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MicroRNA-338 inhibits proliferation, migration, and invasion of gastric cancer cells by the Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Emerging evidence suggests aberrant microRNAs (miRNAs) expression is involved in cancer development through multiple. Although miR338 has shown to have tumor suppression ability and anti-migration effects in some cancers, its regulatory role and molecular mechanism in the development of gastric cancer cells yet remains little known. This work aims to investigate miR-338 in regulating Wnt/β-catenin pathway in epithelial-mesenchymal transition (EMT) in gastric cancers.

MATERIALS AND METHODS: Human gastric cancer cells were transfected with either miR-338 mimic or erythropoietin-producing hepatocellular (Eph)A2-targeting siRNA. The biological function of miR-338 in gastric cancer cells was investigated using a MTT assay and invasion assay. Western blot assay was used to measure the levels of EphA2, GSK-3β, phospho-GSK-3β^{Ser9}, c-Myc, E-cadherin, Vimentin, and β-catenin of at protein level.

RESULTS: Our data showed that miR-338 inhibited proliferation, migration and invasion of human gastric cancer cells. miR-338 affected the Wnt/β-catenin pathway by increasing p-GSK-3β^{Ser9} and decreasing GSK-3βSer9 and c-Myc at protein levels. EphA2 protein level was downregulated and positively correlated with EMT markers. Both silencing of EphA2 and transfection with miR-338 mimic resulted in the up-regulation of the EMT molecular marker E-cadherin and down-regulation of Vimentin and β-catenin at protein levels.

CONCLUSIONS: This study indicated that miR-338 is a potential tumor suppressor in gastric cancer and miR-338 inhibited EMT of gastric cancer cells through deactivation of Wnt/β-catenin signaling targeting at EphA2.

Key Words:
Gastric cancer, miR-338, Wnt/β-catenin, Epithelial-mesenchymal transition, EphA2.

Introduction

Gastric cancer (GC) remains a big clinical challenge due to its poor prognosis. Most gastric cancers are diagnosed in stage IV, where tumor has spread to distant organs with no effective treatment[1]. Previous studies indicated that various populations were in poor health, suffering from it[2,3]. Epidemiological findings suggest that environmental factors play a major role in the carcinogenesis of GC. Diet and infection with *H. pylori* are the two leading risk factors for developing gastric cancers[4]. In addition to environmental factors, emerging evidence demonstrates that GC is a multifactorial disease involving deregulation of canonical oncogenic pathways and epigenetic abnormalities[5-7]. Epithelial-mesenchymal transition (EMT) is a process where cells lost epithelial differentiation and gain mesenchymal phenotypes, which enable newly transformed mesenchymal cells to migrate, invade, resist apoptosis and release lytic enzymes to degrade the extracellular matrix and basement membrane. EMT is known to be essential in both embryonic development and the progression of carcinomas. It can be characterized by the loss of E-cadherin together with the up-regulation of vimentin expression. Studies revealed several distinct signaling pathways and

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transcription factors participating in triggering and completing EMT process[8-10]. Cytoplasmic catenins have been proved to be essential in cadherin-mediated cell adhesion. The E-cadherin/β-catenin complex allows for epithelial integrity and stable cell junctions transcriptions. In addition to its role in maintaining cell adhesion, β-catenin is believed to be a major protein of the Wnt pathway. Wnt signaling has been known to regulate several biological processes including cell proliferation, cell polarity and cell fate determination during embryonic development and tissue homeostasis[11]. It is thought to affect or be affected by the E-cadherin expression through the Wnt/β-catenin pathway. Evidence suggests that canonical Wnt signaling affects cadherin-mediated cell adhesion by down regulating E-cadherin, up regulating adhesion molecules that favor cell motility and inducing proteases and other EMT promoters. Scholars have investigated the involvement of erythropoietin-producing hepatocellular (Eph) receptors in the EMT of numerous cancers. The Eph family member EphA2 has been suggested to promote by enhancing the Wnt/β-catenin pathway in gastric cancer cells. MicroRNAs (miRNAs) are an evolutionarily conserved class of endogenous, small, noncoding RNAs (19-25 nucleotides) which function in transcriptional and post-transcriptional regulations or gene expression[12,13]. Li et al[14] reported seven miRNAs, including miRNA-338, which were significantly correlated with clinical outcome in gastric cancer patients. Down-regulation of miR338-3p has frequently been observed in gastric cancer[15] and colorectal carcinoma[16]. These findings imply that miRNA-338 may be involved in the regulation of the development and progression of gastric cancer. However, few studies have been conducted to elucidate how miRNA338 functions as a regulator during the EMT procedure. In this study, we sought to characterize the role of mi-338 in inducing EMT in GC and its interplays with Wnt/β-catenin pathway and EphA2.

