Research Paper

# Tramadol Suppresses the Proliferation, Migration and Invasion of SNU-1 Gastric Cancer Cells by regulating the microRNA-212-5p/Wnt/β-Catenin Pathway

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**Cao *et al.*: Tramadol in the Oncogenic Phenotypes of Gastric Cancer Cells**

**The study includes the function of tramadol on the oncogenic phenotypes of SNU-1 gastric cancer cells and its mechanism. SNU-1 cells were divided into control group, tramadol group, microRNA-negative control group, microRNA-212-5p group, 300 μg/ml tramadol+anti-microRNA-negative control group and 300 μg/ml tramadol+anti-microRNA-212-5p group. SNU-1 cell proliferation, invasiveness and migration were quantified. Reverse transcription-quantitative polymerase chain reaction and Western blot examined the microRNA-212-5p and beta-catenin expression. The proliferation, invasiveness and migration of SNU-1 cells in tramadol (75 μg/ml, 150 μg/ml or 300 μg/ml) group were significantly reduced. Tramadol elevated microRNA-212-5p level in SNU-1 cells, and microRNA-212-5p elevation suppressed SNU-1 cell proliferation, migration and invasion. Moreover, microRNA-212-5p deficiency reversed the anticancer activity of tramadol in SNU-1 cells. Additionally, tramadol declined beta-catenin protein in SNU-1 cells, while microRNA-212-5p deficiency abolished this effect of tramadol. Tramadol inhibited SNU-1 gastric cancer cell proliferation, invasiveness and migration by the microRNA-212-5p/Wnt/β-catenin pathway.**

**Key words: Tramadol, gastric cancer, microRNA-212-5p, Wnt/β-catenin, chemotherapy**

Currently, gastric cancer remains the main cause of deaths worldwide, even the improvement in treatment strategies such as surgical resection and chemotherapy[1]. Moreover, the high recurrence rates and tumor metastasis is still the main obstacles for prolonging the survival rate of gastric cancer patients[2]. Accordingly, identifying the regulatory mechanisms for gastric cancer progression is crucial for developing new treatment strategies.

Tramadol is a well-tolerated nonsteroidal anti-inflammatory drugs widely used for the treatment of various pain syndromes and postoperative pain, including cancer-related pain[3,4]. Clinical application has proved that the analgesic effect of tramadol is better than most non-opioid drugs, and the toxicity is lower than morphine and pethidine. Tramadol is the only non-narcotic analgesic drug in the guidelines of the Ministry of Health's three-step analgesic therapy for cancer patients with moderate pain. Recent studies have manifested that anesthetic analgesics such as morphine and propofol, can impair malignant behaviors of tumor cells, meanwhile, the application of them in cancer surgery is expected to reduce the risk of postoperative recurrence and metastasis[5-7]. In addition, Tramadol was proved to suppress cell mobility and survival in lung adenocarcinoma *via* blocking Phosphoinositide 3-Kinase (PI3K)/Protein Kinase B (AKT) through PTEN[8]. Tramadol repressed the metastasis and enhanced cytotoxic effects in breast cancer[9]. Nevertheless, effects of tramadol on gastric cancer cell malignant behaviors are till vague. microRNA (miR)-212-5p is a tumor-regulated miRNA. It was found to miR-212-5p overexpression reduced proliferation in gastric cancer cells, which might be related to long non-coding RNA (lncRNA) MIF-AS1/miR-212-5p

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axis[10]. Through the preliminary experiment, we found tramadol treatment significantly increased miR-212-5p content in gastric cancer cells. Here, we speculated that tramadol might exert its anticancer function in gastric cancer by affecting miR-212-5p level.

Here, this study analyzed the functions of tramadol and miR-212-5p on gastric cancer cell oncogenic phenotypes *in vitro*, aiming to lay a theoretical foundation for the application of tramadol in gastric cancer therapy.

## MATERIALS AND METHODS

### Cell culture and treatment:

SNU-1 cells (Procell, Wuhan, China) were maintained in Roswell Park Memorial Institute (RPMI)-1640 (BIOSUN, Shanghai, China) plus 10 % Fetal Bovine Serum (FBS) (BIOSUN) with 5 % Carbon dioxide ($CO_2$) at 37°.

