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Original Article

Stathmin is a potential molecular marker and target for the treatment of gastric cancer

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Abstract: Objective: This study is to investigate the expression levels of stathmin in tissues of gastric cancer, and evaluate the therapeutic effects of stathmin antisense oligodeoxynucleotide (ASODN) and/or docetaxel in human gastric cancer cells. Methods: Immunohistochemistry was performed to detect the expression levels of stathmin in gastric cancer and adjacent tissues. Stathmin ASODN was transfected into gastric cancer SGC 7901 cell lines. The cell proliferation was assessed with the MTT assay, and the inhibitory rates were calculated. RT-PCR and Western blot analysis were performed to detect the mRNA and protein expression levels of stathmin, respectively. The synergistic effects of stathmin ASODN and docetaxel were evaluated. The efficacy and clinical benefit rates of the treatment of docetaxel combined with stathmin evaluation were investigated and compared. Results: Our results showed that the expression of stathmin was elevated in gastric cancer tissues, indicating a possible association between the stathmin expression and the disease occurrence. The MTT assay and tumor growth experiment revealed that stathmin ASODN significantly inhibited the proliferation of gastric cancer cells, both *in vitro* and *in vivo*. Furthermore, stathmin ASDON enhanced the inhibitory effects of docetaxel on the proliferation of gastric cancer cells, indicating a synergistic effect for the combination treatment. Importantly, docetaxel treatment was more effective for stathmin-negative gastric cancer patients, compared with stathmin-positive patients. Conclusion: Stathmin expression provides evidence for the treatment planning for gastric cancers. Stathmin might be a potential molecular marker and target for the treatment of gastric cancer.

Keywords: Gastric cancer, treatment, stathmin, docetaxel

Introduction

Gastric cancer is one of the most common gastrointestinal malignancies worldwide, with high morbidity and mortality [1]. The treatments of the disease mainly focus on the molecular targets involved in the regulation of the differentiation and proliferation of these cancer cells. Stathmin (also known as Op18, p18, p19, or metablastin), a 19-kDa soluble phosphoprotein, is related to cell differentiation and proliferation, especially in cancer cells. At different stages in the cell cycle, stathmin regulates the dynamic equilibrium of the microtubule system via phosphorylation and/or dephosphorylation. The expressions of stathmin are up-regulated in a variety of malignant tumors, including acute leukemia, lymphoma, lung cancer, thyroid cancer, nervous system tumor, breast cancer, prostate cancer, and ovarian cancer [2-7]. The over-expression of stathmin may influence the efficacy of anti-microtubule chemotherapy drugs, and the inhibition of stathmin expression can interfere with mitosis in cancer cells, affecting tumor growth and metastasis. Blocking the biological function of stathmin may become an alternative therapeutic treatment for cancers [8].

Antisense gene therapy is a promising therapeutic strategy for cancers, referring to the application of antisense oligonucleotides (ASODN) to inhibit the transcription and translation of abnormally expressed genes. ASODN could block the abnormal signal transduction in tumor cells, leading to normal differentiation or cellular apoptosis. Mistry *et al.* [9] have designed ribozymes with ideal stathmin RNA cleavage activity. Furthermore, inhibition of stathmin has been shown to affect the cell spin-

Table 1. Analysis of stathmin expression and pathological data in gastric cancer patients

	N	Stathmin expressions		*P* value
		Positive	Negative	
Age				
≥ 60 yr	27	19 (70.4%)	8 (29.6%)	> 0.05
< 60 yr	29	19 (65.5%)	10 (34.5%)	
Gender				
Male	31	18 (58.1%)	13 (41.9%)	> 0.05
Female	25	14 (56.0%)	11 (44.0%)	
TNM stage				
I-II stage	14	6 (42.9%)	8 (57.1%)	< 0.05
III-IV stage	42	31 (70.5%)	11 (29.5%)	
Differentiation				
Mid-high	27	16 (59.3%)	11 (40.7%)	< 0.05
Low	29	24 (82.8%)	5 (17.2%)	
Infiltration				
Serosa intact	10	2 (20.0%)	8 (80.0%)	< 0.05
Serosa penetrated	46	38 (82.6%)	8 (17.4%)	
Lymphatic metastasis				
Yes	44	40 (90.9%)	4 (9.1%)	< 0.05
No	12	3 (25.0%)	9 (75.0%)	

dle formation, and the impaired phosphorylation of stathmin would induce cell arrest at the G_2/M checkpoint, thus inhibiting cell growth. Other researchers have utilized stathmin ASODN to effectively inhibit the growth of K562 cells [10].

