) secondary antibody working solution was added and incubated for 30 min at 37°C. The sections were washed with phosphate buffered saline (PBS), followed by addition of streptomycin anti-biotin peroxidase solution (Beijing Zhongshan Biotechnology Co., Beijing, China) and incubation at 37°C for 30 min. Positive diaminobenzidine (DAB) (Beijing Bioss Biotechnology Co. Ltd., Beijing, China) was used for coloration at room temperature. The sections were then soaked in hematoxylin for 5 min, washed with tap water, immersed in 1% hydrochloric acid for 4 s and rinsed in tap water for 20 min to return to blue. The average optical density (OD) value of NGAL positive staining was detected by Image-Proplus image analysis software (Media Cybernetics, Silver Springs, MD, USA) under high magnification for quantitative analysis. The standard of protein positive cells was that normal positive cells were brown yellow [15]. Five fields of vision under high magnification (× 400) were randomly selected in each section with 100 cells per field of vision, followed by calculation of the percentage of positive cells in total cells. The experiment was independently repeated 3 times.

Cell culture and treatment

Human gastric cancer MGC-803 cell lines were provided by Research Institute of Oncology of Hunan Medical University. Cells were cultivated in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS, HyClone, Logan, Utah, USA), 100 μg/μL penicillin and 100 μg/μL streptomycin) in a humidified thermostat (Thermo Scientific 8000, Thermo Fisher Scientific, Massachusetts, USA) with 5% CO_2 at 37°C. They were passaged every 3 ~ 4 d, and cells in the logarithmic phase of growth were harvested.

Cell transfection

Cell transfection was finished with Lipofectamine™ 2000 reagent based on the requirements of manufacturer's protocol (Invitrogen Inc., Carlsbad, CA, USA). NGAL-small interference RNA (siRNA) sequence is 5'-ACATCCGGCAGGACAATGA-3', and the negative control (NC) sequence is 5'-AACGUACGCGGAAUACUUCGA-3'. The NGAL recombinant plasmids were purchased from Proptech Company and all primers were synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). MGC-803 cells were divided into NGAL-siRNA group (transfected with NGAL-siRNA sequence), NGAL group (transfected with NGAL-siRNA recombinant protein), control group (transfection of control sequence) and blank group (no transfection). Effects of RNA interference were detected 48 h after transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

After cells were washed by PBS three times, the total RNA of the cells was extracted using Trizol method. The cDNA was reverse-transcribed following the instructions of cDNA synthesis kit. The sequences of NGAL and GAPDH (internal reference) were amplified. The forward primer and reverse primer of NGAL were 5'-GAAGACAAAGACCCGCAAAAG-3' and: 5'-CTGGCAACCTGGAACAAAAG-3' respectively, and its amplified product was 795 bp. The forward primer of GAPDH was 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer was 5'-TCCACCACCCTGTTGCTGTA-3', with its amplified product being 509 bp. The PCR amplification conditions were as follows: pre-denaturation at 94°C for 4 min, and 30 cycles of denaturation at 95°C for 15s, renaturation at 55°C for 30 s as well as extension at 72°C for 45s. Next, 2% agarose gel electrophoresis was performed, with the pressure of stacking gel and separation gel at 80 V and 100 V respectively. Absorbance was detected using Gel Electrophoresis Image Analysis System (V1 Company, France). The ratio of absorbance of NGAL to that of GAPDH was regarded as the relative expression of NGAL mRNA. The above experiment was repeated 3 times.

Western blot analysis

The protein of MGC-803 cells was extracted and determined as follows. The harvested cells were lysed, mixed with 3 × loading buffer, stewed in boiling water for 10 min, and then centrifuged at 10000 r/min for 5 min (r = 400 mm). The protein concentration was tested with ultraviolet spectrophotometric method. After 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the MGC-803 protein was transferred to nitrocellulose (NC) membrane using semi-dry electro-transfer method (12 V, 20 min). Subsequently, the protein was pre-stained with ponceau S and its molecular weight was marked. After washing with Tris-buffered saline-Tween (TBST) buffer, the membrane was incubated with primary antibodies, including rabbit anti-human NGAL and Bcl2-associated X protein (Bax) polyclonal antibodies, nude mice anti-human β-actin polyclonal antibody, cysteine-aspartic acid specific protease-9 (caspase-9), B-cell lymphoma-2 (Bcl-2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), caspase-3 and p53 (ClickSoftware Technologies Ltd, USA), at 4°C overnight. Thereafter, the membrane was incubated with horseradish peroxidase-labeled secondary antibodies (goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP,; 1 : 5000; Abcam Inc., Cambridge, MA, USA) at 37°C for 2 h, and washed three times by TBST (10 min each time) as well as once by TBS for 10 min. Then, the membrane was developed with Lumi-Light electrochemiluminescence (ECL) kit (Roche Holding Ltd., Basel, Switzerland), tabled, filmed and fixed with X-ray. After clean water washing, the expressions of apoptosis-associated proteins were determined. Image J software analysis was used for the grey value of the western blot bands. The ratio of the grey value of the target gene to that of the reference gene was taken as the relative protein expression.