Tramadol was obtained from Huarenrizhao (Shandong, China). SNU-1 cells were treated with 75 μg/ml, 150 μg/ml, or 300 μg/ml tramadol for 48 h. 300 μg/ml tramadol was used for further analysis.

### Cell transfection:

The miR-212-5p mimic, inhibitor (anti-miR-212-5p) and the negative control (miR-NC and anti-miR-NC) were designed by RiboBio (Guangzhou, China). Cell transfection was conducted followed in full compliance with the commercial instructions of Lipofectamine 3000.

### Cell Counting Kit-8 (CCK-8) assay:

SNU-1 cells ($1\times10^4$ cells/well) were inoculated in a 96-well plates with 10 μl CCK-8 solution, followed by recoding the absorbance at 450 nm after 2 h incubation.

### Colony formation assay:

SNU-1 cells were incubated for 14 d in a 5 % $CO_2$ incubator. Cell colonies were stained with 0.1 % crystal violet (Beyotime, Shanghai, China) and colonies (≥50 cells) were counted.

### Transwell assay:

Place the 24-well plate on flat ice and gently move it into the 24-well plate by holding the edge of the transwell chamber with sterile tweezers. Take an appropriate amount of medium in to the chamber and then suction it out to make the chamber wet and easy to spread glue. The Matrigel diluted by medium was spread on the transwell cell polycarbonate film in a volume of 35 μl for invasion assay, and uncoated polycarbonate film was used for migration. Accurately $1\times10^5$ cells/well were put into the upper chamber, and the cell suspended fluid volume is 200 μl. 24 h later, the images of migrated cells were collected and migrated cells were counted after dyeing with 0.1 % crystal violet.

### Western blot:

SNU-1 cells are cleaved in Radioimmunoprecipitation Assay (RIPA) buffer to collect supernatant. After separating by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferring to nitrocellulose membranes, membranes were incubated with primary antibodies at 4° all night, and following secondary antibody for 2 h at 37°. After Electrochemiluminescence (ECL) incubation, Quantity One software was adopted to measure the gray value of protein bands.

### Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR):

TRIzol reagent (TaKaRa, Beijing, China) was adopted for the isolation of total Ribonucleic Acid (RNAs). The extracted RNA was reversely transcribed into complimentary Deoxyribonucleic Acid (cDNA), and cDNA was subsequently quantified using the SYBR-Green (TaKaRa) with following conditions; 94° 5 min, 94° 30 sec for 30 cycles, 57° 45 sec and 72° 45 sec. miR-212-5p levels were assayed by the $2^{-\Delta\Delta Ct}$ method with U6 as an internal reference. The sequences of primers; miR-212-5p forward 5'-GGA AAC ATC CTC GAC TG-3' and reverse 5'-ATT GAA CGT GCC TCC GTG TTG AGG-3'.

### Statistical analysis:

The data were presented by $\bar{x}\pm s$. The comparison of the two groups or multiple groups was conducted using t-test or Analysis of Variance (ANOVA). $p<0.05$ meant significant difference.

## RESULTS AND DISCUSSION

Compared with the NC group, the viability and DNA synthesis activity, as well as levels of Ki-67

were significantly reduced in SNU-1 cells with 75 µg/ml, 150 µg/ml, or 300 µg/ml tramadol treatment as shown in fig. 1A and fig. 1B, and Table 1. The migratory and invasion abilities of SNU-1 cells were notably repressed in 75 µg/ml, 150 µg/ml or 300 µg/ml tramadol treated group relative to the NC group as shown in fig. 2 and Table 2.

