MicroRNA-25 contributes to cisplatin resistance in gastric cancer cells by inhibiting forkhead box O3a

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Abstract. Gastric cancer (GC) is a common type of malignancy worldwide, and chemotherapeutic resistance accounts for the majority of the failures in clinical treatment. MicroRNAs (miRs) are a class of small non-coding RNAs, which serve essential roles in GC. The present study aimed to investigate the potential role of miR-25 in the cisplatin sensitivity of GC cells. The expression level of miR-25 was significantly upregulated in the cisplatin-resistant GC cell line SGC-7901/DDP compared with the SGC-7901 parental cell line. Overexpression of miR-25 significantly enhanced cell cycle progression and decreased the sensitivity of SGC-7901 cells to cisplatin, whereas inhibition of miR-25 in the SGC-7901/DDP cisplatin-resistant cells resulted in cell cycle arrest at the G_0/G_1 phase and significantly increased drug sensitivity. Furthermore, the tumor suppressor forkhead box O3a (FOXO3a) was identified as a direct target gene of miR-25 by luciferase assay and western blot analysis, and was shown to mediate the drug-resistance phenotype of GC cells. These findings suggest that upregulation of miR-25 is important for GC cells to establish a cisplatin-resistant phenotype via a FOXO3a-dependent mechanism. Therefore, targeting miR-25 may be a promising therapeutic approach to treat patients with cisplatin-resistant GC.

Introduction

Gastric cancer (GC) is a common type of malignancy worldwide (1). Although there have been significant improvements in the clinical management of GC, chemotherapy remains one of the most important therapeutic strategies for advanced GC. However, due to the heterogeneity in the etiology and genetic basis of GC, the efficacy of chemotherapeutic drugs varies among the different subtypes of patients. A substantial proportion of patients eventually develop low chemoresponsiveness to chemotherapeutic drugs, including cisplatin, and this is one of the main reasons for GC-associated mortality (2).

Numerous mechanisms have been proposed to explain the phenomenon of drug resistance in cancer cells. For example, the enhanced expression of multidrug resistance protein 1 (P-glycoprotein) facilitates drug efflux from cancer cells (3), and alterations of cell cycle, autophagy and apoptosis regulators may also serve critical roles in cellular responsiveness to anticancer drugs (4,5). In recent decades, research into microRNAs has greatly expanded our understanding of chemotherapy resistance (6). MicroRNAs are single-stranded, non-coding RNAs that negatively regulate gene expression by binding to the 3'-untranslated region (UTR) of a specific mRNA. A number of microRNAs, which are implicated in the processes of DNA damage and repair, apoptosis regulation, epigenetic regulation and cell cycle regulation, have been revealed to produce diverse effects on the response of cells to chemotherapeutic drugs (6). Despite earlier studies demonstrating the oncogenic role of miR-25 in GCs (7-10), the exact role of microRNA-25 (miR-25) in cisplatin-resistant GC cells has not yet been well investigated.

The present study demonstrated that miR-25 was highly expressed in SGC-7901/DDP cisplatin-resistant GC cells compared with in the parental cell line, SGC-7901. Overexpression of miR-25 in the parental cell line led to decreased cisplatin sensitivity, whereas inhibition of miR-25 in SGC-7901/DDP cells partially decreased the cisplatin resistance. Subsequently, the tumor-suppressive transcriptional factor forkhead box O3a (FOXO3a), which controls a number of genes involved in cell cycle regulation, was established as a direct target of miR-25. Therefore, to the best of our knowledge, the present study revealed for the first time that miR-25 is a major contributor to the cisplatin resistance of GC cells.