Materials and Methods

Cell Culture

Human gastric cancer AGS cell line was purchased from the Cell Bank (Shanghai, China). The cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT, USA) and antibiotics in a humidified (37°C, 5% CO_2) incubator. The medium was changed every 2 to 4 days. When cells reached an estimated confluence of 80%, they were trypsinized, centrifuged and reconstituted in 2 ml RPMI-1640 supplemented with 10% (v/v) FBS at a concentration of 1×10^5 cells/ml. Then, cells were kept in (37°C, 5% CO_2) incubator for 24 h.

Cell Transfection

The cells were harvested and washed with Opti-MEM reduced serum medium twice. A miR-338 mimic, EphA2 siRNA or negative control was used for transfection in this study. Cells were transfected with Lipofectamine 2000 Reagent (Carlsbad, CA, USA) following the manufacturer's protocol. The primer sequences used for miR-338 were as follows: forward 5'- TGACATGCCGATCTACATG-3' and reverse 5'- ATAAGCTTGATATCG-3' and for the primer sequences used for EphA2 siRNA were as follows: forward 5'- TGACATGCCGATCTACATG-3' and reverse 5'- CATGTAGATCGGCATGTCA-3'. Plasmids were constructed by GeneChem Biomedical (Shanghai, China).

Invasion Assay

Cell invasion assay was performed using according to the manufacturer's instructions (Chemi-Con, ECM550, Shinagawa, Tokyo, Japan). Briefly, 2.5×10^5 100-200 μL cells were seeded in the upper compartment of the Transwell chambers. The lower compartment contained 500 μl of medium, supplemented with 10% (v/v) fetal bovine serum (FBS). The cells were maintained in humidified incubator for 16 h. After incubation, the non-migrating cells were removed and cells invading the membrane were first fixed and stained with Giemsa, then they were quantified. The results were observed and photographed by Leica DC 300F microscope (Wetzlar, Germany), randomly selecting 6 views.

MTT Assay

Cell proliferation was evaluated by methylthiazolyl tetrazolium bromide (MTT). The AGS cells were harvested after 24 h transfection and re-seeded in 96-well plates at a density of 4×10^3 per well. Six replicated samples were assayed in each group. 20 μL MTT (Beyotime, Shanghai, China) was added to each well. After 4 h culture, the supernatant was discarded, 200 μL dimethyl sulfoxide (DMSO) was added to each well, and the plate was placed on a shaker for 10

min to allow the DMSO completely dissolution. The absorbance was measured at wave-lengths of 570 nM using UV1902P spectrophotometer (Shanghai Aucy Technology Instrument Co., Ltd, Shanghai, China). The half maximal inhibitory concentration (IC50) was calculated using Origin, version 8.5 (Wellesley Hills, USA).

Cell Extracts and Western Blotting

The cells in each group were harvest after 6, 12, 24 h transfection and lysed with lysis buffer for 50 min. The cells were homogenized and centrifuged at 20000 r/min for 20 min at 4°C. The protein concentration in the supernatant was determined with the Bio-Rad protein assay kit (Active Motif, Carlsbad, CA, USA). The protein (40 μg) was subjected to 12-15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking nonspecific binding sites with 5% fat dry milk for 1 h, it was incubated with the anti-EphA2 (1:1800), anti-E-cadherin (1:1500), anti-Vimentin (1:1000), anti-β-catenin (1:1000), anti-GSK-3β (1:800), anti-p-GSK-3β (1:800), anti-c-Myc (1:1500). The membrane was incubated overnight at 4°C with primary antibodies. Monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Next, the membrane was washed with Tris-buffered saline (TBS) 3 times (5 min each), and later incubated with secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 2 h. After washing membrane, the signals were detected using ImmobilonWestern HRP substrate (Millipore, Billerica, MA, USA) and band densities were quantified using BandScan, version 5.0 (Ulvales, Chlorophyta).