Flow cytometry

Cells treated with 0.25% trypsin were centrifuged at 1000 r/min for 5 min to remove culture medium (r = 17 cm). The MGC-803 cells were washed by PBS, after which supernatant was discarded and cells were collected simutaneously. They were washed two times with ice-cold PBS, and fixed in 80% ethanol at 4°C overnight. After washing three times with PBS, cells were re-suspended in 0.1 mg/ml propidium iodide for staining at room temperature for a half hour without light. Cell cycle was detected by flow cytometry (Beckman Coulter Inc., Fullerton, California, USA). The above experiment was repeated 3 times.

Water soluble tetrazolium-1 (WST-1) assay

Cells in the NGAL-siRNA, control and blank groups were selected and were adjusted to a density of 1×10^4 cells/mL. A total of 200 μL of the cells was seeded in a 96-well plate. After 24, 48 and 72 h of incubation, 20 μL of WST-1 reagent (Fuzhou Maixin Biotech., Co., Ltd., Fuzhou, China) was put into the plate as well as incubated for 4 h. OD at 450 nm (OD_{450}) of each well was measured by microplate reader in triplicate. Inhibition rate of proliferation (%) = (1 - OD_{450} value in the NGAL-siRNA group / OD_{450} value in the control group) × 100%.

Flow cytometry

After transfection for 48 and 72 h, non-transfected cells in the NGAL-siRNA, NGAL, control and blank groups were collected, mixed with 60 mL of deionized water and 20 mL of binding buffer and washed with PBS. A total of $5 \sim 12.5 \times 10^4$ cells were suspended with 250 μL of binding buffer to reach a final cell concentration of $2 \sim 5 \times 10^4$ cells/mL. Subsequently, 195 μL of cell suspension together with 5 μL of Annexin V/fluorescein isothiocyanate (FITC; 20 μg/ml) (Dako North American Inc., CA, USA) was incubated at room temperature for 10 min without light. Thereafter, 190 μL of mixed solution was used to rinse and re-suspend the cells, and the cells were stained by 10 μl of 20 μg/mL propidium iodide (PI). Apoptosis of cells was detected with flow cytometry.

Xenograft in nude mice

Forty-five BALB/c nude mice weighing 18-20 g and aged 6-7 weeks were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., (Shanghai, China). Mice were housed under specific-pathogen-free conditions in Experimental Animal Center of Chongqing Medical University. Mice were randomly divided into the following 3 groups (n = 15): NGAL-siRNA group, control group and blank group. Mice in the NGAL-siRNA group were hypodermically injected with same volume of MGC-803 cells stably expressing NGAL-siRNA; mice in the control group were hypodermically injected with the MGC-803 cells stably expressing random control sequence; and mice in the blank group were subcutaneously injected with MGC-803 cells.

The major diameter (a) and minor diameter (b) of tumors in mice were measured, and the tumor volume (TV) was calculated by TV = 1/2 × a × b^2. Growth conditions of tumors in nude mice were observed and growth curve was plotted. Nude mice were sacrificed on the 30th day. Their tumor tissues were weighed, fixed in neutral formalin, dehydrated, embedded in paraffin and sectioned. The levels of proliferative cell nuclear antigen (PCNA) in each group were determined using immunohistochemical staining. The sections were added with serum blocking buffer, washed three times by PBS (5 min each time), incubated in 3% H_2O_2 for 10 min as well as washed three times by PBS (5 min each time). Then the sections were incubated with PCNA primary antibody (1: 1000; ClickSoftware Technologies Ltd, USA) at 4°C overnight, rewarmed at room temperature for 45 min the next day, and washed three times by PBS (5 min each time), followed by adding secondary antibody (1: 5000; ClickSoftware Technologies Ltd, USA), incubation at room temperature for 20 min and three PBS washes (5 min each time). The experiment results were evaluated using double-blind method. Five high power fields of vision (× 400) were randomly selected in every section, and 100 cells were counted in each field. DAB staining was yellowish-brown and positive 3-Amino-9-ethylcarbazole (AEC) staining was red. The percentage of positive cells to total tumor cells was calculated.