In the present study, we measured the expression levels of stathmin in tissues of gastric cancer, and evaluated the therapeutic effects of stathmin ASODN and chemotherapy, alone and in combination, in human gastric cancer cell lines. Our findings provide the theoretical basis for the investigation of stathmin as the molecular marker and therapeutic target in the treatments of gastric cancer.

Materials and methods

Patients

Totally 56 patients with gastric cancer were enrolled in this study. None of these patients had received radiotherapy, chemotherapy, or minimally invasive treatments before surgery (**Table 1**). Cancers and adjacent tissues were collected and used for the investigation. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Shandong Provincial Qianfoshan Hospital.

Thirty-two of these 56 patients were subjected to the detection of stathmin expression and the following combination treatment of stathmin ASDON and docetaxel. There were no significant differences in age, gender, pathology, clinical stage, and PS score, between these patients (**Table 2**). Chemotherapy treatments started 1 m after surgery, with the following DC protocols: Day 1, 75 mg/m^2 docetaxel; Day 1-3, 25 mg/m^2 cisplatin, intravenous infusion, 21 d for a cycle of treatment. Evaluation of toxicity was performed every week, and the therapeutic effects were evaluated every 2 cycles, according to WHO evaluation criteria. Treatments continued until disease progression or intolerable toxicity occurred. Each patient received at least 6 treatment cycles.

The efficacy and clinical benefit rate were calculated and compared between these two groups. According to RECIST 1.0 standards, the responses could be divided into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). The efficacy was expressed as CR + PR, and the clinical benefit rate was calculated as CR + PR + SD. One-year survival rate was also calculated and compared between these groups.

Cell lines and reagents

Gastric cancer SGC 7901 cell lines were purchased from KeyGEN BioTECH (Nanjing, Jiangsu, China). Stathmin ASODN and sense oligodeoxynucleotide (SODN) were synthesized by Boya Biotechnology Co., Ltd. (Shanghai, China). The sequences were as follows: stathmin ASOND, 5'-CCTGGATATCAGAAGAAGCCAT-3', and stathmin SOND, 5'-CCTGGATATCAGAAGAAGCCAT-3'. Docetaxel and cisplatin were purchased from Qilu Pharmaceutical Co., Ltd. (Jinan, Shandong, China). RPMI 1640 medium was from Hyclone (Logan, Utah, USA). Lipofecter liposome transfection reagents were purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). MTT was purchased from Sigma (St. Louis, MO, USA). TUNEL kits were from Roche (Basel, Switzerland). Im-

Table 2. Relationship between stathmin expression and efficacy of chemotherapy

Groups	N	Efficacy (CR + PR)	Clinical benefit rate (CR + PR + SD)	One-year survival rate
Stathmin positive group	19	10.5% (2)	52.6% (10)	68.4% (13)
Stathmin negative group	13	23.1% (3)	69.2% (9)	69.2% (4)

munohistochemistry PV9000 kits and DAB chromogenic kits were from ZSGB-BIO (Beijing, China). Total RNA purification kits, TIANScript cDNA synthesis kits, and PCR kits were purchased from TianGen Biotech Co., Ltd. (Beijing, China). Peroxidase enzyme-labeled anti-rabbit IgG antibody was from Cellway-Lab (Luoyang, Henan, China). PCR instrument was from Biometra (Gottingen, Germany). Microplate Reader was from Rayto (RT-2100C; Shenzhen, China).

Immunohistochemistry

Tissue sections were deparaffinized. Primary antibody (rabbit IgG) was used to incubate the sections at 4°C overnight. Biotinylated secondary antibody was further added into the sections. Then DAB chromogenic reagent was used, and hematoxylin counterstaining was performed. The staining was observed under a microscope. Five fields with high-magnification were randomly selected from every section, and 200 cancer cells were counted for each field. The percentages of positive cells were then calculated. The protein expression levels were analyzed as described by Wachters *et al.* [11]. Percentage of positive cells < 10% was considered as negative, and > 10% was considered as positive.

Cell transfection

The gastric cancer SGC 7901 cell were transfected when 70% to 80% confluence was reached. Stathmin ASODN or SODN were transfected with the Lipofecter liposome transfection reagents, according to the manufactures' instructions.