Materials and methods

Cell lines, transfection and drug treatment. The human GC cell line SGC-7901 and the cisplatin-resistant variant SGC-7901/DDP were obtained from Nanjing KeyGen

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Biotech Co., Ltd. (Nanjing, China). Cells were cultured in Gibco RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2, and cells were passaged every other day. To maintain the cisplatin resistance of SGC-7901/DDP cells, 1 μg/ml cisplatin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to the culture medium. The cisplatin was dissolved in PBS and applied to cells at a final concentration of 0.01, 0.1, 1, 10 or 100 μg/ml for 48 h. The mimics for miR-25 (cat no. miR10000081-1-5) and its inhibitor strand (cat no. miR20000081-1-5) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Negative controls were also provided by Guangzhou RiboBio Co., Ltd. Cells were transfected using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The concentration of mimics/inhibitors used for transfection was 100 nM, and the transfection time was 48 h at 37˚C. The small interfering RNA (siRNA) against human FOXO3a (si-FOXO3a; 5'-ACUCGGGUCCAGCUCCAC-3') was also purchased from Guangzhou RiboBio Co., Ltd. The negative control was also provided by the manufacturer (cat no. siN05815122147-1-5), and the transfection protocol was identical to that of the microRNA mimic transfection.

Drug sensitivity assay (MTT assay). An MTT assay was used to detect the proportion of surviving cells following cisplatin treatment. Cells were equally seeded at 1.5x10^5/ml at 37˚C, grown in 96-well plates, and transfected with the miR-25 mimic, miR-25 inhibitor or si-FOXO3a. Subsequently, cells were treated with cisplatin at the indicated concentrations, as described above, 48 h after transfection. Cells were allowed to incubate for 48 h, and 20 μl MTT reagent (5 mg/ml; Sigma-Aldrich; Merck KGaA) was then added to each well, 4 h prior to the assay being performed. Cisplatin medium was replaced at this point. Following a 4-h incubation with the MTT reagent, the formazan in each well was dissolved in dimethyl sulfoxide, and the absorbance value at 490 nm was detected using a spectrophotometer.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from the cells was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA was purified by incubation with 75% ethanol at room temperature for 5 min. First-strand synthesis was performed with 1.5 μg RNA using the stem-loop primer kit Bulge-Loop™ miRNA qRT-PCR Starter kit (cat no. C10211-1) provided by Guangzhou RiboBio Co., Ltd., and PCR amplification of the cDNA was performed using the SYBR Premix Ex Taq II kit (Takara Biotechnology Co., Ltd., Dalian, China) and specific primer sets (cat no. miRQ0000081-1-1; Guangzhou RiboBio Co., Ltd.) for miR-25 and U6 (which was amplified as the internal control). The thermocycler conditions for the PCR reaction were as follows: 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 30 sec, for 35 cycles. The relative expression level of miR-25 was determined by the $2^{-\Delta\Delta Cq}$ method (11), and the experiments were repeated in triplicate.

Cell cycle analysis. Cell cycle distribution was analyzed by flow cytometry. Briefly, cells were fixed with 70% ethanol at -20˚C overnight. Following rehydration with 1.8 ml PBS on the following day, cells were treated with 100 μl 100 μg/ml RNase (cat no. RT405; Tiangen Biotech Co., Ltd., Beijing, China) for 30 min at 37˚C, and stained with 400 μl 50 μg/ml propidium iodide (cat no. st512; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at room temperature. Subsequently, cells were analyzed using a FACSort™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Luciferase activity assay. The putative binding site was searched with miRanda database (www.microrna.org). The wild type and mutant 3'UTR sequences of FOXO3 that contain the potential target of miR-25 were synthesized by Shanghai Shenggong Biology Engineering Technology Service, Ltd. (Shanghai, China). The predicted binding sequence in wild type 3'UTR (5'-GUGCAAU-3') was mutated to 5'-ACAUGGC-3'. The sequences were subcloned into a pMIR-REPORT miRNA Expression Reporter Vector system (Thermo Fisher Scientific, Inc.). The pMIR-REPORT-3'UTR constructs were transfected into HEK293 cells with miR-25 mimics and *Renilla* luciferase constructs (Promega Corporation, Madison, WI, USA) at 37˚C for 24 h with Lipofectamine® 2000 (Thermo Fisher Scientific., Inc.). At 24 h after transfection, luciferase activity was determined by a Dual-Luciferase Reporter Assay system (Promega Corporation), according to the manufacturer's protocol.

