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mir-3168 targeted inhibition of TP53 promotes malignant transformation and cisplatin resistance of AGS and AGS/DDP gastric cancer cells

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ABSTRACT

Objective: To investigate the effect of mir-3168 on the malignant transformation and cisplatin resistance of AGS and AGS/DDP gastric cancer cells, and to verify its target gene. **Methods:** The expression of mir-3168 in AGS and AGS/DDP gastric cancer cells was detected by qPCR, and mir-3168 mimic, inhibitor and negative control were synthesized. They were transfected into AGS and AGS/DDP gastric cancer cells, respectively. The expression of mir-3168 and TP53 mRNA was detected by qPCR. Cell viability was detected by CCK8 under gradient cisplatin treatment and non treatment, apoptosis was detected by flow cytometry, cell invasion was detected by Transwell, and TP53 protein expression was detected by western blot, The database predicted the binding sites of mir-3168 and TP53. According to the binding sites, the double luciferase experiment was used to verify the binding of *mir−3168* and *TP53*. **Results:** Compared with cisplatin sensitive gastric cancer cell AGS, mir-3168 was significantly overexpressed in cisplatin resistant gastric cancer cell AGS/DDP; mir-3168 mimic promotes cisplatin resistance, proliferation and invasion of AGS and AGS/DDP gastric cancer cells, and inhibits apoptosis of AGS and AGS/DDP gastric cancer cells; mir-3168 inhibitor inhibits cisplatin resistance, proliferation and invasion of AGS and AGS/DDP gastric cancer cells, and promotes apoptosis of AGS and AGS/DDP gastric cancer cells; mir-3168 mimic inhibits the expression of TP53 mRNA and protein, and mir-3168 inhibitor promotes the expression of TP53 mRNA and protein; Targetscan database predicted that there was a binding point between mir-3168 and TP53, and the double luciferase experiment suggested that mir-3168 was bound to TP53 through the predicted binding site. **Conclusion:** mir-3168 may promote the malignant transformation of AGS and AGS/DDP gastric cancer cells and cisplatin resistance by targeting TP53.

1. Introduction

Gastric cancer is currently the fourth most common malignancy and the third leading cause of tumor-related death in the world[1]. Gastric cancer has the highest incidence and mortality in East Asia, especially South Korea, Mongolia, Japan and China, and has become the second deadliest cancer in China[2]. Poor prognosis for gastric cancer is often due to late detection, aggressive features, and adverse reactions to existing therapies. Although gastrectomy, radiotherapy and chemotherapy have improved the survival rate of gastric cancer patients, the resistance of chemotherapy drugs is still a clinical problem in the treatment of gastric cancer[3]. Especially in patients with advanced gastric cancer, most of them have a poor prognosis after drug resistance, and more than half of patients with advanced gastric cancer die of recurrence[3]. Cisplatin (DDP) is considered first-line for patients with advanced gastric cancer. Cisplatin exerts antitumor effects by activating multiple apoptosis

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pathways[4]. In most patients with gastric cancer, cisplatin resistance is inevitable, leading to treatment failure, and the mechanism of resistance is not well understood[5]. Therefore, there is an urgent need to find new targets to reverse drug resistance and elucidate potential molecular mechanisms to improve the clinical challenges of cisplatin resistance.

Micro RNA (micro RNA) is a noncoding R about 22nt long NA, which functions by targeting mRNA 3' UTR terminal inhibitory protein translation, is associated with many types of tumors[6-8] and is involved in regulating a variety of important tumor processes, including cisplatin resistance in gastric cancer[9-11]. miR-3168 is one of the micro RNA (microRNA), and studies have shown that miR-3168 can promote hepatocellular carcinoma progression by downregulating p53[12]. It can also be used as a potential predictor of nephrotoxicity in cisplatin-induced head and neck cancer patients[13]. and significantly upregulated in serum exosomes in patients with new coronary pneumonia[14]. However, the function and mechanism of miR-3168 in gastric cancer have not been reported. This study mainly explores the effect of miR-3168 on the malignant progression of gastric cancer cells and cisplatin resistance, and verifies the molecular mechanism of targeting TP53. To further understand the molecular mechanism of miR-3168 on gastric cancer progression.