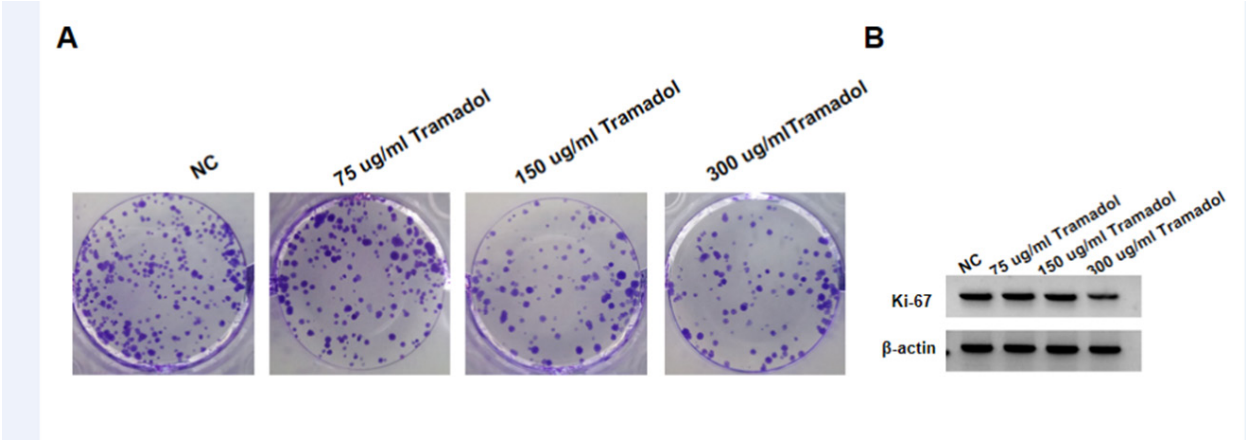


**Fig. 1: Tramadol suppresses SNU-1 cell proliferation, (A): Colony formation assay for cell proliferation and (B): Western blot for Ki-67 protein levels**

**TABLE 1: TRAMADOL SUPPRESSES SNU-1 CELL DNA SYNTHESIS ACTIVITY (x̄±s, n=9)**

| Group | A values | Colonies | Ki-67 |
|---|---|---|---|
| NC | 1.135±0.10 | 124±10.36 | 0.83±0.08 |
| 75 µg/ml | 0.923±0.08* | 105±8.37* | 0.71±0.06* |
| 150 µg/ml | 0.635±0.07* | 73±5.96* | 0.57±0.05* |
| 300 µg/ml | 0.584±0.05* | 57±5.03* | 0.44±0.04* |
| F | 100.716 | 138.975 | 73.085 |
| p | 0.000 | 0.000 | 0.000 |

Note: Relative to NC group, *p<0.05

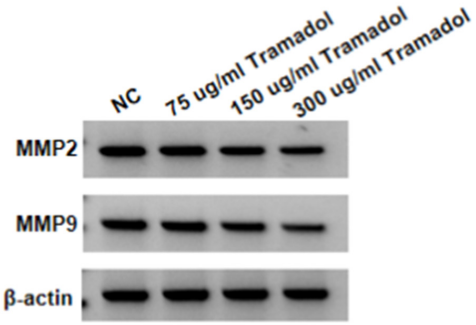


**Fig. 2: Western blot for the protein levels of MMP2 and MMP9**

**TABLE 2: TRAMADOL SUPPRESSES SNU-1 CELL MIGRATION AND INVASION (x̄±s, n=9)**

| Group | Migration number | Invasion number | MMP2 | MMP9 |
|---|---|---|---|---|
| NC | 218±18.15 | 184±15.02 | 0.92±0.09 | 0.75±0.07 |
| 75 µg/ml | 182±15.03* | 154±13.28* | 0.72±0.07* | 0.62±0.05* |
| 150 µg/ml | 141±10.37* | 121±10.24* | 0.54±0.06* | 0.48±0.04* |
| 300 µg/ml | 105±8.35* | 81±7.34* | 0.45±0.04* | 0.37±0.03* |
| F | 118.348 | 125.716 | 85.5 | 99.515 |
| P | 0 | 0 | 0 | 0 |

Note: Relative to NC group, *p<0.05

Relative to the NC group, we found miR-212-5p levels were markedly elevated in SNU-1 cells with 75 μg/ml, 150 μg/ml, or 300 μg/ml tramadol treatment (Table 3). As exhibited in fig. 3 and Table 4, miR-212-5p mimic introduction markedly elevated miR-212-5p expression, functionally, forced expression of miR-212-5p suppressed cell viability, DNA synthesis activity, migration and invasion, as well as reduced the levels of Ki-67, Matrix Metalloproteinase (MMP)-2 and MMP9 in SNU-1 cells.

SNU-1 cells were transduced with anti-miR-212-5p or anti-miR-NC, followed by treating with 300 μg/ml tramadol. As expected, anti-miR-212-5p introduction reduced tramadol-evoked elevation of miR-212-5p levels in SNU-1 cells (Table 5). Functionally, the suppression of tramadol on SNU-1 cell viability, DNA synthesis activity, migration and invasion, as well as the reduction of tramadol on Ki-67, MMP2 and MMP9 protein levels in SNU-1 cells were abolished by anti-miR-212-5p introduction (Table 5, fig. 4A and fig. 4B). Tramadol treatment reduced the protein levels of β-catenin, which was rescued by miR-212-5p silencing (fig. 5 and Table 6).