RT-PCR

MCG803 and SGC 7901 cells were transfected with 20 μmol/L ASODN and SODN. After 72 h, 3 × 10^6 cells were collected, and total RNA was extracted with the TrizolRNA extraction kit (Sangon, Shanghai, China). The mRNA expression of stathmin was detected with the RT-PCR detection kit (Sangon), according to the manufactures' instructions. The primer sequences were as follows: stathmin, forward 5'-ATGGCTTCTTCTGATATCCAG-3' and reverse 5'-GAGGGGAAAGGGGGAATTCTG-3'; internal reference β-actin, forward 5'-TCCTGTGGCATCCACGAAACT-3' and reverse 5'-GAAGCATTTGCGGTGGACGAT-3'. The products were subjected to electrophoresis on 20 g/L agarose gel, and the bands were imaged and analyzed with the BandScan software.

Western blot analysis

Total proteins were harvested from cells, separated on 10% SDS-PAGE gels. The primary antibodies against stathmin and β-actin were purchased from Santa Cruz, Santa Cruz, CA, USA (anti-stathmin, cat # sc-374209, 1:200 dilution; anti-β-actin, cat # sc-130301, 1:10000 dilution). Secondary antibodies were goat anti-mouse IgG-HRP (cat # sc-2005, 1:10000 dilution, Santa Cruz). Bands were detected using the ECL system (Pierce Biotechnology, Rockford, IL, USA). The experiments were repeated for at least three times.

In vivo experiments

Forty 6-8-week old male nude mice were randomly divided into two groups. For the ASODN-treated group, 11 × 10^7 gastric cancer cells (0.2 mL) transfected with ASODN were intraperitoneally inoculated into the nude mice. For the control group, gastric cancer cells without transfection were inoculated. After 3 months, the tumor sizes were determined, and the effects of antisense stathmin on *in vivo* tumor growth were assessed.

MTT assay

Cells were planted onto 96-well plates at a density of 5 × 106 cells/L in 200 μL medium, and cultured in a 37°C, 5% CO_2 incubator. After 72 h, 20 μL 5 g/L MTT was added into each well for a further incubation for 4 h. The supernatant was aspirated, and 150 mL DMSO