Western blotting. Cells grown in 6-well plates for 24 h at 37˚C were transfected and treated with cisplatin as aforementioned, and then were analyzed by western blotting. Total protein from the cells was collected using SDS lysis buffer supplemented with protease inhibitor (Beyotime Institute of Biotechnology, Haimen, China). Appropriate quantities of cell lysates were denatured by heating in sample buffer (Beyotime Institute of Biotechnology) at 100˚C for 3 min, and then 50 μg protein for each sample was separated by 10% SDS-PAGE and blotted onto polyvinylidene membranes. Membranes were blocked with 5% skimmed milk for 1 h at room temperature, followed by an overnight incubation at 4˚C with primary antibodies against β-actin (cat no. sc-8432; 1:1,000) and cyclin-dependent kinase (CDK) inhibitor 1B (p27Kip1; cat no. sc-528; 1:300) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and FOXO3a (cat no. 12,829; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA). The samples were then incubated with the secondary antibodies [goat anti-rabbit horseradish peroxidase (HRP); cat no. sc-2004; 1:2,000; goat anti-mouse HRP; cat no. sc-2005; 1:2,000 (both from Santa Cruz Biotechnology, Inc.)] for 1 h at room temperature. Following a series of washes with PBST (0.5% Tween-20), protein bands were detected using the SuperSignal West Pico Chemiluminescent Substrate chemiluminescence visualization kit (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation. The comparisons were performed using Student's t-test. Two tailed $P<0.05$ was considered to indicate a statistically significant difference. All the experiments were performed ≥3 times.

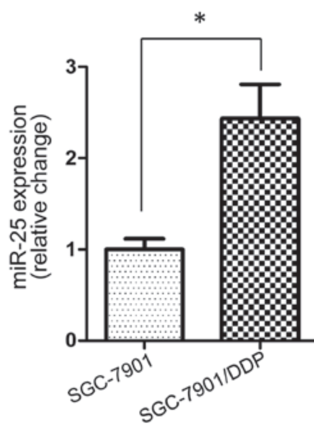

Figure 1. miR-25 is upregulated in the DDP-resistant GC cell line SGC-7901/DDP. The expression level of miR-25 in SGC-7901/DDP cells relative to its parental cells was determined by reverse transcription-quantitative polymerase chain reaction. *P<0.05, n=3. DDP, cisplatin; miR-25, microRNA-25.

Results

miR-25 expression is upregulated in the cisplatin-resistant SGC-7901/DDP cell line. To investigate the potential role of miR-25 in cisplatin resistance in GC cells, the present study first examined the expression levels of miR-25 in the SGC-7901 cell line and its cisplatin-resistant variant SGC-7901/DDP. RT-qPCR demonstrated that miR-25 had a significantly higher expression level in SGC-7901/DDP cells compared with in the parental SGC-7901 cell line (P<0.05; Fig. 1).

Overexpression of miR-25 decreases the sensitivity of SGC-7901 cells to cisplatin. The observed upregulation of miR-25 expression in SGC-7901/DDP cells prompted the hypothesis that miR-25 may serve an important role in the development of cisplatin resistance, and this was investigated by modulating miR-25 expression levels in SGC-7901 cells by transfection with miR-25 mimics. The transfection efficacy was verified by RT-qPCR, which demonstrated significant upregulation of miR-25 in the mimic-transfected cells (P<0.05; Fig. 2A). Subsequently, flow cytometric analysis revealed fewer cells in the G_0/G_1 cell cycle phase in mimic-transfected compared with negative control cells, indicating enhanced cell cycle progression concomitant with increased miR-25 levels (Fig. 2B). Furthermore, an MTT assay revealed that transfection with miR-25 mimics resulted in significantly decreased sensitivity of SGC-7901 cells to cisplatin at doses of 1-10 μg/ml (P<0.05; Fig. 2C).