Statistical analysis

All data were analyzed by SPSS 21.0 software (IBM Corporation, New York, NY, USA). Measurement data were expressed as mean ± standard deviation. Differences between two groups were examined with *t*-test. Analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were used for multiple group comparisons. $p < 0.05$ was considered to represent significant differences.

Results

NGAL is highly expressed in gastric cancer tissues

Initially, we observed positive protein expression of NGAL in the gastric cancer tissues and adjacent normal tissues. The results (Fig. 1) showed that positive cells of NGAL protein expression were stained brown yellow. The positive protein expression of NGAL and mRNA expression of NGAL in the gastric cancer tissues were significantly higher than those in the adjacent normal tissues ($p < 0.05$). These results demonstrated that NGAL is highly expressed in gastric cancer tissues.

NGAL gene silencing inhibits the mRNA and protein expressions of NGAL in MGC-803 cells

We performed RT-qPCR and western blot analysis to determine the mRNA and protein expressions of NGAL in different transfection groups. The expression of NGAL in the NGAL-siRNA group was significantly lower than that in the blank and control groups (both $p < 0.05$). The expression of NGAL in the NGAL group was significantly higher than that in the

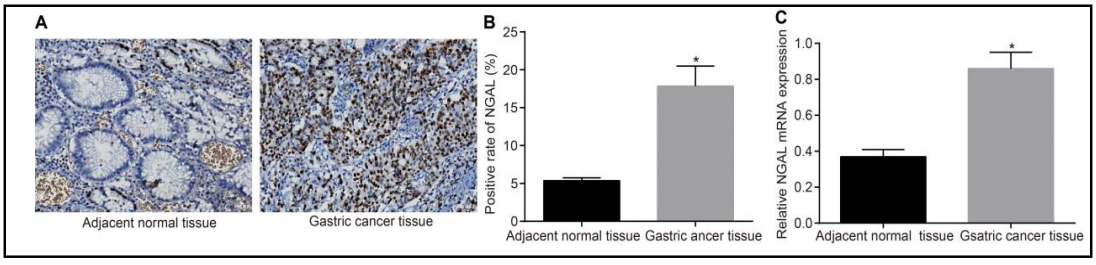

Fig. 1. Immunohistochemistry and RT-qPCR reveal that NGAL is highly expressed in gastric cancer tissues. Note: Panel A-B, immunohistochemistry (× 400) shows that positive protein expression of NGAL in the gastric cancer tissues is significantly higher than that in the adjacent normal tissues; Panel C, RT-qPCR shows that mRNA expression of NGAL in the gastric cancer tissues is significantly higher than that in the adjacent normal tissues; *, p<0.05 vs. the adjacent normal tissues; NGAL, neutrophil gelatinase-associated lipocalin; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. The data are presented as mean ± standard deviation, analyzed by t test. The experiment was independently repeated three times.