2. Materials and methods

2.1 Cell culture

Human gastric cancer cell line AGS and human gastric cancer cisplatin-resistant cell line AGS/DDP were purchased from Wuhan Prosa Life Science and Technology Co., Ltd. Cells are cultured in Ham's F-12 medium with the addition of 10% fetal bovine serum and 1% biclonal antibody. Place in a 5% CO_2 cell culture incubator at 37 ℃ with fluid change 3 times a week.

2.2 Vector synthesis and transfection

miR-3168 mimic, miR-3168 inhibitor and negative control (NC) were synthesized by Huzhou Hippo Biotechnology Co., Ltd. using Lipo6000™ transfection reagent (Biyuntian, C0526) according to the reagent instructions, cells are transfected at 70% confluency, and transfected cells are harvested 48 h after transfection for subsequent experiments.

2.3 RNA extraction with q-PCR detection

RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Shanghai, China). Perform real-time RT-PCR analysis of miR-3168 using RT Reagent Kit (Chengdu Fuji Biotechnology Co., Ltd., catalog number: RT-02011) according to the kit instructions, 3 per sample duplicates. Briefly, 500 ng of total RNA was reverse-transcribed into complementary DNA and used using SYBR Green Master Mix (Vazyme, MQ101-01) was quantified on qPCR instruments (Model 7900HT) RT-PCR. Expression of *miR-3168* in each group was determined relative to U6, or *TP53* in each group was determined relative to *GAPDH* and calculate the fold change by relative quantification ($2^{-\triangle\triangle Ct}$). The primers were synthesized by Shanghai Shenggong Biotechnology Co., Ltd., and the sequences are shown in Table 1.

Tab 1

qPCR primer sequence

Primer name	Primer sequences (5' 3')
TP53-F	TGTGACTTGCACGTACTCCC
TP53-R	ACCATCGCTATCTGAGCAGC
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
miR-3168-F	GCCGAGGAGTTCTACAG
miR-3168-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTCTGA
GAPDH-F	GCTGTAGCCAAATCGTTGT
GAPDH-R	CCAGGTGGTCTCCTCTGA

2.4 CCK8 experiment to detect cell viability

The cell count Kit-8 (Beijing Quanzhijin Biotechnology Co., Ltd., catalog number: FC101-03) assay is used to determine cell viability. Cells are seeded in 96-well plates at a density of 2 000 per well and transfected for 48 h as described above. After 1, 2, 3, 4, and 5 days of incubation, CCK-8 is added to each well and then incubated 2 hours. The absorbance at a wavelength of 450 nm was then determined by spectrophotometer (Shimadzu, Japan, model: UV-1900) with 3 biological replicates per set of experiments.

2.5 Flow cytometry for apoptosis

According to the Annexin V-FITC/PI kit (Biolegend, 640914) instructions, apoptosis was detected by flow cytometry. For staining, add 5 μL Annexin V-FITC, 5 μL PI, and 400 μL 1× to the transfected cell sample. Binding buffer and incubate for 15 min in the dark at room temperature. Samples are analyzed by flow cytometry (BD Corporation USA, FACSAria) within 1 h. Perform 3 biological replicates per set of experiments.

2.6 Transwell experiment to detect cell invasion

Transwell analysis is used for invasion analysis. For the invasion assay, migrate the lower chamber of the chamber (Coring, 3422) with 100 μL of Matrigel (Coring, 354248) 30 before adding medium to the chamber. Transfected cells (1×10^6 cells/mL) are resuspended in the medium. The upper chamber holds 100 μL of cells containing 5% serum medium and 600 μL of complete medium added to the bottom chamber. After 24 h incubation at 37 ℃ with 5% CO_2, fix the cells with 4% paraformaldehyde (Biyuntian, P0099). 0.1% crystal violet (Biyuntian, C0121) solution staining. Invading cells are photographed and counted in five randomly selected regions by inverted fluorescence microscopy (Nikon, DS-Fi3).

2.7 Diluciferase experiment

The binding site was predicted by the Targetscan database (http://www.targetscan.org/), and the TP53 wild type (WT) and mutant type (Mut) were designed according to the binding site sequence and construct the corresponding double luciferase plasmid vector, seed AGS cells in 96-well plates, and then use Lipo6000™ transfection reagent (Biyuntian, C0526) at 70% confluency of cells with TP53 wild type (WT) and mutant type (MUT) plasmid and miR-3168 mimic or miR-NC co-transfection after 48 h incubation, according to the kit's instructions (Promega, E1910) measures luciferase activity. Each set of experiments is repeated three times.