**TABLE 3: TRAMADOL ELEVATES miR-212-5p EXPRESSION IN SNU-1 CELLS (x̄±s, n=9)**

| Group | miR-212-5p |
|---|---|
| NC | 1.00±0.10 |
| 75 μg/ml | 1.76±0.15* |
| 150 μg/ml | 2.38±0.21* |
| 300 μg/ml | 2.72±0.25* |
| F | 147.994 |
| p | 0.000 |

Note: Relative to NC group, *p<0.05

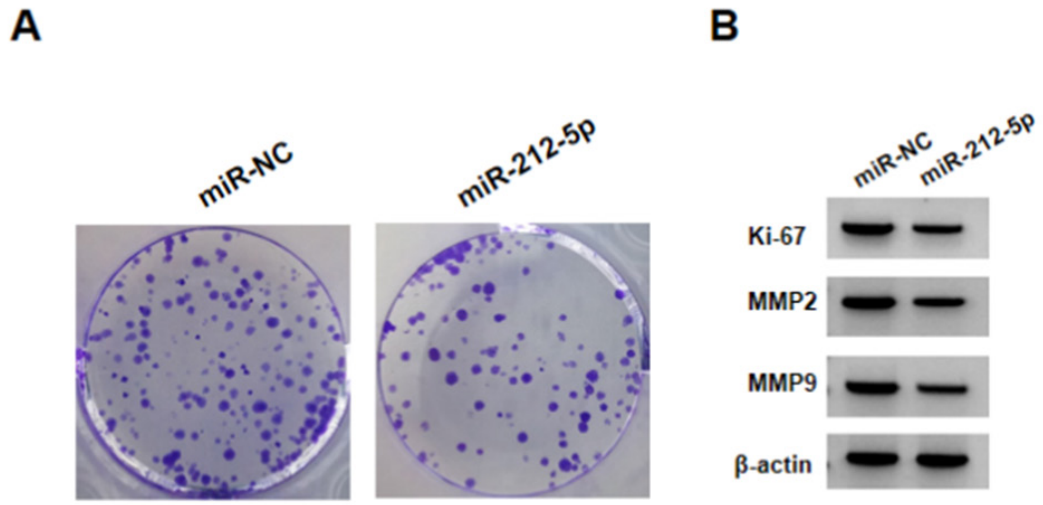


**Fig. 3: miR-212-5p overexpression suppresses the proliferation, migration and invasion of SNU-1 cells, (A): Colony formation assay for cell proliferation and (B): Western blot for Ki-67, MMP2 and MMP9 protein levels**

**TABLE 4: miR-212-5p OVEREXPRESSION SUPPRESSES THE PROLIFERATION, MIGRATION AND INVASION OF SNU-1 CELLS**

| Group | miR-212-5p | A values | Colonies | Ki-67 | MMP2 | MMP9 | Migration number | Invasion number |
|---|---|---|---|---|---|---|---|---|
| miR-NC | 1.00±0.11 | 1.134±0.11 | 128±9.67 | 0.84±0.07 | 0.93±0.09 | 0.73±0.07 | 214±17.63 | 181±14.33 |
| miR-212-5p | 2.34±0.19* | 0.594±0.05* | 61±5.34* | 0.41±0.04* | 0.46±0.02* | 0.39±0.03* | 97±8.31* | 76±6.51* |
| t | 18.311 | 13.407 | 18.196 | 16 | 15.294 | 13.393 | 18.009 | 20.013 |
| p | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

Note: Relative to the miR-NC group, *p<0.05

**TABLE 5: miR-212-5p SILENCING REVERSES THE EFFECTS OF TRAMADOL ON SNU-1 CELLS**

| Group | miR-212-5p | A values | Colonies | Ki-67 | MMP2 | MMP9 | Migration number | Invasion number |
|---|---|---|---|---|---|---|---|---|
| Tramadol+anti-miR-NC | 1.00±0.10 | 0.581±0.04 | 59±5.14 | 0.43±0.04 | 0.47±0.04 | 0.36±0.03 | 103±9.17 | 83±7.12 |
| Tramadol+anti-miR-212-5p | 0.41±0.04* | 1.096±0.09* | 127±10.66* | 0.86±0.08* | 0.94±0.09* | 0.78±0.07* | 228±20.06* | 191±15.37* |
| t | 16.434 | 15.687 | 17.238 | 14.423 | 14.316 | 16.545 | 17.002 | 19.127 |
| P | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