Inhibition of miR-25 reverses the cisplatin resistance of SGC-7901/DDP cells. miR-25 expression was significantly inhibited in SGC-7901/DDP cells via transfection with an miR-25 inhibitor (P<0.05; Fig. 3A). In contrast to miR-25 overexpression, the inhibition of miR-25 resulted in cell cycle arrest, with a greater proportion of cells in the G_0/G_1 phase in inhibitor-transfected cells than in the negative control group (Fig. 3B). Analysis of cisplatin sensitivity by MTT assay revealed that cells transfected with miR-25 inhibitor exhibited significantly decreased cell viability following treatment with cisplatin at doses of 0.1-10 μg/ml (Fig. 3C), which suggested that miR-25 inhibition may reverse the cisplatin resistance of SGC-7901/DDP cells.

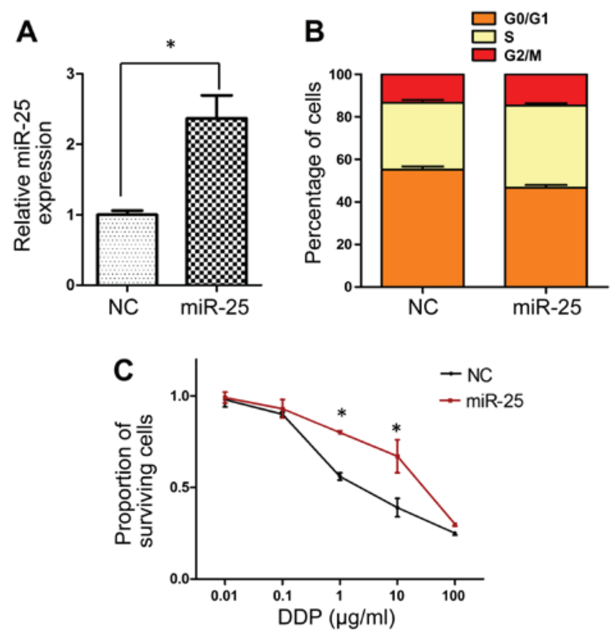

Figure 2. Overexpression of miR-25 in SGC-7901 cells decreases their sensitivity to DDP. Cells were transfected with miR-25 mimics (miR-25 group) prior to analysis. (A) Relative expression of miR-25 was increased following miR-25 mimic transfection. (B) Cell cycle distribution was determined by flow cytometric analysis. (C) The relative cell viability was determined by MTT assay following treatment of the cells with various doses of DDP (0.01, 0.1, 1, 10, 100 μg/ml). *P<0.05 vs. NC, n=3 replicates per group. miR-25, microRNA-25; DDP, cisplatin; NC, negative control.

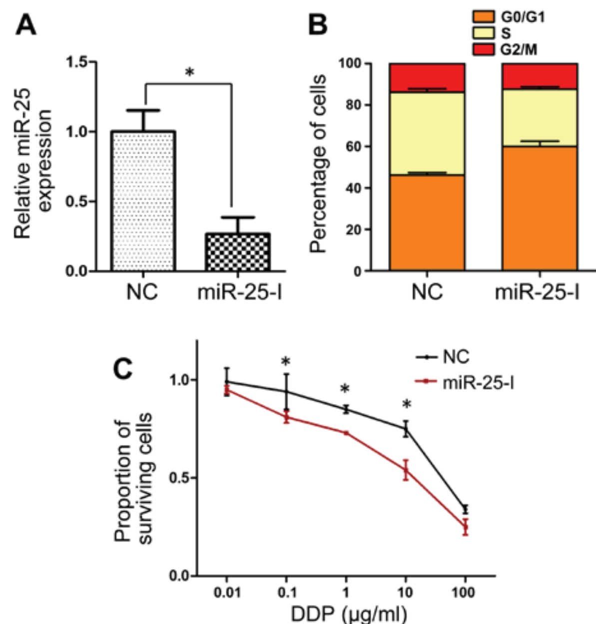

Figure 3. Inhibition of miR-25 in SGC-7901/DDP cells reverses their resistance to DDP. Cells were transfected with miR-25 inhibitors (miR-25-I group) prior to analysis. (A) Relative expression of miR-25 was decreased following miR-25-I transfection. (B) Cell cycle distribution was determined by flow cytometric analysis. (C) Relative cell viability was determined by MTT assay following treatment of the cells with various doses of DDP (0.01, 0.1, 1, 10, 100 μg/ml) following transfection. *P<0.05 vs. NC, n=3 replicates per group. miR, microRNA; DDP, cisplatin; miR-25-I, microRNA-25-inhibitor; NC, negative control.