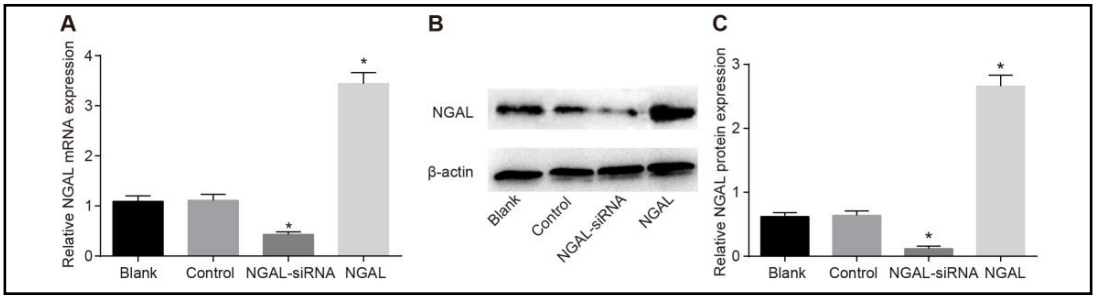

Fig. 2. RT-qPCR and western blot analysis demonstrate that NGAL gene silencing inhibits the mRNA and protein expressions of NGAL in MGC-803 cells. Note: Panel A, the histogram shows that NGAL gene silencing inhibits the mRNA expression of NGAL in MGC-803 cells, determined by RT-qPCR; Panel B-C, western blot analysis shows that NGAL gene silencing inhibits the protein expression of NGAL in MGC-803 cells; *, $p<0.05$ vs. the control and blank groups; NGAL, neutrophil gelatinase-associated lipocalin; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. The data are presented as mean ± standard deviation, analyzed by one-way ANOVA. The experiment was independently repeated three times.

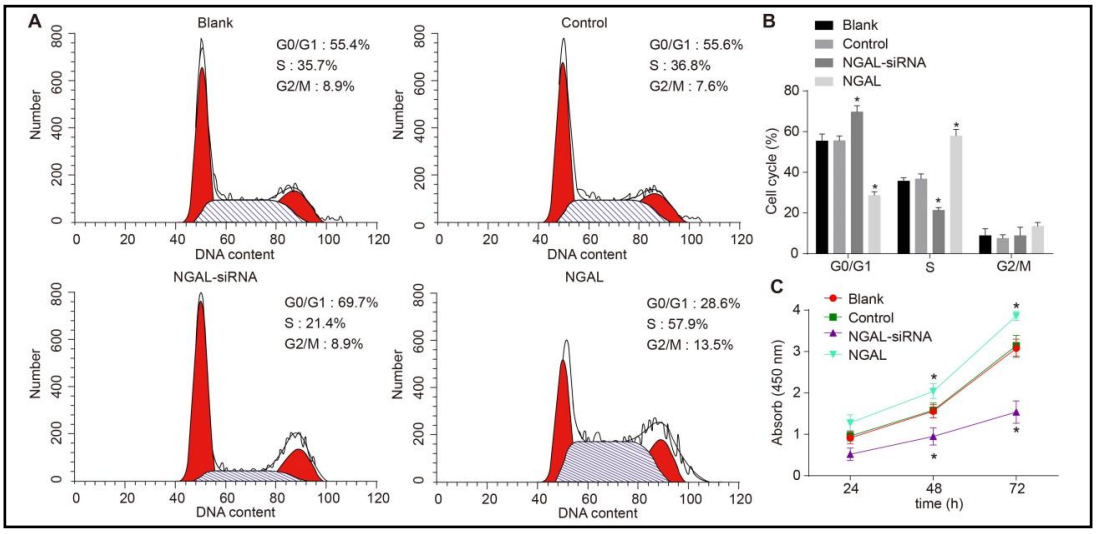

Fig. 3. Flow cytometry demonstrates that NGAL gene silencing inhibits the progression of cell cycle and proliferation of MGC-803 cells. Notes: Panel A-B, flow cytometry illustrates that the amount of MGC-803 cells at G_0/G_1 phase is obviously increased by NGAL-siRNA but that at S phase is decreased; Panel C, WST-1 assay illustrates that NGAL gene silencing inhibits the proliferation of MGC-803 cells; *, $p<0.05$ vs. the control and blank groups; NGAL, neutrophil gelatinase-associated lipocalin; siRNA, small interfering RNA. The data are presented as mean ± standard deviation, analyzed by one-way ANOVA. The experiment was independently repeated three times.

blank and control groups (both $p < 0.05$) (Fig. 2). These results suggested that NGAL gene could have a comparatively high interfering efficiency.

NGAL gene silencing inhibits the progression of cell cycle and proliferation of MGC-803 cells

Flow cytometry was used for analysis of cell cycle, which exhibited that the amount of cells at G_0/G_1 phase was obviously increased in the NGAL-siRNA group compared to the control and blank groups, while the number of cells at S phase was obviously decreased (all $p < 0.05$). The amount of cells at G_0/G_1 phase was obviously decreased in the NGAL group compared to the control and blank groups, while the number of cells at S phase was obviously increased (all $p < 0.05$) (Fig. 3A-B). The data indicated that NGAL gene silencing

can arrest MGC-803 cells at G_0/G_1 phase in the progression of cell cycle. We further observed the relationship between NGAL gene silencing and cell proliferation of MGC-803 cells. The results showed that NGAL-siRNA could notably inhibit the proliferation of MGC-803 cells while NGAL could induce this proliferation in a time-independent manner (Fig. 3C). The number of cells at G_2/M phase showed no significant difference among the three groups (all $p > 0.05$). The cell cycle and proliferation of MGC-803 cells were not significantly different between the blank and control group (all $p > 0.05$). Taken together, NGAL gene silencing could inhibit the progression of cell cycle and proliferation of MGC-803 cells.