2.8 Western blot detects protein expression

Transfect cells as described above and lyse on ice for 30 min. After 15 min of centrifugation, protein concentration is determined by BCA method (Biyuntian, P0012) and then separated by 10% SDS-PAGE (Biyuntian, P0012A). Subsequently, the protein is electrically transferred to the nitrocellulose membrane. Combine the membrane with TP53 antibody (1:1 000; proteintech; 60283-2-Ig) and GAPDH (1:5 000; proteintech; 60004-1-Ig), incubate overnight at 4 ℃. and secondary antibodies conjugated to horseradish peroxidase at room temperature (1: 5 000; KPL; 074-1807) incubation for 1 h, ECL (Biyuntian, P0018S) after development exposure, save the picture after exposure and export the picture, using image analysis software Image J (National Institutes of Health, version 1.8.0) to perform grayscale analysis on the image.

2.9 Statistical analysis

Data are represented as mean ± standard deviation, using Prism 8.0 software, The difference between groups was analyzed by the after the t-test, and $P<0.05$ was regarded as statistically significant the difference between the two groups.

3. Results

3.1 miR-3168 was significantly expressed in gastric cancer cisplatin-resistant cells AGS/DDP cells

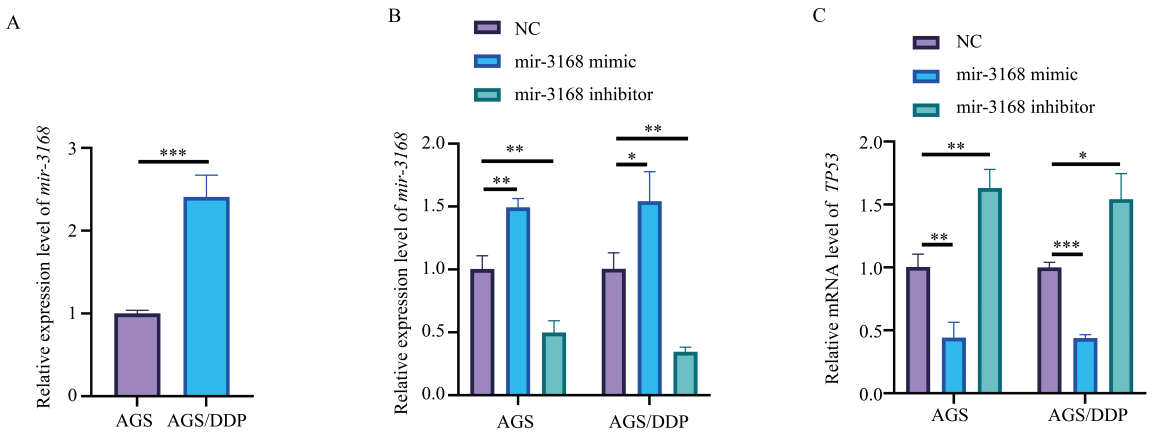
qPCR was used to detect the expression of *miR-3168* in gastric cancer cisplatin-sensitive cells A GS and gastric cancer cisplatin-resistant cells AGS/DDP, and the results showed that it was relative to AGS Cells (1.00±0.04), relative expression of *miR-3168* in AGS/DDP cells (2.41 ±0.26) was significantly improved (see Figure 1A, t=9.21, $P<0.001$).

3.2 miR-3168 mimic promotes miR-3168 expression and inhibits TP53 mRNA expression

mir-3168 mimic, miR-3168 inhibitor and negative control (NC) were used transfection of AGS or AGS/DDP cells with detection of *miR-3168* by qPCR With TP53 mRNA expression, the results showed that AGS cells, relative to the NC group (1.00±0.00), miR-3168 expression in the mimic group (1.49±0.00) was significantly adjusted upwards (Figure 1B, t=6.70, $P<0.01$), *TP53* mRNA expression (0.44±0.00) was significantly downregulated (Figure 1C, t=6.12, $P<0.01$); mir-3168 expression (0.50±0.00) in the *miR-316* inhibitor group was significantly downregulated (Figure 1B, t=6.18, $P<0.01$), *TP53* mRNA expression (1.63±0.00) was significantly adjusted upwards (Figure 1C, t=6.04, $P<0.01$); AGS/DDP cell, relative to the NC group (1.00±0.00). *miR-3168* expression (1.54±0.00) in the mimic group was significantly upregulated (Figure 1B, t=3.49, $P<0.05$), *TP53* mRNA expression (0.44±0.00) was significantly downregulated (Figure 1C, t=20.17, $P<0.001$); *mir-3168* expression (0.35±0.00) in the miR-316 inhibitor group was significantly downregulated (Figure 1B, t=8.69, $P<0.01$), TP53 mRNA expression (1.54±0.00) was significantly adjusted upwards (Figure 1C, t=4.54, $P<0.05$), consistent with AGS cells. The results showed that the miR-3168 mimic and inhibitor were successfully transfected, and the *miR-3168* mimic could promote the expression of miR-3168 and inhibit *TP53* mRNA expression.