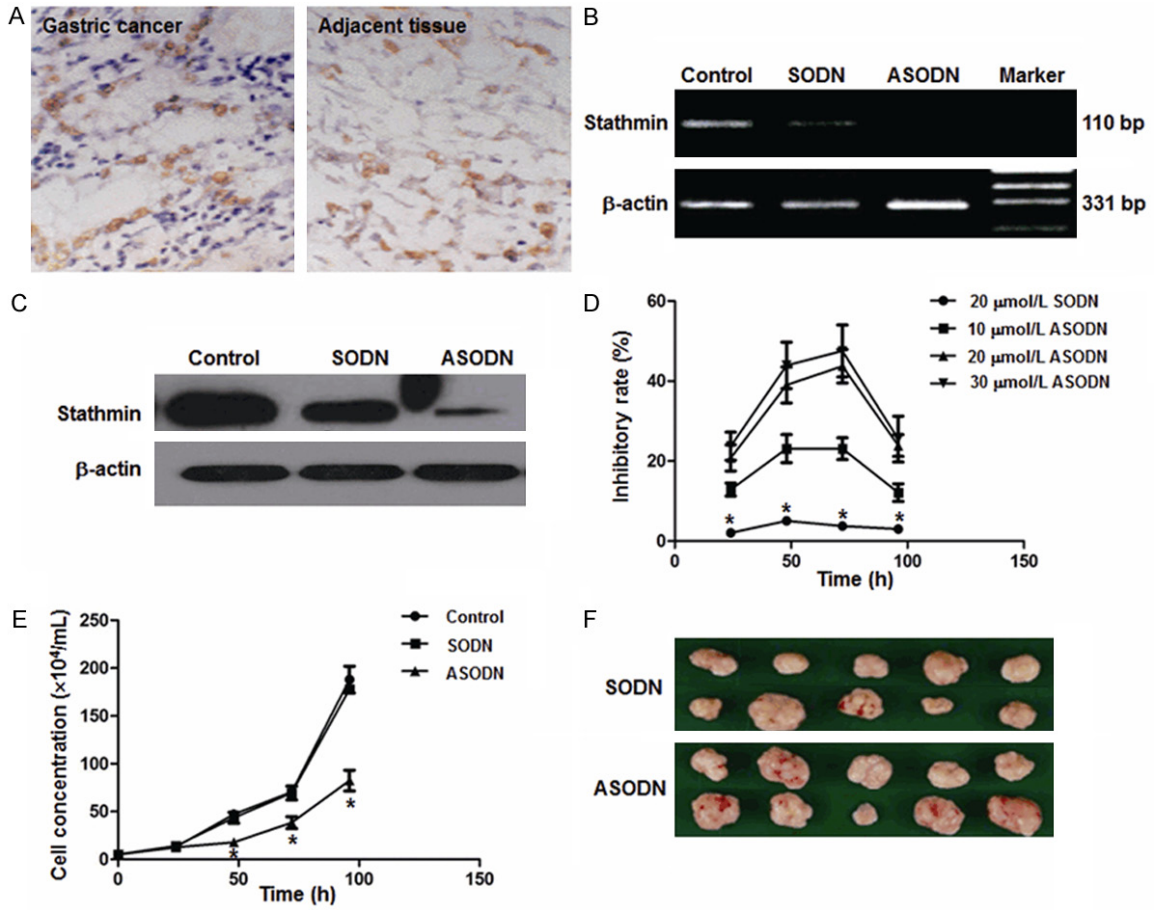

Figure 1. Effects of Stathmin ASODN on the proliferation of gastric cancer cells *in vitro* and *in vivo*. A. The expressions of stathmin in gastric cancer and adjacent tissues were detected with immunohistochemisty (× 400). B. The mRNA expression levels of stathmin were detected with RT-PCR after cells were transfected with stathmin ASODN or SODN. C. The protein expression levels of stathmin were detected with Western blot analysis after cells were transfected with stathmin ASODN or SODN. The experiments were repeated for at least three times. D. The MTT assay was performed to detect the proliferation of gastric cancer SGC 7901 cells. The inhibitory rates were calculated to assess the effects of the treatments of stathmin SODN (20 μmol/L), or ASODN, at indicated concentrations (10, 20, and 30 μmol/L), for 24 h, 48 h, 72 h, and 96 h, respectively, on the cell proliferation. Compared with cells treated with SODN, $^{*}P < 0.05$. E. Doubling time was measured in stathmin ASODN-, SODN-treated cells, as well as untransfected SGC 7901 cells. Compared with control at 48 h, 72 h and 96 h, $^{*}P < 0.05$. F. The diameters of the gastric tumors in nude mice were measured to evaluate the effects of stathmin ASODN on gastric cancer cell growth *in vivo*. Tem representative tumors from each group were shown.

was added into each well. The plate was gently shaken for 10 min, until the crystals were fully dissolved. The absorbance at 570 nm was read on a microplate reader.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed with the SPSS 15.0 software. One-way ANOVA and x^2 test was used for the comparison between groups. Factorial analysis was performed for the interaction analysis. $P < 0.05$ was considered statistically significant.

Results

Expression of stathmin in gastric cancer and adjacent tissues

To investigate the expression of stathmin in gastric cancer and adjacent tissues, immunohistochemistry was performed. Our results showed that majority of the cells in the cancer tissues were positive for stathmin, while the staining was obviously less intense in the adjacent regions (**Figure 1A**). In these samples, the positive expression rate for stathmin in the can-

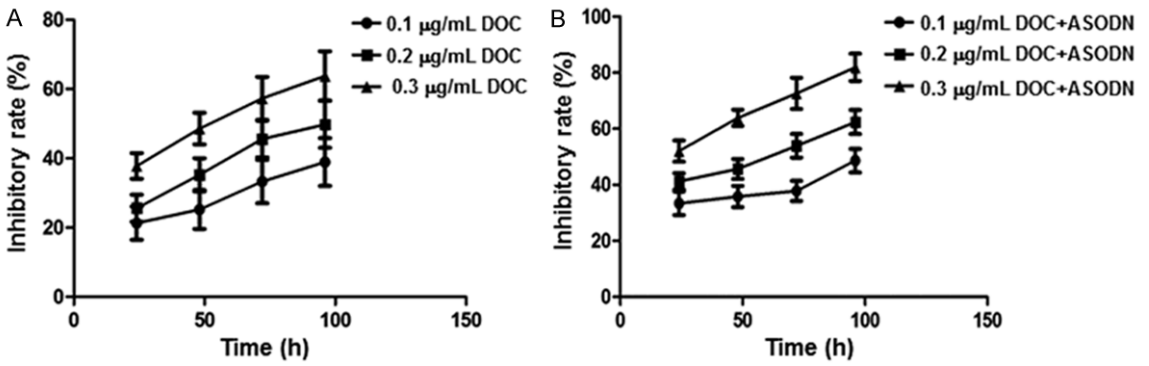

Figure 2. Effects of combination of stathmin ASDON and docetaxel on the proliferation of gastric cancer cells. A. Effects of docetaxel, at indicated concentrations (0.1, 0.2, and 0.4 μg/mL), on the proliferation of gastric cancer SGC 7901 cells. B. Effects of combination of stathmin ASDON and docetaxel on the proliferation of gastric cancer cells. Cell proliferation was assessed with the MTT assay.