NGAL gene silencing promotes apoptosis of MGC-803 cells

We conducted additional flow cytometry to investigate the effect of NGAL on MGC-803 cell apoptosis. The results displayed that the apoptosis rate of MGC-803 cells in the NGAL-siRNA group was obviously higher in comparison to the control and blank groups (both $p < 0.05$), while the apoptosis rate of MGC-803 cells in the NGAL group was obviously lower (both $p < 0.05$). There were no significant differences in the apoptosis rate of MGC-803 cells between the control and blank groups ($p > 0.05$) (Fig. 4). The results implied that NGAL gene silencing could promote the apoptosis of human gastric cancer MGC-803 cells.

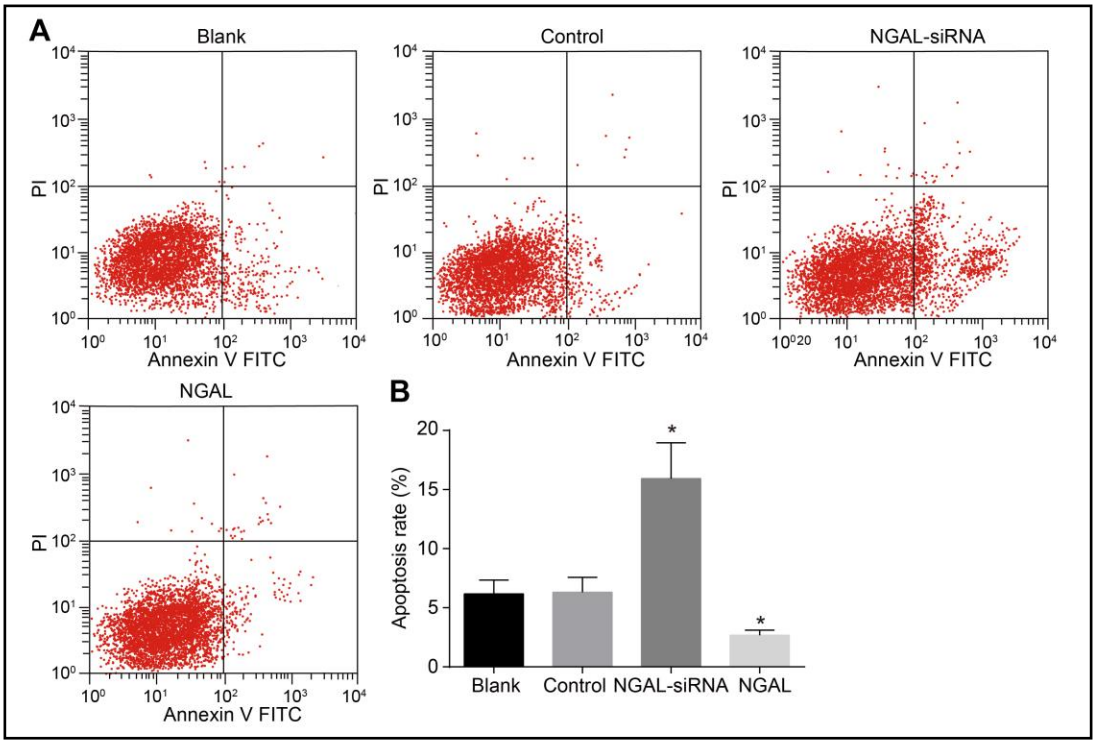

Fig. 4. Flow cytometry demonstrates that NGAL gene silencing promotes apoptosis of MGC-803 cells. Notes: Panel A, apoptosis detection images reveal that NGAL gene silencing promotes apoptosis of MGC-803 cells; Panel B, histogram of MGC-803 cell apoptosis displays that NGAL gene silencing promotes apoptosis of MGC-803 cells; *, p<0.05 vs. the control and blank groups; NGAL, neutrophil gelatinase-associated lipocalin; siRNA, small interfering RNA. The data are presented as mean ± standard deviation, analyzed by one-way ANOVA. The experiment was independently repeated three times.