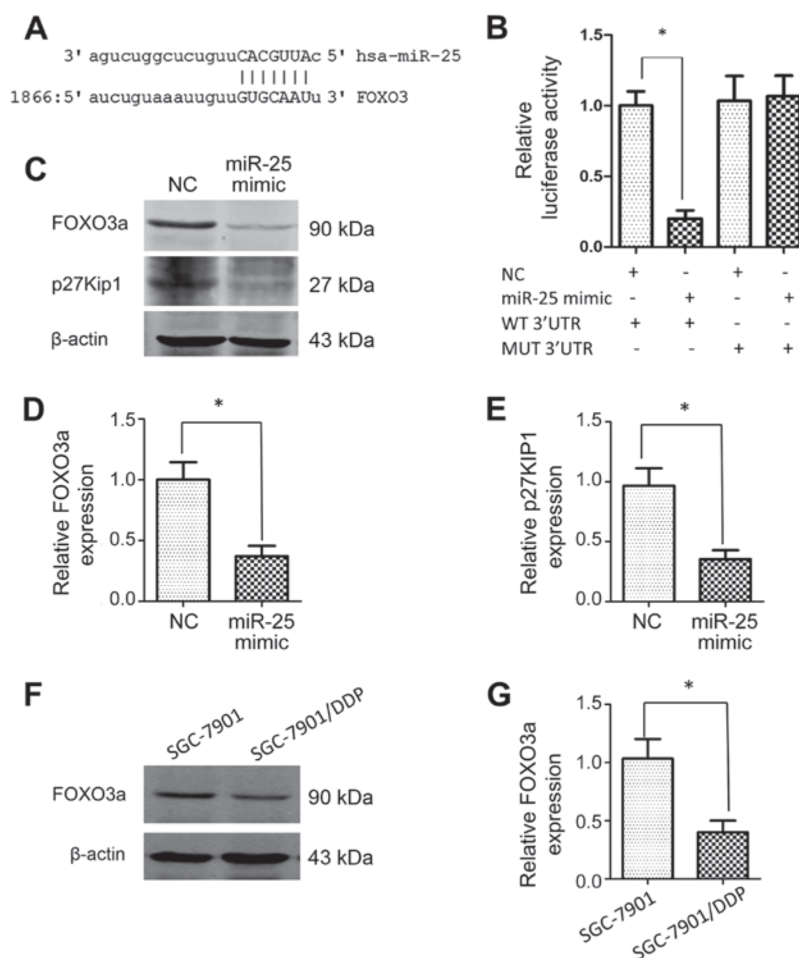

Figure 4. FOXO3a is a target of miR-25. (A) The predicted binding site, as determined using the miRanda database, is shown. (B) The relative luciferase activity levels of the reporters containing WT or MUT 3'UTR sequences of FOXO3a were measured following miR-25 transfection in HEK293 cells. (C) Western blotting was used to assess the effect of miR-25 mimics on the expression levels of FOXO3a and p27Kip1 in SGC-7901 cells. (D and E) Graphs showing the quantification of the integrated protein band densities following western blotting (as shown in C). (F) Western blotting was used to assess the expression level of FOXO3a in the SGC-7901 cell line and its DDP-resistant variant, SGC-7901/DDP. (G) Graphs showing the quantification of the integrated protein band densities following western blot analysis of FOXO3a (as shown in F). β-actin was used as the loading control for all western blot analyses. *P<0.05, n=3 in each group. FOXO3a, forkhead box O3a; miR-25, microRNA-25; WT, wild-type; MUT, mutant; 3'UTR, 3' untranslated region; NC, negative control; p27Kip1, cyclin-dependent kinase inhibitor 1B.