3.3 miR-3168 promotes cisplatin resistance and proliferation in A GS and AGS/DDP gastric cancer cells

The results of CCK8 after transfection with cisplatin or control treatment showed that in AGS cells, relative to the NC control group [(5.67±0.00)%], miR-3168 mimic group at 40μg/mL cisplatin The relative cell viability [(19.67±0.00)%] was significantly increased (t=3.90, $P<0.05$), suggesting a significant increase in cisplatin resistance. In AGS/DDP, relative to the NC control group [(29.67±0.00)%], miR-3168 relative cell viability [(56.00±0.00)%] in the mimic group under the action of cisplatin at 40 μg/mL cisplatin significantly increased, indicating a significant increase in cisplatin resistance (Figure 2A, t=4.91, $P<0.01$).



A: *miR–3168* was detected in the cells themselves; B: *miR–3168* was detected after transfection; C: *TP53* detection after transfection. $^{**}P<0.01$, $^{***}P<0.001$.

Fig 1 Expression of *mir–3168* and *TP53* in AGS and AGS/DDP cells detected by qPCR

The results of CCK8 without cisplatin treatment after transfection showed that in AGS cells, relative to the NC control group (1.91±0.00), The OD_{450} value of miR-3168 mimic group (2.72±0.00) increased significantly (t=11.74, P<0.001). It was suggested that miR-3168 mimic promoted the activity of AGS cells; The OD_{450} value (1.28±0.00) of miR-3168 inhibitor group was significantly reduced (t=29.84. P<0.001), suggesting that miR-3168 inhibitor significantly inhibits AGS cells activity. In AGS/DDP cells, relative to the NC control group (1.99±0.00), miR-3168 mimic group OD_{450} values (2.66±0.00) increased significantly (t=10.45, P<0.001), prompt miR-3168 mimic significantly promoted AGS/DDP cell activity, miR-316 inhibitor group the value of OD450 (1.62±0.00) decreased significantly (t=6.80, P<0.001), indicating that the value was significantly reduced miR-316 inhibitor inhibits A-GS/DDP cell activity, consistent with AGS cells, as shown in Figure 2B.

The results of apoptosis after transfection showed that in AGS cells, relative to the NC control group (10.81% ± 0.21%) The apoptosis rate (6.32% ± 0.49%) was significantly reduced in the miR-3168 mimic group (t=14.56, P<0.001), suggesting that miR-3168 mimic inhibits AGS apoptosis, the apoptosis rate (6.32% ±0.49%) in the miR-316 inhibitor group was significantly reduced (t=30.96, P<0.001), prompts that miR-316 inhibitor promotes AGS apoptosis; In AGS/DDP cells, miR-3168 mimic relative to NC control (10.69±0.11). The apoptosis rate (5.53±0.29) was significantly reduced (t=28.51, P<0.001). Suggesting that miR-3168 mimic inhibits apoptosis in AGS/DDP cells, miR-316 inhibitor group The apoptosis rate (15.92±0.10) increased significantly (t=60.78, P<0.001). Suggest that miR-316 inhibitor promotes apoptosis in AGS/DDP cells, with AGS The cells are consistent, Figure 2C.