NGAL gene silencing decreases expressions of apoptosis-inhibiting proteins NF-κB and Bcl-2 and increases expressions of pro-apoptotic proteins caspase-9, Bax, caspase-3 and p53 in MGC-803 cells

Western blot assay was used to explore whether NGAL could influence the protein expressions of apoptosis-related proteins. The results showed that the protein expressions of NF-κB and Bcl-2 in MGC-803 cells in the NGAL-siRNA group were significantly reduced compared with the control and blank groups, while the protein expressions of caspase-9, Bax, caspase-3 and p53 were significantly increased (all $p < 0.05$). In comparison to the control and blank groups, the protein expressions of NF-κB and Bcl-2 in MGC-803 cells in the NGAL-siRNA group were significantly increased, while the protein expressions of caspase-9, Bax, caspase-3 and p53 were significantly decreased (all $p < 0.05$). However, the expressions of NF-κB, Bcl-2, caspase-9 and Bax in the control and blank groups had no significant differences (all $p > 0.05$) (Fig. 5). Therefore, it was concluded that NGAL gene silencing could decrease expressions of apoptosis-inhibiting proteins and increase expressions of pro-apoptotic proteins.

NGAL gene silencing suppresses tumor growth in nude mice

By comparing the tumor growth curve and tumor tissue weight in nude mice among the NGAL-siRNA, control and blank groups at 30 d after tumorigenesis (Fig. 6), we found that the tumor growth rate in the NGAL-siRNA group was significantly lower than the blank and control groups ($p < 0.05$). The weight of tumor tissues in the blank group, NGAL-siRNA group and control group was (1.65 ± 0.18) g, (0.82 ± 0.11) g and (1.68 ± 0.21) g, respectively, suggesting that the tumor in blank and control groups was significantly larger than that in the NGAL-siRNA group (both $p < 0.05$). Furthermore, the comparison of PCNA level among the three groups exhibits that the compared with the blank and control groups, the NGAL-siRNA group showed significantly decreased PCNA level (both $p < 0.05$) (Fig. 7). The results further verified that NGAL gene silencing could inhibit the proliferation of the human gastric cancer MGC-803 cells *in vivo,* thereby reducing the tumorigenesis in nude mice.

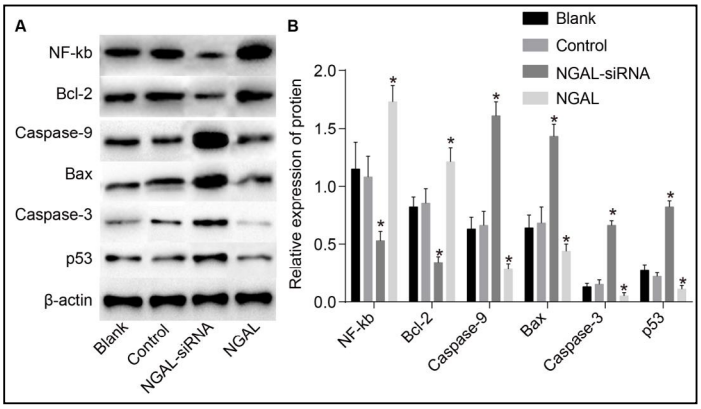

Fig. 5. Western blot assay shows that NGAL gene silencing decreases expressions of apoptosis-inhibiting proteins NF-κB and Bcl-2 and increases expressions of pro-apoptotic proteins caspase-9, Bax, caspase-3 and p53 in MGC-803 cells. Notes: Panel A, protein bands of the apoptosis-related proteins show that NGAL gene silencing decreases expressions of apoptosis-inhibiting proteins NF-κB and Bcl-2 and increases expressions of pro-apoptotic proteins caspase-9, Bax, caspase-3 and p53 in MGC-803 cells; Panel B, statistical chart shows that NGAL gene silencing decreases expressions of apoptosis-inhibiting proteins NF-κB and Bcl-2 and increases expressions of pro-apoptotic proteins caspase-9, Bax, caspase-3 and p53 in MGC-803 cells; *, $p<0.05$ vs. the control and blank groups; NGAL, neutrophil gelatinase-associated lipocalin; siRNA, small interfering RNA; Bcl-2, B-cell lymphoma-2; Bax, Bcl2-associated X protein; caspase-9, cysteine-aspartic acid specific protease-9. The data are presented as mean ± standard deviation, analyzed by one-way ANOVA. The experiment was independently repeated three times.

