RETRACTED: AnnexinA5 Might Suppress the Phenotype of Human Gastric Cancer Cells via ERK Pathway

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Gastric cancer is one of the most fatal diseases around the world. However, the mechanism of the development of gastric cancer is still not clarified. In addition, the anticancer drugs have cytotoxicity with different degrees. AnnexinA5, a member of the annexin family, has a great binding ability with the membrane phospholipid in a calcium dependent manner and is involved in the development of various cancers. This study aims to explore the influence of annexinA5 on human gastric cancer cells and whether it has the potential to be an auxiliary treatment to gastric cancer. In this study, the role of annexinA5 was detected from both the endogenous and the exogenous aspects on the gastric cancer cell lines MGC-803 and MKN-45. The cells were divided into a knockdown group in which RNA interference technique was used to suppress annexinA5 expression and a protein-supplementing group in which annexinA5 protein was added in the culture supernatant. After the suppression ratio of RNA interference was determined and the IC50 of annexinA5 protein was decided respectively, the cells’ proliferation was detected by MTT assay, colony formation assay, and the expression of PCNA. FCM assay and PI staining methods were applied to test cell apoptosis and necrosis. To investigate whether ANXA5 influence cell metastasis, wound healing assay and transwell assay were employed. To further detect the mechanism of annexinA5 action, the signal pathway was examined with Western Blot method. When ANXA5 gene was knocked down, cell proliferation and metastasis were promoted, while cell apoptosis was suppressed. On the other hand, after the annexinA5 protein was applied to the gastric cancer cells, cell proliferation and metastasis were inhibited, while cell apoptosis and necrosis were promoted. AnnexinA5 played its role via ERK signal pathway. ANXA5 acted as tumor suppressor gene in the gastric cancer by suppressing ERK signal pathway and has the potentiality to be an auxiliary anticancer agent.

Keywords: AnnexinA5, gastric cancer, proliferation, apoptosis, invasion, migration, ERK pathway

INTRODUCTION

Gastric cancer is the fourth fatal tumor around the world. There are about 850 thousands new cases and 650 thousands death per year (1). The mechanism of gastric cancer is so complex that the conventional treatment operation and chemotherapy are not ideal for the recurrence and the toxic side effects. Since biotherapy is popular for the less toxic side effects, it is urgent to detect the mechanism of gastric cancer...
development to find out the biological target on which to conduct biological therapy. AnnexinA5 (ANXA5) is a kind of calcium-dependent lipid binding protein which can be secreted in the extracellular matrix and is often used to detect cell apoptosis (2–4), drug transport or as the adjuvant drug of the chemotherapy for its high binding ability with the phosphatidylserine (PS) (5). Recently, accumulating researchers find that ANXA5 plays different roles in the development of various neoplasms such as ovary carcinoma (6), uterine cervical carcinoma (7), and colorectal carcinoma (8) where it is regarded as the diagnosis and prognosis marker. It promotes cell proliferation and metastasis, suppressing cell apoptosis in cholangiocarcinoma (9), murine hepatocellular carcinoma (8) where it is regarded as the diagnosis and prognosis marker. ANXA5 also plays its oncogene action in renal cell carcinoma (12) and hepatocellular carcinoma via the ERK signal pathway (10). Contrarily, large amounts of research demonstrated ANXA5 acts as tumor suppressor. For instance, ANXA5 can suppress cell proliferation and metastasis in uterine cervical carcinoma (13); it transfers to the mitochondria, suppressing the voltage-dependent anion channel (VDAC) oligomerization which leads to prostate cancer cell apoptosis (14). ANXA5 suppresses murine neuroblastoma cell proliferation by blotting PS which results in the enhanced T cell-dependent tumor immunity (15). It suppresses cell metastasis, promotes apoptosis, and enhances the result of CHOP chemotherapy of diffuse large B-cell lymphoma via the PI3K/Akt signal pathway (16). In addition, when ANXA5 protein was applied to the murine melanoma xenograft model, tumor size and angiogenesis were remarkably reduced by suppressing VEGF expression (17), which implies that ANXA5 protein might be used as a kind of treatment agent; when ANXA5 was managed to form a complex with TRAIL, it can promote the TRAIL-induced apoptosis and increase cell sensibility to TRAIL (17, 18). All these imply that ANX5 has the potential to be used as a cancer therapy. However, whether ANXA5 plays any role during the development of gastric cancer is still unclear. In this study, we detected the phenotypes of the gastric cancer cells by inhibiting endogenous ANXA5 expression with RNA interference. In addition, we added exogenous ANXA5 protein to the cells to detect whether ANXA5 could act as extracellular auxiliary drug and has the potential ability of biological therapy. Our data indicated that ANXA5 acts as oncogene suppressor in gastric cancer which might be through the MAPK/ERK pathway and has the potential to be an auxiliary treatment to gastric cancer.