Statistical Analysis

Data are presented with the means ± standard deviations. Data were analyzed by the student's *t*-test (Two-tailed) using the probability value of $p < 0.05$ to define statistically significant differences. All analysis was performed using SPSS19.0 (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA).

Results

miR-338 Inhibits GC Cell Growth and Invasion

We began with inhibitory effect of miR-338 on cell viability using the MTT assay (Figure 1A). The potential effect of miR-338-3p on cell invasiveness by the transwell invasive assays (Figure 1B) was investigated. The results showed that increased levels of miR-338 significantly inhibited the growth ($p < 0.05$) and repressed the invasiveness of AGS cells ($p < 0.05$). These findings indicate that elevated miR-338 levels in GC cells markedly reduce their ability to form and migrate tumors.

miR-338 Regulated the Wnt/β-Catenin Pathway and Downregulated EphA2 in Gastric Cancer Cells

Since Wnt/β-catenin signaling is known to participant in both tumorigenesis and EMT, we then

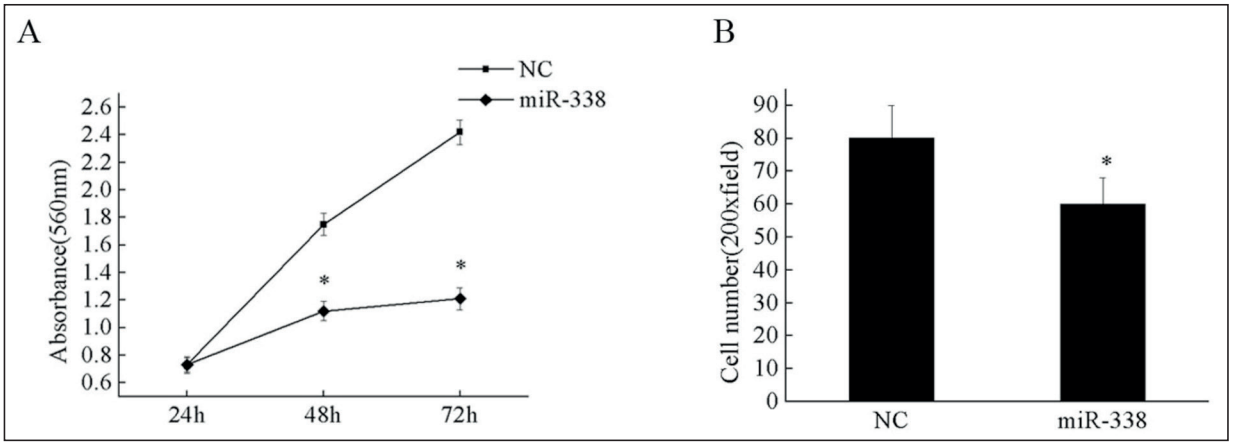

Figure 1. Increased miR-338 inhibits growth human GC cell growth and invasion. Cell proliferation was assessed by the MTT assay in AGS. Cells ***(A)*** 24 h, 48 h, and 72 h after transfection with miR-338 mimic. Cell invasion ***(B)*** was determined by Transwell invasion assay; $*p < 0.05$ (compared with miR-normal controls).

investigated whether miR-338 potentially modulate Wnt/β-catenin signaling pathway. Western blotting was used to assess the protein levels of several Wnt/β-catenin pathway's targets including GSK-3β, P-GSK-3β^{Ser9} and c-Myc. Alteration of p-GSK-3β could reflect altered Wnt/β-catenin signaling activities and the up-regulation p-GSK-3β is a marker of hyper activation of this signaling. Figure 2A showed that the expression levels of GSK-2P were increased in cells transfected with miR-338 mimics while p-GSK-3P and c-Myc were decreased ($p < 0.05$). The results suggest that miR-338 functioned as a suppressor of the Wnt/β-catenin pathway in GC cells. Also, we tested the EphA2 expression at protein level by Western blotting (Figure 2B). Notably, the level of the EphA2 was decreased in GC cells transfected with miR-338 mimic ($p < 0.05$).