Note: Relative to the tramadol+anti-miR-NC group, *p<0.05

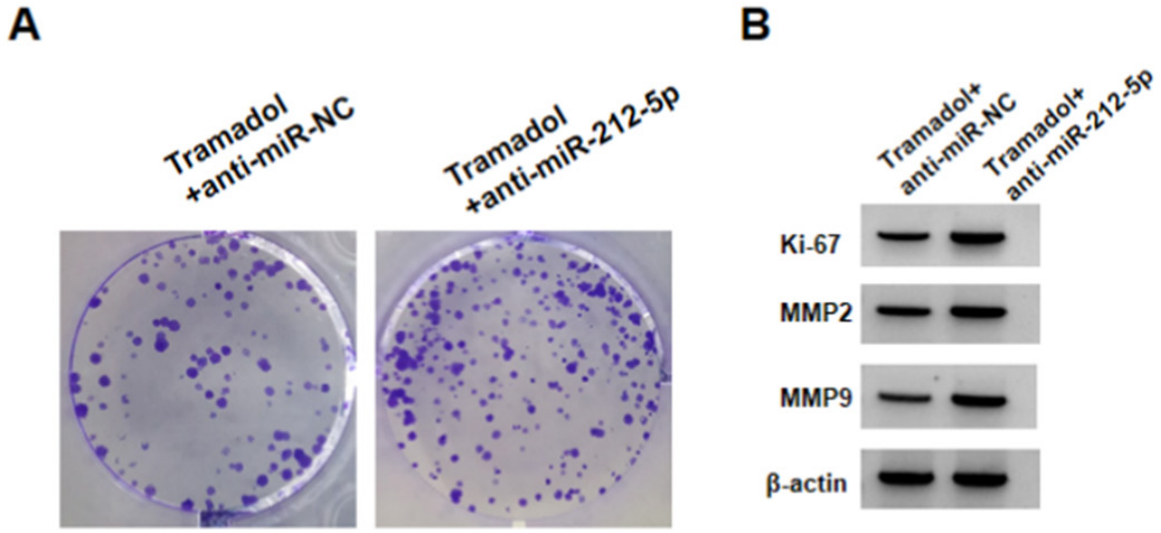


**Fig. 4: miR-212-5p silencing reverses the effects of tramadol on SNU-1 cells, (A): Colony formation assay for cell proliferation and (B): Western blot for Ki-67, MMP2 and MMP9 protein levels**

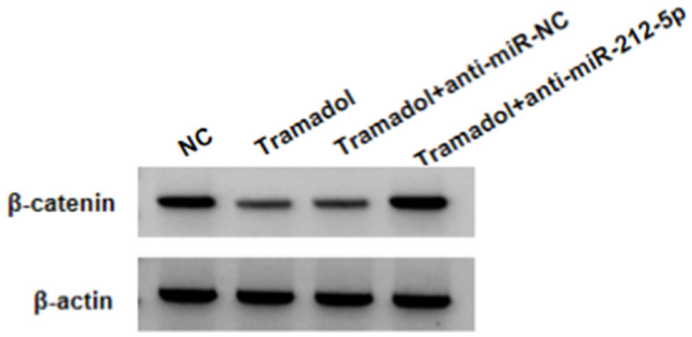


**Fig. 5: Western blot for β-catenin protein level**

**TABLE 6: TRAMADOL INACTIVATES WNT/β-CATENIN PATHWAY BY miR-212-5p**

| Group | ß-catenin |
|---|---|
| NC | 0.71±0.07 |
| Tramadol | 0.32±0.04* |
| Tramadol+anti-miR-NC | 0.34±0.03 |
| Tramadol+anti-miR-212-5p | 0.75±0.06# |
| F | 175.636 |
| p | 0.000 |

Note: Relative to the NC group, *p<0.05 and relative to the tramadol+anti-miR-NC group, #p<0.05