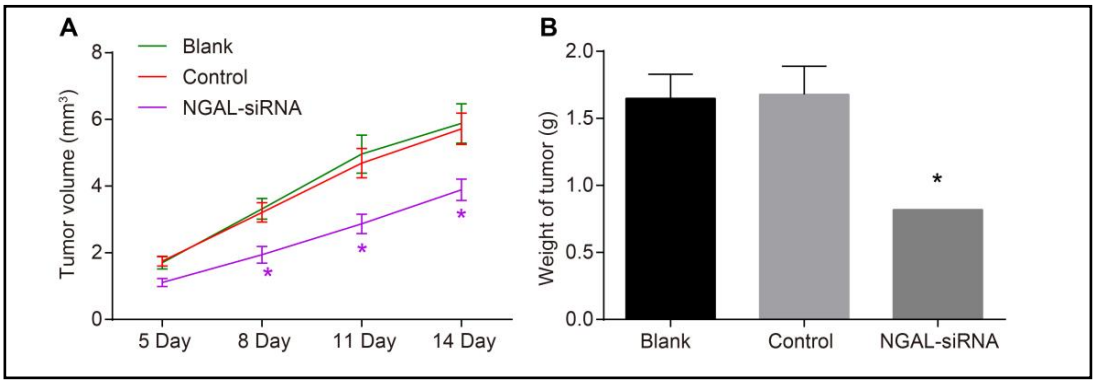

Fig. 6. NGAL gene silencing suppresses tumor growth rate and tumor tissue weight in nude mice at 30 d after tumorigenesis. Notes: Panel A, tumor growth curve shows that tumor growth rate in the NGAL-siRNA group is significantly lower than the blank and control groups; Panel B, tumor tissue weight in nude mice among the NGAL-siRNA, control and blank groups was measured at 30 d after tumorigenesis, showing that the tumor in blank and control groups was significantly larger than that in the NGAL-siRNA group; *, $p<0.05$ vs. the control and blank groups; NGAL, neutrophil gelatinase-associated lipocalin; siRNA, small interfering RNA. The data are presented as mean ± standard deviation, analyzed by one-way ANOVA. The experiment was independently repeated three times.

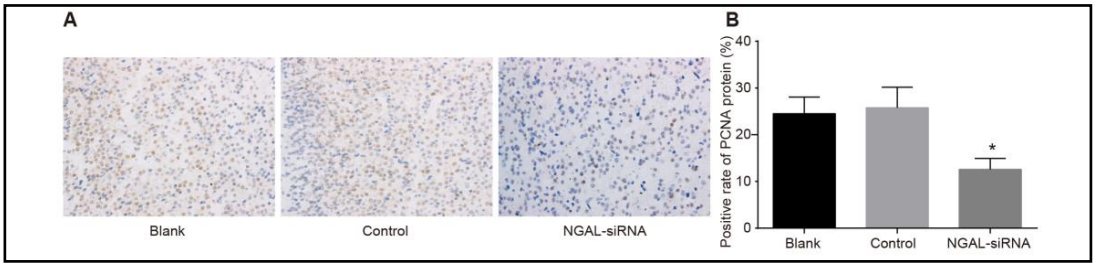

Fig. 7. NGAL gene silencing decreases PCNA level in nude mice. Notes: Panel A, immunohistochemical staining (× 400) shows that the NGAL-siRNA group have significantly decreased PCNA level in comparison to the control and blank groups; Panel B, histogram suggests that the NGAL-siRNA group have significantly decreased PCNA level in comparison to the control and blank groups; *, $p<0.05$ vs. the control and blank groups; NGAL, neutrophil gelatinase-associated lipocalin; siRNA, small interfering RNA; PCNA, proliferating cell nuclear antigen. The data are presented as mean ± standard deviation, analyzed by one-way ANOVA. The experiment was independently repeated three times.