**MATERIALS AND METHODS**

**Cell Culture**

Human gastric cancer cell lines MGC-803 and MKN-45 were obtained from Shanghai Academy of Medical Sciences (Shanghai, China). The cells were incubated at 37°C in 5% CO₂ in plastic tissue culture flasks (Corning Inc., Acton, MA, USA) with complete Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL). ANXA5, PCNA, Bcl-2, Bax, MMP-9, E-cadherin, β-actin, GAPDH, MEK1/2, pMEK1/2, ERK1/2, and pERK1/2 monoclonal antibody were purchased from Sigma (St. Louis, MO, USA) and the ANXA5 protein was bought from novoprotein (Shanghai, China); the Trizol reagent, protein assay kit, the siRNA targeted to ANXA5 and the negative control siRNA were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA); the gel image analysis system and electrophoresis apparatus were purchased from Bio-Rad (Hercules, CA, USA). The digital image analysis system and the fluorescence microscope were from Nikon (Tokyo, Japan), and the ultraviolet analyzer was from Beckman Coulter (Miami, FL, USA).

**Database Consulting**

To determine ANXA5 protein expression in gastric cancer tissues, we consulted the database “THE HUMAN PROTEIN ATLAS” (https://www.proteinatlas.org/) and “The Cancer Genome Atlas” (https://www.cancer.gov/) to do the statistical analysis.

** AnnexinA5 RNA Interference**

Cells were classified into two groups. Cells transfected with the si-RNAs targeted ANXA5 was regarded as A5-si group. A non-silencing control sequence was designed according to the sequence of a negative control and the cells transfected with it is Neg group. The selected siRNA duplex sequences specifically targeted ANXA5 (GenBank accession number NM_001154.3) showed no homology to any other sequences by a blast search. The target sequence was as follows: TTCCAGGAGTGAGATGAGT. Transfection was carried out using Lipofectamine 3000 reagent with a molar ratio between siRNA and lipid of about 1:2.5. 48 h after transfection, cells were collected and used for functional assays.

**The Determination of ANXA5 Protein Concentration**

Cells were classified into two groups. Cells without any treatment are the blank group; Cells added with the ANXA5 protein are named A5-add group. The ANXA5 protein freeze-dried powder was prepared into 0.1 mg/ml storage solution. Cells were implanted into 96-well plates and were treated with 10, 20, 40, 80, and 160 nM ANXA5 protein respectively. OD value was tested, and IC50 was calculated 24 h later with the determination of 40 nM as the ANXA5 protein concentration to treat the cells.

**Real Time PCR**

Total RNA was isolated from cells with a Trizol reagent kit according to the manufacturer’s instructions. cDNA was generated with the Invitrogen Thermoscript RT-PCR System (Invitrogen) and digested with Rnase as suggested by the supplier. Samples without reverse transcriptase were used as negative controls to confirm the absence of genomic DNA. Quantitative real-time PCR was performed using the iCycler iQ Multi-Color Real-time PCR Detection System and the SYBR Green Supermix (Bio-Rad). β-actin was used as housekeeping gene for normalization. Samples were analyzed in triplicate, and a dilution series was used in each run to determine the PCR efficiency for each pair of primers. The following primers were chosen to generate the PCR fragments:
ANXA5: AGACCCCTGTAGCTGGAATTG and TCGTGTTCCAAGATGGTGA. β-actin: TGGCACCCAGCACAATGAA and CTAAGTCATAGTCCGCCTAGA. PCR results were quantified using the ΔΔCt method according to the formula: Expression ratio = 2−ΔΔCt, where ΔΔCt = ΔCt target gene − ΔCt endogenous control gene (β-actin) (19).

Western Blot Analysis
The cells were homogenized in a single detergent lysis buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1% Triton X-100; and 0.5% each of protease and phosphatase inhibitor cocktails). The supernatants were transferred into new tubes, assayed for protein content using a protein assay kit, aliquoted at a concentration of 5 µg/20 µl in lysis buffer. Then the samples were mixed with loading buffer (100 mM Tris, pH 6.8; 200 mM DTT; 4% SDS; 20% glycerol; and 0.2% bromophenol blue) at a 1:1 dilution, boiled for 5 min and then separated on 10% SDS-polyacrylamide Tris-glycine gels. The proteins were then transferred onto polyvinylidene difluoride membranes and reacted with monoclonal antibodies at 1:1,000 dilution overnight at 4°C and then reacted with HRP-conjugated goat anti-mouse antibodies for 1 h at room temperature. The bound antibodies were detected by chemiluminescence according to the manufacturer’s instructions and assayed with ImageJ software. All experiments were performed three times in triplicate.