miR-338 Leads to EMT Associated Markers Dysregulation and β-catenin Down-regulation

To further explore the inhibitory effect of miR-338 on the invasion and migration of GC cancer, we tested the effects of miR-338 elevation on the protein expression levels of the epithelial marker, E-cadherin and the mesenchymal marker, Vimentin. Based on the importance role of E-cadherin/β-catenin complex in the development of EMT, we also tested whether miR-338 levels affected β-catenin expression at protein level. Western blot analysis was performed on cell lysates by using antibodies E-cadherin, Vimentin, and β-catenin. As shown in Figure 3, miR-338 elevation increased E-cadherin protein level and reduced Vimentin level. Less β-catenin protein was presented in miR-338 transfected cells than that in control cells ($p < 0.05$).

Suppression of EphA2 Protein Leads to EMT Associated Markers Dysregulation and β-Catenin Down-regulation

Previously, it has been suggested EphA2 promote EMT in gastric cancer. Silencing of EphA2 resulted in decreased EMT related markers. We used Western blotting to determine the expression of the EMT-induced markers E-cadherin and Vimentin in cells transfected with siRNA targeting EphA2. As shown in Figure 4, E-cadherin protein level increased while Vimentin level decreased in GC cells with suppressed EphaA2 protein. Less β-catenin protein were present in EphA2 silenced cells than that in control cells ($p < 0.05$).