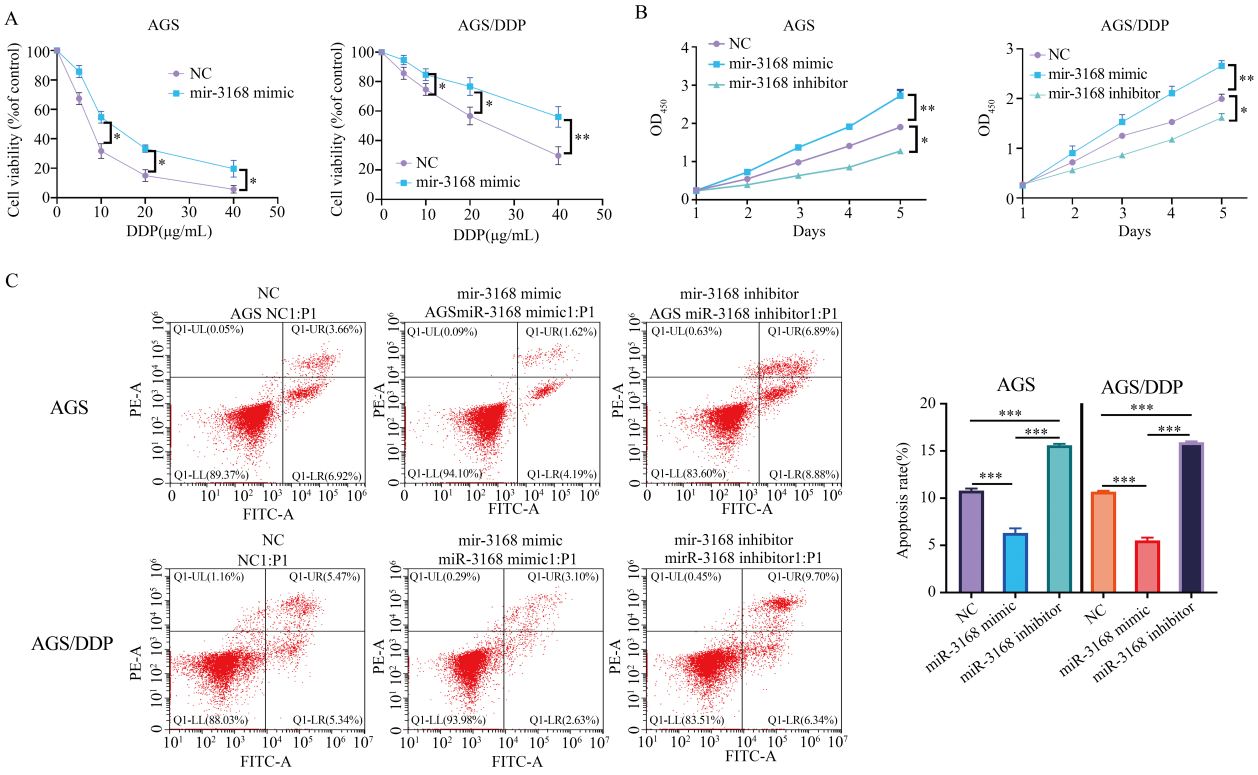
The above results showed that miR-3168 promoted cisplatin resistance and proliferation in A GS and AGS/DDP gastric cancer cells.

3.4 miR–3168 promotes the invasion of AGS and AGS/DDP gastric cancer cells

The results of Transwell invasion after transfection showed that in AGS cells, miR-3168 relative to the NC control group (309.0±0.00). The number of invasive cells (753.3±0.00) increased significantly in the mimic group (t=26.58, P<0.001), suggesting that miR-3168 mimic promotes AGS cell invasion, miR-316 inhibitor group. The number of invading cells (65.33±0.00) decreased significantly (t=19.20, P<0.001), suggesting miR-316 inhibitor significantly inhibits AGS cell invasion. In AGS/DDP cells, miR-3168 mimic relative to NC control (163.3±0.00). The number of invading cells in the group (506.0±0.00) increased significantly (t=28.25, P<0.001), prompting miR-3168 mimic promotes AGS/DDP cell invasion. The number of invading cells (50.33±0.00) in the miR-316 inhibitor group was significantly reduced (Figure 3, t=12.80, P<0.001), suggesting that miR-316 inhibitor inhibits AGS/DDP cells invade, consistent with AGS cells. It was shown that miR-3168 promoted the invasion of AGS and AGS/DDP gastric cancer cells.

3.5 miR-3168 targets binding TP53

Targetscan database predictions show that miR-3168 has a binding site with the 3' UTR end of TP53 Figure 4A). The results of the diluciferase experiment showed that the relative T P53 wild-type and miRNA-negative control co-transfected group (TP53-WT+miR-NC) relative luciferase activity (1.00±0.00), TP53 wild type with miR-3168 Cotransfection set (TP53-WT+miR-3168) was significantly reduced relative to luciferase activity (0.31±0.00) (t=33.27, P<0.001). The relative TP53 mutant and miRNA-negative control co-transfection group (TP53-MUT+mi R-NC) relative luciferase activity (1.00±0.00), TP53 mutant and miR-3168 co-transfection group (TP53-MUT+miR-3168) was not significantly different relative to luciferase activity (0.91±0.00). (Figure 4B, t=2.486, P >0.05). It was shown that miR-3168 targets binding to the TP53 3'UTR end and binds through the predicted target.