Discussion

Multimodal therapy combined with surgery, radiation therapy and systemic chemotherapy has constantly improved the treatment of advanced gastric cancer [16]. However, the prognosis is still poor. Therefore, it is urgent to develop novel targeted therapy. NGAL, a protein belonging to lipocalin family, is expressed by activated neutrophils and various epithelial cells. It functions to protect against apoptosis, oxidative stress, and bacterial infection, and may play a critical role in the treatment of tumor [17]. Overexpression of NGAL enhanced the migration as well as invasion of the tumor cells *in vivo* and *in vitro*. On the contrary, neutralization and knockdown of it reduced the migration and invasion of tumor cells [18]. Therefore, the aim of our study is to explore the effect of NGAL gene silencing on human gastric cancer MGC-803 cells, with the hope of offering a new therapeutic method in the treatment of gastric cancer. The results showed that NGAL gene silencing contributes to controlling gastric cancer progression through inhibiting cell proliferation and enhancing cell apoptosis in MGC-803 cells.

In this study, RNA interference (RNAi) technology, which has a strong effect on post-transcriptional gene silencing and has been widely used to target oncogenes and inhibit

cancer growth, was used to silence NGAL gene [19]. We firstly constructed siRNA sequences targeting the NGAL gene and stable transfectants were selected for investigation, which were indeed effective. Then we assessed the expression of NGAL gene in MGC-803 cells. We found that the expressions of NGAL mRNA and protein were highly suppressed in the NGAL-siRNA group in comparison to the blank and control groups, suggesting that NGAL participates in the development and evolvement of gastric cancer. Similarly, Michalak *et al.* supported that NGAL has been considered to be an oncogene in gastric cancer, and high expression of NGAL was also observed in gastric cancer. It seemed that the intensity of its over-expression was linked to neoplasm [20]. Additionally, it has been found that urine NGAL is significantly increased and related to the degree of albuminuria in type 2 diabetic nephropathy (T2DN) [21]. Besides, contrast-induced acute kidney injury (CIAKI) presented with elevations in plasma biomarker of NGAL 24 h after iopromide injection [22]. Furthermore, the infusion of macrophages that overexpressing NGAL was related to decreased kidney interstitial fibrosis and inflammation [23].

Most importantly, compared with the control and blank groups, we found that the amount of cells arrested at G_0/G_1 phase in the NGAL-siRNA group was significantly increased, while the number of cells at S-phase was significantly reduced. The results demonstrated that silencing of NGAL led to a reduction in the proliferation of MGC-803 cells. Moreover, the inhibitory function of silenced NGAL on the development of MGC-803 cells was time-dependent. After 72 h, the apoptosis rate in the NGAL-siRNA group was significantly lower than that in the blank and control groups. NGAL, one of the lipocalin family members characterized by the ability to bind and transport small lipophilic substances, was found to be overexpressed in many kinds of tumors [24]. It is composed of 178 amino acids and was found to form a complex with the gelatinase as well as matrix metalloproteinase-9, and its overexpression was related to poor prognosis, suggesting that it might be a biomarker for the prognosis of patients [25]. Previous studies have shown that the down-regulation or deficiency of NGAL inhibited cell growth [26, 27].

After 72 h, the expressions of NF-κB and Bcl-2 proteins in the NGAL-siRNA group were significantly lower than those in the blank and control groups, while the expressions of caspase-9 caspase-3, p53 and Bax proteins were significantly higher. NF-κB, an important transcription element, controls various cellular processes under pathologic or normal conditions, including tissue wound healing, inflammation, cell proliferation, angiogenesis and cell differentiation [28]. NF-κB will stimulate cell proliferation in response to cell injury [29]. Bcl-2 is a key player in genetic program of eukaryotic cells favoring survival through inhibiting cell death, whose overexpression has been found in many kinds of cancers, such as carcinomas, leukemia or lymphomas [30]. Caspase-9, a protein mainly localized in mitochondria, is a critical regulatory player in mitochondria-mediated apoptosis pathway. Activated caspase-9 cleaves and activates caspase-7 and -3 directly, leading to the biochemical destruction of the cells [31]. Bax is one of the pro-apoptotic genes, the overexpression of which results in apoptosis in a number of cells [32]. Bax expression seems to play a critical role in suppressing the development of cancer, and the decrease in Bax expression has been found in lots of cancers [33]. The apoptosis-related proteins, such as caspase3 and p53 could interact with NGAL [34, 35].

Conclusion

In conclusion, our results demonstrated that NGAL silencing contributes to inhibiting the proliferation of MGC-803 cells and promoting cell apoptosis. However, this study also has some drawbacks including small size of specimens. Consequently, we really look forward to having more projects to further verify our conclusion. In addition, we will further study the mechanisms regarding NGAL and apoptosis-related proteins using protein interaction method.

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Disclosure Statement

No conflict of interests exists.

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