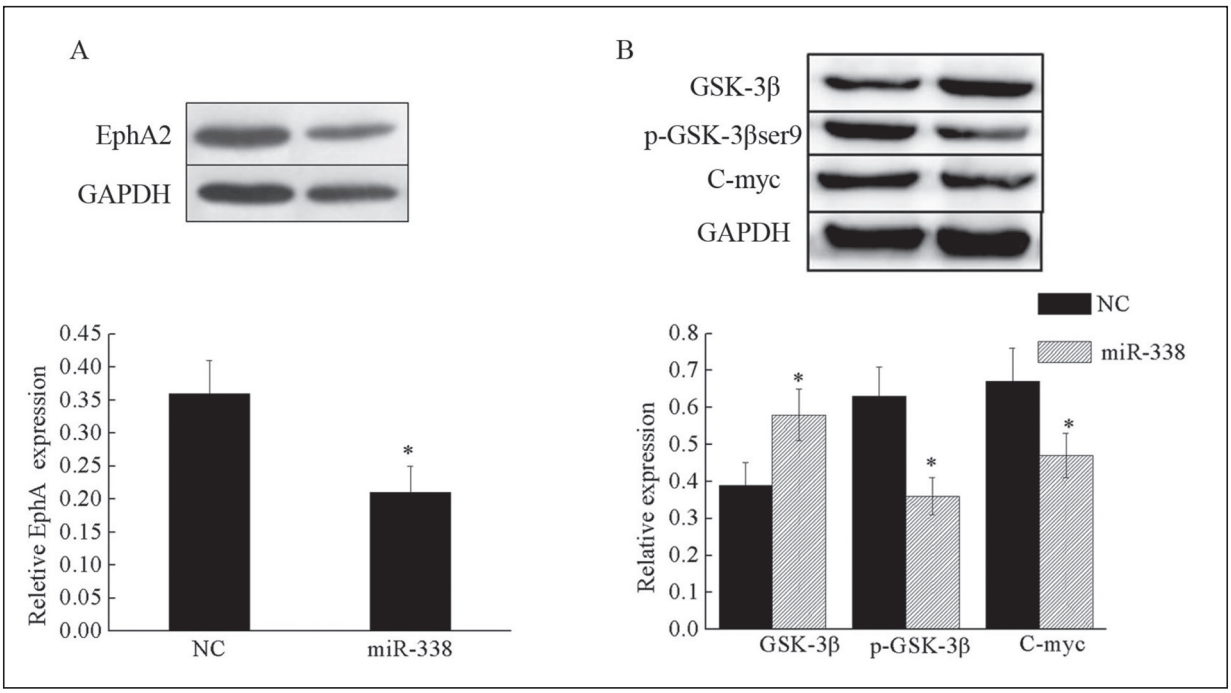

Figure 2. miR-338 regulated the Wnt/β-catenin pathway and downregulated EphA2 in gastric cancer cells. EphA2 ***(A)***, GSK-3β, phospho-GSK-3β^{Ser9} and c-Myc ***(B)*** expression was analyzed by Western blotting in AGS cells transfected with miR-33 mimic; GAPDH as reference; *$p < 0.05$ (compared with normal controls).

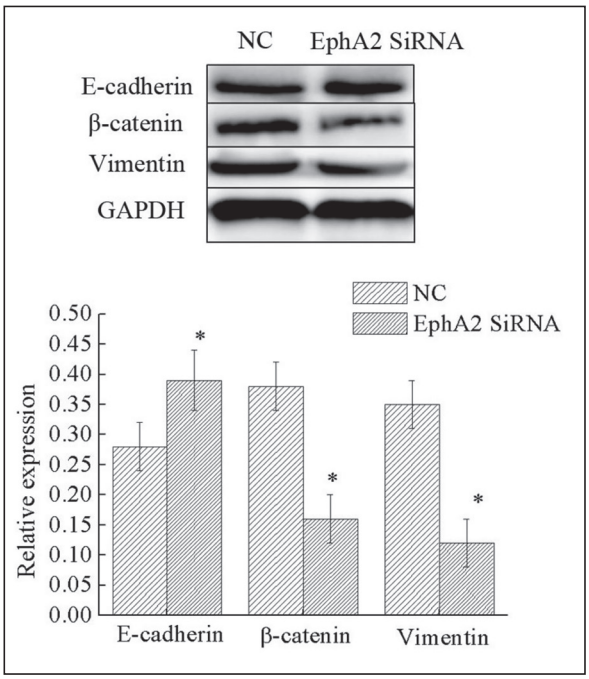

Figure 3. miR-338 leads to β-catenin down-regulation and EMT associated markers dysregulation in gastric cancer cells. E-cadherin, β-catenin and Vimentin expression was analyzed by Western blotting in AGS cells transfected with EphA2-targeting siRNA. GAPDH as reference; $*p < 0.05$ (compared with normal controls).

Discussion

In this study, we found that miR-33 functioned as a potential tumor suppressor by inhibiting Wnt/β-catenin signaling and inducing EMT in cultured human gastric cancer cell. Our findings strongly indicated that the signal of miR-338 for inducing gastric cancer cells to undergo EMT might pass through down-regulation of EphA2. To our knowledge, this is the first research revealing interplays between miR-338 and EphA2 in miRNA induced EMT. Gastric cancer is a complex and multifactorial disease resulting from interactions between environmental and deregulation of oncogenic cell pathways[17]. Various vulnerable groups suffered from it[18,19]. EMT has been mainly discussed and studied in the diffuse type gastric carcinoma and is believed to be essential for tumor invasion and metastasis[8,10,20]. Evidence has shown that miRNAs are wildly involved in inducing EMT post-transcriptional process. In addition, it has been proved that these small, noncoding RNAs could indirectly alter the function of EMT transcription factors or target the involved signaling pathways. MiR-200 family is found to be the negative regulator of the Wnt/β-catenin by inhibiting Zeb1/2 and Snail1. Moreover, WNT components such as FBXW11 and FZD5, were found to be repressed by miR-199-3p and miR200b, and downregulated by miR-30 family[21-24]. miR-338 has been reported to be associated with carcinogenesis and angiogenesis in different tumors. In malignant melanomas, expressions of miR-33 along with miR-193a and miR-565 were found to be decreased[25]. Research has shown that miR-338 regulated EMT procedure via smoothened in liver cancer and pulmonary fibrosis[26,27]. Downregulated miR-338 was also observed in human gastric cancer tissues and cultured gastric cell lines[28]. Similar to existing findings, we also found that miR-338 could act as an inhibitor in gastric cell migration, invasion and proliferation[28,29]. Wnt/β-catenin is well-known to be a critical signaling pathway in maintaining an epithelial cell phenotype, proper cell to cell junctions, and tissue homeostasis.