A: CCK8 detection after transfection with cisplatin; B: CCK8 test after transfer; C: Apoptosis detection. *P<0.05; **P<0.01, ***P<0.001.

Fig 2 mir-3168 promotes cisplatin resistance and proliferation of AGS and AGS/DDP gastric cancer cells

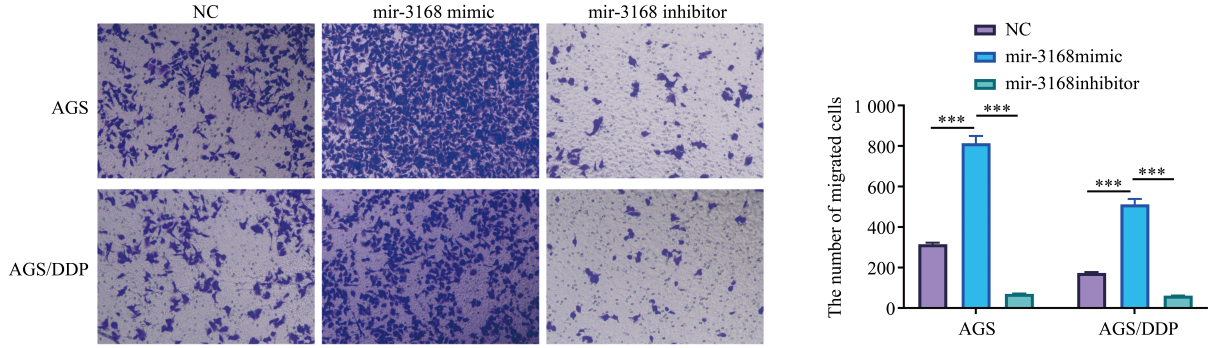
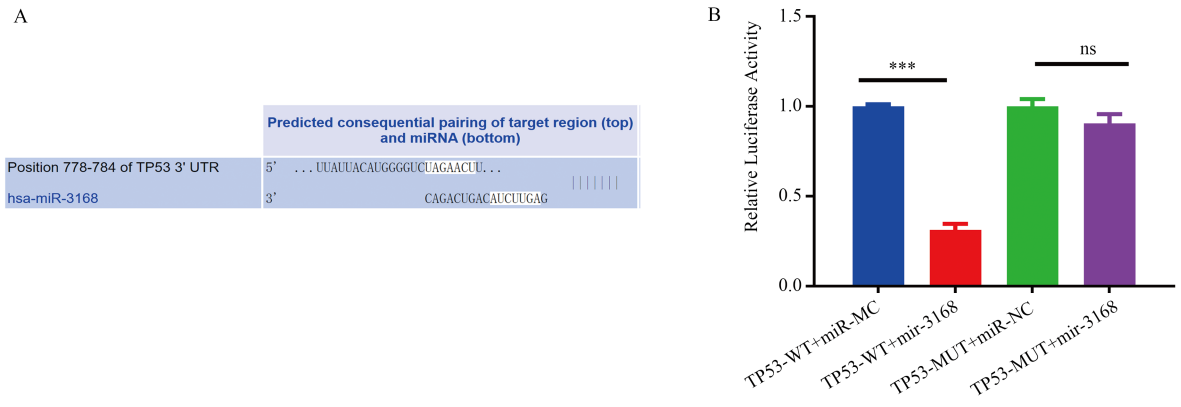


Fig 3 mir-3168 promotes invasion of AGS and AGS/DDP gastric cancer cell. ***P<0.001.



A: Targetscan database prediction; B: Double luciferase experiment. ***P<0.001.