MTT Assay
Cell suspensions (100 µl) were dispensed into 96-well round-bottomed microtiter plates (3,000 cells/well) and incubated for 12–72 h at 37°C in 5% CO2. At every time point, 5 mg/ml of sterile MTT was added into each well and incubated at 37°C for 4 h. After aspiration of the medium, 150 µl of DMEM (Sigma, St. Louis, MO, USA) was added and mixed, and absorbance was determined at 490 nm with a spectrophotometer (Spectramax 190; Molecular Devices, Sunnyvale, CA, USA). Growth curves were generated from the average values of eight wells in each group.

Colony Formation Assay
The cells (5 × 10²/well) were seeded in 12-well plates and incubated in DMEM medium containing 10% FBS at 37°C atmosphere containing 5% CO2. After incubation, non-adherent cells were removed with a cotton bud. Cells that invaded into the lower chambers were completely removed with a cotton bud. Cells that invaded into the lower chambers were fixed in 95% methanol for 15 min, stained with 0.1% crystal violet for 10 min prior to washing with water, and counted in five random fields with an inverted microscope.

Analysis of Apoptosis by FCM Assay
Cell apoptosis was analyzed by flow cytometry (FACSAria II; BD Biosciences, Franklin Lakes, NJ, USA). Cultured cells were washed twice with phosphate-buffered saline (PBS) and resuspended in binding buffer at 1 × 10⁶ cells/ml. Cell suspensions (1 × 10⁷ cells/100 µl) were transferred to 5 ml culture tubes, and 5 µl Annexin V-FITC (eBioscience, San Diego, CA, USA) and 5 µl PI (eBioscience) were then added. The cells were gently vortexed and incubated at room temperature in the dark for 15 min. Subsequently, another 400 µl binding buffer was added. Flow cytometry was performed within 4 h staining.

Wound Healing Assay
When the cells reached 75–80% confluence, they were scratched with a micropipette tip in the cell monolayer. After 48 h incubation, recovery of the wound was observed and images were captured by a phase-contrast microscope.

Cell Invasion Assay
The cell invasion assay was performed using the QCM 24-well cell invasion assay kit (Millipore, USA). Each lower chamber contained an additional 600 µl of 0.5% FBS as the chemoattractant. Cells (1 × 10⁵) were placed into the upper chamber and then incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO2. After incubation, non-invading cells in the top chambers were completely removed with a cotton bud. Cells that invaded into the lower chambers were fixed in 95% methanol for 15 min, stained with 0.1% crystal violet for 10 min prior to washing with water, and counted in five random fields with an inverted microscope.

Statistical Analysis
Data are presented as the means ± standard deviation (SD). Statistical analysis was performed by using the One-way ANOVA to two cell group data analysis while two-way ANOVA to more than two groups with GraphPad Prism 7.0 software and values of P < 0.05 were considered to indicate statistically significant differences.

RESULTS
Endogenous ANXA5 Expression in Gastric Cancer Tissues
With the data preserved in the databases, we found that endogenous ANXA5 expression decreased significantly in the
gastric cancer tissues when compared with the adjacent normal tissue. It was unaccountable that the overall survival rate of the GC patients with high ANXA5 expression was significantly lower than that of low ANXA5 expression. To detect whether ANXA5 expression was associated with clinical stages, we analyzed its expression in each stage and the results showed that with the stage increase, ANXA5 expression decreased except stage IV which showed abnormal increased expression. Further, with the clinical stage increase, the survival rate decreased except that of stage IV (Figures 1A–I).

**Efficiency of ANXA5 Interference**

The ANXA5 suppression ratio was examined by western blot analysis. 48 h after RNA interference, the ANXA5 suppression
rate was about 78.3% (Figures 1J, K) which was significantly decreased compared with that in the Neg group.

ANXA5 Regulated Gastric Cancer Cell Proliferation

The effects of ANXA5 on the proliferation of MGC-803 and MKN-45 cells were assessed by MTT assay and colony formation assay respectively. In the MTT experiment, subsequent to culture for 12, 24, 36, 48, and 72 h, the *in vitro* growth of the A5-si cells was significantly faster than that of the Neg group at all the time points (*P* < 0.0001), while that of the A5-add group was much slower than that of the blank group (Figure 2A). To confirm this result, we performed a colony formation assay (Figure 2B) which showed that the number of cell colony was significantly more in the A5-si group and less in the A5-add group (*P* < 0.0001). Further, the proliferation marker PCNA expression was also detected by immunohistochemistry with the results of increased expression in the A5-si group and decreased expression in the A5-add group (Figure 2C).