In traditional drugs, painkillers are mostly narcotic analgesics and some psychotropic drugs, which are easy to produce a certain degree of dependence and tolerance, resulting in medical personnel having certain concerns when using pain-relieving drugs, and they are reluctant to use them easily, resulting in many pain symptoms not being controlled in time. Tramadol is a type of artificially synthesized central analgesic drug that acts on μ-opioid receptors, as well as norepinephrine and serotonin systems, are used to alleviate moderate to severe pain[11]. The main problem with the use of opioid painkillers is their side effects, especially in outpatient patients and patients who need to take long-term medication, which can cause nausea, vomiting, dizziness, drowsiness, and cardiovascular and respiratory depression, as well as drug dependence. Tramadol has fewer side effects than the former, and has no inhibitory effect on the respiratory and cardiovascular systems, and rarely produces body dependence and tolerance. At present, the effects of anesthetic analgesics on tumor growth and metastasis are currently one of the hotspots in oncology research. Research shows that tramadol repressed the mobility and survival of breast cancer cells *via* blocking α2-adrenoceptor signaling[12]. At the same time, tramadol could induce apoptosis and reduce Reactive Oxygen Species (ROS) production in endometrial cancer cells, and enhanced the cytotoxic effects of cisplatin or doxorubicin in endometrial cancer[13]. In addition, tramadol had cytotoxic effects on colon cancer stem cells and triggered cell apoptosis[14]. Thereafter, this study displayed that tramadol treatment significantly impaired the proliferative, migratory and invasive abilities of gastric cancer cells. Ki-67 protein is a widely used marker for proliferation in cancer cells, and has function in mitotic and interphase cells[15]. MMP-2 and MMP-9 participate in the degradation of key components of the basement membrane and extracellular matrix, leading to tumor metastasis[16]. We observed tramadol treatment reduced Ki-67, MMP2 and MMP9 levels, further implying the anticancer activity of tramadol in gastric cancer.

Research displayed that miR-212-5p overexpression was able to impede breast cancer cell Epithelial-to-Mesenchymal Transition (EMT)[17]. The clear renal cell carcinoma showed decreased miR-212-5p level, and its elevation impaired cell growth and migration[18]. Glioma cells also displayed low miR-212-5p expression, which elevation could hinder glioma tumor growth in nude mice[19]. miR-212-5p performed tumor suppressing activity in breast cancer by impairing cell glycolysis, growth and metastasis, which might be related to circWHSC1/miR-212-5p/AKT3 axis[20]. Propofol suppressed ovarian cancer cell glycolysis and growth by elevating miR-212-5p through decreasing circ-ZFR expression[21]. In our work, gastric cancer cell showed increased miR-212-5p after tramadol treatment, moreover, forced expression of it restrained gastric cancer cell mobility and proliferation, importantly, and its silencing reversed the anticancer action of tramadol on tumor cells.

The Wnt/β-catenin pathway is implicated in diverse physiological processes, and the abnormal activation of this pathway is closely linked with the occurrence and metastasis of tumors, and targeting inhibition of Wntn/β-catenin pathway has been recognized as a potential cancer treatment strategy[22]. In gastric cancer, research reports that noncoding RNAs, such as LINC01133[23] and miR-6838-5p[24], inactivated Wnt/β-catenin pathway to impair multiple malignant processes of gastric cancer. The aberrant Wnt/β-catenin activation could enhance the arrest of ferroptotic death in gastric cancer cells *via* promoting the transcription of GPX4[25]. SERPINH1 evoked Wnt/β-catenin activation to boost the metastasis of gastric cancer[26]. In addition, the apoptosis induction and proliferation arrest activity of miR-212-5p in acute myeloid leukemia were achieved by the blockage of Wntn/β-catenin pathway[27]. Besides that, LINC00115 activated Wnt/β-catenin axis in prostate cancer cell by increasing FZD5 through absorbing miR-212-5p, thereby promoting the invasiveness and survival of prostate cancer cells[28]. Also, this study confirmed tramadol treatment mediated the blockage of Wnt/β-catenin pathway *via* miR-6838-5p.

In summary, tramadol impaired the proliferative, migratory and invasive abilities of gastric cancer cells *via* modulating miR-212-5p/Wntn/β-catenin axis. These data provide important evidences for tramadol application in surgical treatment of gastric cancer.

**Conflict of interests:**

The authors declared no conflict of interests.

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