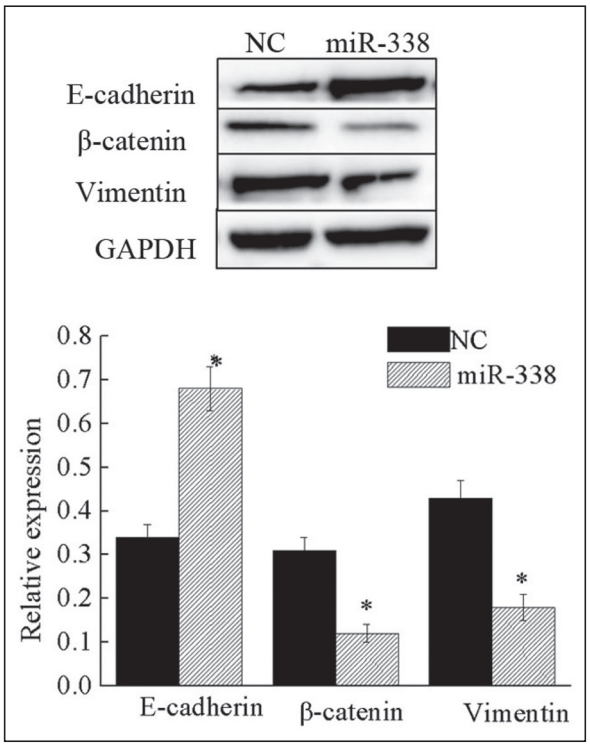

Figure 4. Suppression of EphA2 protein leads to EMT associated markers dysregulation and β-catenin down-regulation, E-cadherin, β-catenin, and Vimentin expression was analyzed by Western blotting in AGS cells transfected with miR-338 mimic. GAPDH as reference; $*p < 0.05$ (compared with normal controls).

Alteration of the members of this signaling pathway is involved in the development of cancer and the EMT process, which is required for cancer metastasis. Cytoplasmic β-catenin levels are regulated by a complex containing GSK-3/APC/Axin. When cells are exposed to a Wnt signal, the degradation pathway of β-catenin is inhibited and this protein is accumulated in cytoplasm and nucleus. Then, nuclear β-catenin interacts with transcription factors (TCF) such as c-Myc or Cyclin-D1 associated with cell proliferation[30]. Dysregulation of Wnt/β-catenin signaling and the E-cadherin cell adhesion system have been implicated as important events in the initiation and/or progression of several forms of cancers. In our work we demonstrated that transfection with miR-338 leads to decreasing phosphorylate GSK3β (ser9 site) implying inhibition of the Wnt/β-catenin pathway. This was further demonstrated by increased expression of E-cadherin and decreased β-catenin and Vimentin at protein levels shown in our research. Recently, EphA2 has been found to function as an activator of EMT in carcinogenesis, which could be a mechanism of EphA2-mediataed cancer invasion and metastasis[31]. Huang et al[32] reported that EphA2 promoted EMT by enhancing the Wnt/β-catenin in gastric cancer at both protein and RNA levels. In this study, we confirmed that EphA2 could promote EMT by silencing EphA2 with EphA2 siRNA. EMT specific markers were regulated in the EphA2-silenced group with increased E-cadherin and decreased Vimentin and β-catenin. Interestingly, same effects were observed in cells transfected with miR-338 in this study. Taken together with the other finding in this work, decreasing expression of EphA2 at protein level in cells with miR338 transfection, these results suggest that miR-338 inhibited GC proliferation, invasion, and migration through targeting EphA2.

Conclusions

We observed the inhibitory effect of miR-338 on the growth, migration, and invasion in gastric cancer by Wnt/β-catenin pathway. Moreover, our study showed interplays between miR338 and EphA2 in the EMT process. Further investigation into genetic level would facilitate a better understanding of the complexity of the underlying mechanism and may contribute to a novel targeted therapy for GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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