cer tissues was 71.4%, while expression rate was only 37.5% in the adjacent regions ($P < 0.05$). These results suggest a possible association between the expression of stathmin and the occurrence and development of gastric cancer.

Effects of stathmin ASODN on proliferation of gastric cancer SGC 7901 cells in vitro

To investigate the functions of stathmin in the proliferation of gastric cancer cells, stathmin ASODN or sense oligodeoxynucleotides (SODN) was transfected into SGC 7901 cells. At 72 h after transfection, the relative stathmin mRNA expression levels in the ASODN-, SODN-transfected cells, and control cells were 0.21 ± 0.08, 1.01 ± 0.43, and 1.44 ± 0.54, respectively (**Figure 1B**). Significant differences were observed between the ASODN- and SODN-transfected groups ($P < 0.01$). The protein expression levels were determined by Western blot analysis. As shown in **Figure 1C**, the stathmin protein levels were decreased in ASODN-transfected cells in comparison with the SODN-transfected cells ($P < 0.01$).

The proliferation of these cells was then assessed with the MTT assay. The cells were first treated with stathmin SODN (20 μmol/L), or ASODN at indicated concentrations (10, 20, and 30 μmol/L), for 24 h, 48 h, 72 h, and 96 h, respectively. The proliferation was detected, and the inhibitory rates were calculated. Our results showed that SGC 7901 cell proliferation was significantly inhibited by stathmin ASODN at studied concentrations, compared with the SODN-treated group ($P < 0.05$). The inhibitory effects of stathmin ASODN peaked at 72 h, and the optimal treatment concentration was 20 μmol/L (**Figure 1D**).

The population doubling time was also measured in stathmin ASODN-, SODN-treated cells, as well as untransfected SGC 7901 cells. The results showed that in the untransfected control group, cells proliferated in an exponential manner, and the population doubling time was around 20-21 h. The changes in the cell proliferation doubling time were not observed when these cells were treated with 20 μmol/L stathmin SODN (**Figure 1E**; $P > 0.05$). However, at 24 h after the transfection with 20 μmol/L stathmin ASODN, the cell proliferation started to be inhibited, and the doubling time was prolonged to 25-28 h. At 48 h, 72 h, and 96 h after transfection, the cell numbers were significantly lower than the control group (**Figure 1E**; $P < 0.01$). Taken together, these results suggest that stathmin ASODN could significantly inhibit the proliferation of gastric cancer SGC 7901 cells *in vitro*.

Effects of stathmin ASODN on gastric cancer cell growth in vivo

The effects of stathmin ASODN on gastric cancer cell growth *in vivo* were then investigated. Our results indicated that the growth of gastric cancer cells transfected with stathmin ASODN in nude mice were obviously slower than the control group. The mean diameter of gastric tumors transfected with stathmin ASODN in nude mice was 4.04 ± 0.17 mm^3, which was

much smaller than the SODN-treated group (8.01 ± 0.21 mm^3) (**Figure 1F**; $P < 0.05$). These results suggest that stathmin ASODN could inhibit the proliferation of gastric cancer cells *in vivo*.

Effects of combination of stathmin ASDON and docetaxel on the proliferation of SGC 7901 cells

To investigate the effects of the combination of stathmin ASODN and docetaxel on gastric cancer cells, these cells were first treated with docetaxel alone, and then in combination with stathmin ASODN. The proliferation assessment showed that docetaxel inhibited the proliferation of SGC 7901 cells, in a dose- and time-dependent manner (**Figure 2A**). When combined with the stathmin ASODN, the inhibitory effects on the cell proliferation were enhanced, which were significantly stronger than either treatment alone (**Figure 2B**; $P < 0.05$). These results suggest that there is a synergistic effect for the combination of stathmin ASDON and docetaxel on the proliferation of gastric cancer SGC 7901 cells.