Fig 4 mir-3168 targeted binding TP53

4. Discussion

As one of the most common digestive tract tumors, gastric cancer seriously threatens the healthy life of people all over the world[15]. Gastric cancer has an insidious onset and is usually advanced at the time of clinical consultation[16]. In recent years, the important role of miRNAs in gastric cancer has been widely studied, and the analysis of miRNAs appears to be superior to mRNA in terms of gastric cancer prognosis analysis[17]. The current report presents prognostic features for gastric cancer, including four risk miRNAs (miR-10b, miR-21, miR-223, and miR-338). ;Risk ratio >1) and three protective miRNAs (miR-30a-5p, miR-126, and let-7a; Risk ratio < 1) and clinically associated with clinical pathology[18]. Another study showed that low expression of miR-21 and miR-181b correlated with overall survival in patients treated with S-1 and doxyfluuridine[19]. The low expression of miR-125a-3p is associated with tumor size, invasion, metastasis and advanced clinical stage, and is an independent prognostic marker for gastric cancer[20]. This study showed that miR-3168 promoted the proliferation and invasion of AGS and AGS/DDP gastric cancer cells, and inhibited AGS and AGS/DDP apoptosis of gastric cancer cells suggests that miR-3168 may be the key miRNA to promote the malignant transformation of gastric cancer, and may also be a key auxiliary diagnostic index and drug inhibitory target for gastric cancer.

Cisplatin (DDP) has been widely used clinically as an anticancer drug and exhibits broad-spectrum antitumor effects. However, its side effects, especially cisplatin resistance, seriously affect the clinical efficacy of cisplatin[21]. Therefore, it is necessary to identify the mechanism of cisplatin resistance and seek possible combination therapy to improve its efficacy in gastric cancer. Studies have shown that secretion of exosomes containing miR-21 by tumor-associated macrophages promotes cisplatin resistance in gastric cancer cells[22]. miR-149 can reverse cisplatin resistance in gastric cancer SGC7901/DDP cells by targeting FoxM1[23]. miR-101 can alleviate incidental resistance in gastric cancer cells by targeting ANXA2[24]. miR-21 can modulate cisplatin resistance in gastric cancer cells by inhibiting autophagy through the PI3K/Akt/mTOR pathway[11]. This study showed that miR-3168 promotes cisplatin resistance in A GS and AGS/DDP gastric cancer cells by transfecting miR-3168 The results of inhibitor suggest that by inhibiting the expression of miR-3168, it can reverse the cisplatin resistance of AGS and AGS/DDP gastric cancer cells, which may become the treatment of gastric cancer cisplatin, one of the potential ways to reverse after the emergence of resistance.

TP53, also known as p53, is one of the most common tumor suppressor genes and is frequently mutated in human cancers[25]. Studies have shown that TP53 mutation is a key event in the early stage of gastric cancer and promotes the development of gastric cancer[26]. TP53 mutations and MET amplification of circulating tumor DNA also predict poor prognosis in patients with advanced gastric cancer[27]. Boswellic acid can sensitize gastric cancer cells to cisplatin-induced apoptosis through the p53-mediated pathway[28]. Exosome-delivered miR-769-5p promotes cisplatin resistance in gastric cancer by targeting CASP9 and promoting ubiquitination degradation of p53[29]. The experimental results of this study showed that miR-3168 targeted binding to the TP53 3'UTR terminal binding site and inhibited the expression of TP53 mRNA and protein. This may be a key mechanism by which miR-3168 promotes cisplatin resistance in AGS and AGS/DDP gastric cancer cells.

Of course, there are some shortcomings in this study, this study did not detect the expression of miR-3168 in clinical samples of gastric cancer, and analyzed the clinical significance of miR-3168 in gastric cancer; Secondly, this study mainly carried out cell experiments, and did not carry out experimental discussions at the *in vivo* level of animals. Finally, after miR-3168 targets binding to TP53, no further experimental verification was carried out on the downstream pathway of p53. These will be explored further in our follow-up research.

In conclusion, our study shows that miR-3168 targeted inhibition of TP53 promotes malignant transformation of AGS and AGS/DDP gastric cancer cells and cisplatin resistance. This may be one of the key mechanisms of gastric cancer progression and cisplatin resistance, which provides a theoretical basis for further understanding the mechanism of gastric cancer development and reversing cisplatin resistance.

Authors′ contributions

Wujun Wei: Cell detection and writing; Chunfang Wang: experimental guidance; Qi Jiang: material procurement and accounting; Guidan Xu: carrier construction; Jingjing Huang: Cell culture and transfection, Cheng Lin: CCK8 detection, Rentong Hu: Diluciferase experiment; Zhengyi Chang: Experimental Design and Work Organization.

Author's statement of conflict of interest

All authors have no conflict of interest and agree to submit this manuscript.

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