ANXA5 Regulated Gastric Cancer Cell Death

Flow cytometry (FCM) and PI staining were employed to find out whether ANXA5 could modulate cell death. As shown in Figure 3A, apoptosis of A5-si cells decreased while A5-add group increased significantly. To evaluate the overall cell death, cells were visualized under fluorescence microscopy after paraformaldehyde fixation and staining with PI and we got the same results as shown in Figure 3B. Meanwhile, we examined the expression of the most popular apoptosis related genes bcl-2 and bax with the results that Bcl-2 expression increased in the A5-si group, while it decreased in the A5-add group and that of Bax appeared no remarkable alteration in both cells (Figure 5).

ANXA5 Regulated Gastric Cancer Cell Metastasis

Wound healing assay was applied to detect the migration of the cells. As shown in Figure 4A, the si-ANXA5 cells migrated fast while the A5-add group cells showed reduced migration ability.
ANXA5 Regulate Gastric Carcinoma Cell Proliferation and Metastasis via MAPK/ERK Pathway

Western blot was used to detect the expression of pMEK and pERK, which are the biomarkers of the MAPK/ERK signal pathway. With the down-regulation of ANXA5, the expression of total MEK and ERK was not altered, while pMEK and pERK level was enhanced significantly (P < 0.0001). On the other hand, with the ANXA5 protein added to the cell culture supernatant, the pMEK and pERK level was reduced (Figure 5). To make sure that ANXA5 depends on this pathway, we added the MEK inhibitor U0126 20µM to the A5-si cells to neutralize the increase of pMEK and pERK; on the other hand, the A5-add cells were transfected with the ANXA5 siRNA to offset the decrease of pMEK and pERK. We found the expression of Bcl-2, PCNA, and MMP-9 restored to the original level which demonstrated that ANXA5 influenced the gastric cancer cells through the MAPK/ERK pathway.

DISCUSSION

In this study, we detected the influence of ANXA5, a protein with high affinity to phosphatidylserine (PS), in a calcium dependent manner on gastric cancer cells. As a widely used molecule to probe apoptosis, far less is known about its other multiple properties, especially in cancer therapies.

Depending on the analysis from databases, we found ANXA5 was down-regulated in human gastric cancer which implied ANXA5 might act as a tumor suppressor. However, it is unconventional that the overall survival of the ANXA5 high-expressed group was lower than that of the low-expressed group. So, next we continued to analyze the survival of each clinic stage. First, we found that with the clinic stage increased, except stage IV, ANXA5 expression decreased which was consistent with the reduced ANXA5 expression in gastric cancer patients. It was unusual and ill-defined that in stage IV, ANXA5 expression was
abnormally increased, and the survival of this stage was lower than that of stages I and II. Most of the data above indicated that ANXA5 might be an oncogene suppressor; however, its detailed functions are still misty. Thus, from both endogenous and exogenous aspects, we investigated the role of ANXA5 in gastric cancer cells.

ANXA5 knockdown could prompt cell proliferation and metastasis while suppressing apoptosis in gastric carcinoma cells. Although DT40 cells were found resistant to apoptosis if lacking ANXA5 protein (20) and the interaction of ANXA5 and beta5 integrin can regulate the apoptosis of the growth plate chondrocytes (21), the biological function of endogenous ANXA5 is still unclear. Here, both MTT and colony formation assay suggested that ANXA5 knockdown could promote the gastric cancer cell viability. At the same time, the proliferation biomarker PCNA expression was enhanced when ANXA5 was inhibited. To identify ANXA5 influence on cell death, both FCM assay and PI staining methods were used. Apparently decreasing chromatic cells were observed in the ANXA5 suppression cells. In our previous work, we found ANXA5 could promote apoptosis of uterine cervical cancer cells by suppressing Bcl-2 expression and increasing Bax expression. In this study, we also detected the expression of Bcl-2 and Bax and found that Bcl-2 expression was reduced while Bax did not show apparent alteration. However, the Bcl-2/Bax ratio was still decreased which might explain the apoptosis of gastric carcinoma cells.