Based on these *in vitro* results, the relationship between the prognosis of docetaxel treatment and the stathmin expression in gastric cancer patients was then evaluated. As shown in **Table 2**, the efficacy and clinical benefit rate in the stathmin-positive group were 10.5% and 52.6%, respectively, which were much lower than the stathmin-negative group (23.1% and 69.2%, respectively) ($P < 0.05$). In the one-year follow-up visit, 6 cases of death occurred in the stathmin-positive group, while 4 occurred in the stathmin-negative group. The one-year survival rates were 68.4% and 69.2%, respectively ($P > 0.10$). These results indicate that docetaxel treatment would be more effective for stathmin-negative gastric cancer patients, and screening for stathmin in these patients would provide evidence and/or expectations for the treatment choices.

Discussion

Stathmin is a characteristic gene in lymphoma, whose product is a highly conserved intracellular protein consisting of 149 amino acids. Numerous intracellular and extracellular cytokines, and products of oncogenes and tumor suppressor genes, may induce changes in cellular functions through direct and/or indirect interactions with stathmin. Meanwhile, stathmin is also the substrate of intracellular kinases, including PAK, MAPs, and Cdc. Therefore, stathmin has been considered as a relay in signal transduction. In cell division, extracellular signaling factors could influence the phosphorylation of stathmin, thereby affecting the microtubule system, and subsequent cell division and proliferation [12-15]. These extracellular factors include protein kinase C activator, peptide hormones increasing intracellular CAMP levels, a variety of growth factors (TGF-β and TNF-α), oncogenes and tumor suppressor genes (p53 and N-myc), and heat shock proteins. Alli *et al.* [16] found that stathmin was over-expressed in breast cancer cells containing mutant p53 alleles.

In this study, our results showed that the combination of stathmin ASODN transfection and docetaxel significantly inhibit the proliferation of the SGC 7901 cells, with a much stronger effect than either treatment alone, indicating a synergistic effect of these two treatments. In normal tissues, the expression of stathmin is higher in the tissues with high cellular metabolism than those with low proliferative capacity. Moreover, the expressions of stathmin are higher, in tumors with high proliferative activities. Hanash *et al.* [17] found that, in HL 60 leukemia cells, stathmin expression was up-regulated. When these cells were induced to differentiate, and the proliferation was slowed down, the expression of stathmin was then decreased. Our results indicated that stathmin ASODN could inhibit the proliferation of SGC 7901 cells at studied concentrations, which peaked at 72 h after transfection. On the other hand, stathmin SODN transfection did not lead to obvious changes in cell proliferation. In line with this, *in vivo* results showed that the proliferation of gastric cancer cells transfected with stathmin ASODN was slower than those transfected with SODN in nude mice.

Nowadays, therapeutic treatments specifically targeting stathmin, involving nucleic acids and siRNA, have been under intense investigation, including monotherapy and combination therapies (e.g., combined with docetaxel treatment) [10, 18]. These methods aim at inhibiting the mRNA expression of stathmin, resulting in cell cycle arrest at the G_2/M checkpoint, inhibiting cell proliferation, and increasing apoptosis.

Given that stathmin plays a key role in microtubule dynamics, it is reasonable to combine these treatments with other drugs interfering with the function of microtubules, including docetaxel and vinca alkaloids. Docetaxel stabilize microtubules via preventing tubulin depolymerization, while vinca alkaloids destabilize tubulins by binding with these proteins, delaying the assembly of microtubules. Although these drugs work through different mechanisms, they both can arrest cellular mitosis, and lead to apoptosis. Docetaxel treatment together with stathmin anti RNA plasmid could dramatically inhibit the cell proliferation, accompanied with abnormal aneuploidy and increasing apoptotic processes. In contrast, blocking stathmin could decrease the sensitivities of the cells to the spindle destabilizing drug vinblastine. Taken together, the spindle formation in mitosis depends on the coordination and equilibrium between certain proteins like stathmin.

In conclusion, our results showed that the expression of stathmin was elevated in gastric cancer patients. Stathmin ASODN significantly inhibited the proliferation of gastric cancer cells, both *in vitro* and *in vivo*. Moreover, the combination of stathmin ASDON and docetaxel could exert synergistic effects on the proliferation of gastric cancer cells. Importantly, docetaxel treatment would be more effective for stathmin-negative gastric cancer patients. These results indicate that stathmin expression would provide evidence for the treatment choices, and stathmin is a potential molecular marker and target for the treatment of gastric cancers.

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Disclosure of conflict of interest

None.

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