ANXA5 knockdown cells showed increased metastatic ability. With wound healing assay and transwell assay, we found the cell metastasis ability was apparently increased when ANXA5 expression was suppressed. MMP-9, a biomarker of cell metastasis, showed increased expression which ensured our conclusion. It was reported that ANXA5 can promote cell proliferation and metastasis, while it can suppress apoptosis in the cell lines of cholangiocarcinoma (9), renal carcinoma (12), and glioblastoma multiforme cells (11) which is contrary to our results, suggesting that ANXA5 may play a different role in different tumor cells, and in gastric cancer, it acted as tumor suppressor gene.

In light of the results from the ANXA5 knockdown experiments above, together with that ANXA5 is a kind of secretary protein molecule, we suspected whether ANXA5 protein could suppress cell proliferation by exogenous addition so as to be a potentiate auxiliary treatment on clinic. Therefore, we added ANXA5 protein into the supernatant of the gastric cancer cells and tested the alteration of the cells malignant phenotype. It was a pleasure to see that ANXA5 protein could suppress cell proliferation and metastasis while promoting apoptosis which is in line with the endogenous experiments, as with the report that ANXA5 inhibits neuroblastoma growth in vivo (15) and increased articular chondrocyte apoptosis induced by basic calcium phosphate crystals (22). These results also were consistent with the anti-cancer activity of ANXA5 in murine melanoma model where ANXA5 was regarded as an angiogenesis inhibitor (17).
To detect the mechanism of ANXA5 function, we hope to find out the signal pathway ANXA5 depends on. The most popular signal pathways in cancer development are PI3K/Akt and MAPK pathways. After ANXA5 expression was suppressed in the ANXA5-si group or on the other hand, ANXA5 protein was applied in the ANXA5-add group, we examined the expression of the key molecules in both the pathways and there was no alteration of pPI3K and pAkt, while pMEK and pERK level was reduced. To make sure that ANXA5 depends on this pathway, the MEK inhibitor U0126 20 μM was added to the A5-si cells to neutralize the increase of pMEK and pERK; On the other hand, the A5-add cells were transfected with the ANXA5 siRNA to offset the decrease of pMEK and pERK. With the down-regulation of ANXA5, the expression of total MEK and ERK was not altered, while pMEK and pERK level was enhanced significantly (P < 0.0001). On the other hand, with the ANXA5 protein added to the cell culture supernant, pMEK and pERK level was reduced. To make sure that ANXA5 depends on this pathway, the MEK inhibitor U0126 20 μM was added to the A5-si cells to neutralize the increase of pMEK and pERK; On the other hand, the A5-add cells were transfected with the ANXA5 siRNA to offset the decrease of pMEK and pERK. At the same time, Bcl-2, PCNA, and MMP-9 restored to the original level which demonstrated that ANXA5 influenced the gastric cancer cells through MAPK/ERK pathway.

Of course, this study has limitations as follows: First, the data obtained was from in vitro study, without in vivo study; secondly, we did not find the interacting genes of ANXA5, which should be done in the future study.

Taken together, our results implied that ANXA5 might act as an anti-cancer protein which could suppress cell proliferation and metastasis while it can prompt cell apoptosis via the MEK/ERK signal pathway. In addition, ANXA5 might have the potentiality to be an auxiliary treatment to gastric cancer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article-supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XW and YD conceived and designed the experiments. XW, YD, YZ, ML, JZ, YC, and HW performed the experiments and collected the data. XW performed the statistical analysis. XL designed the experiment, supervised the entire study, and revised the manuscript. YD revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

The present study was supported by the Key Subjects in University of Hebei Province (grant no. 2013-4), by the Science and Technology Agency [grant no.10276142], and by the Education Department of Hebei Province [grant no.Z2014026].

FIGURE 5 | ANXA5 regulates gastric carcinoma cell proliferation and metastasis via the MAPK-ERK pathway. Western blot was used to detect the expression of pMEK and pERK expression which are the biomarkers of the MAPK/ERK signal pathway. With the down-regulation of ANXA5, the expression of total MEK and ERK was not altered, while pMEK and pERK level was enhanced significantly (P < 0.0001). On the other hand, with the ANXA5 protein added to the cell culture supernant, pMEK and pERK level was reduced. To make sure that ANXA5 depends on this pathway, the MEK inhibitor U0126 20 μM was added to the A5-si cells to neutralize the increase of pMEK and pERK; On the other hand, the A5-add cells were transfected with the ANXA5 siRNA to offset the decrease of pMEK and pERK. At the same time, Bcl-2, PCNA, and MMP-9 restored to the original level which demonstrated that ANXA5 influenced the gastric cancer cells through MAPK/ERK pathway. (B) The statistics of gene expression in MGC-803 cells. (C) The statistics of gene expression in MKN-45 cells.