FOXO3a serves as a target of miR-25. Since microRNAs function to block gene expression by inexact base-pair matching with the 3'UTR of the target mRNA, an online database search was performed in the present study to investigate the mechanisms underlying the effects of miR-25. A possible interaction between miR-25 and the tumor suppressive gene FOXO3 was identified (Fig. 4A). Subsequently, a luciferase activity assay revealed that transfection of HEK293 cells with miR-25 mimics decreased the luciferase activity of the reporter containing the wild-type 3'UTR of FOXO3a (Fig. 4B); however, the luciferase activity of the reporter carrying the mutant 3'UTR was not affected by miR-25 mimics, which suggested that this interaction is specific. In addition, western blotting demonstrated that the overexpression of miR-25 in SGC-7901 cells led to decreased protein levels of FOXO3a and, consistently, downregulation of p27Kip1, the transcriptional target of FOXO3a (Fig. 4C-E). Furthermore, FOXO3a protein level was significantly decreased in SGC-7901/DDP cells compared with SGC-7901 cells (Fig. 4F and G). Collectively, these results indicated that FOXO3a is a direct target of miR-25 in GC cells.

Knockdown of FOXO3a decreases the cisplatin sensitivity of SGC-7901 cells. Following the siRNA-mediated knockdown of FOXO3a, western blot analysis confirmed that si-FOXO3a was sufficient to inhibit FOXO3a expression in SGC-7901 cells (Fig. 5A). Subsequently, an MTT assay was used to determine the sensitivity of SGC-7901 cells to cisplatin. As presented in Fig. 5B, cell viability was significantly higher in FOXO3a-knockdown cells compared with the negative control group following treatment with cisplatin at doses of 0.1-10 μg/ml, indicating decreased sensitivity. This result was similar to the data from the miR-25 mimic transfection, in that the overexpression of miR-25 and the knockdown of FOXO3a each decreased the sensitivity of GC cells to cisplatin.

Discussion

Cisplatin is one of the predominant first-line chemotherapeutic drugs used for the treatment of GC in clinical practice. Despite the high sensitivity of patients with GC to cisplatin at initial administration, a substantial number of patients develop drug resistance, which is one of the major causes of

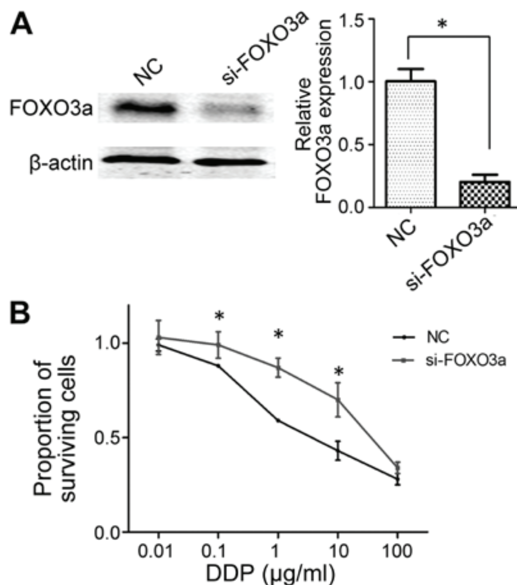

Figure 5. Knockdown of FOXO3a decreases the sensitivity of SGC-7901 cells to DDP. (A) The knockdown efficacy of si-FOXO3a was verified by western blot analysis and relative densitometric quantification of the protein bands (right panel). β-actin was used as the loading control. (B) An MTT assay was used to assess the relative viability of si-FOXO3a-transfected cells following treatment with various doses of DDP (0.01, 0.1, 1, 10, 100 μg/ml). *P<0.05 vs. NC, n=3 replicates per group. FOXO3a, forkhead box O3a; DDP, cisplatin; si-FOXO3a, small interfering RNA against FOXO3a; NC, negative control.

treatment failure and GC relapse (2). Therefore, solving the clinical problem of acquired drug resistance is of considerable importance for the successful treatment of recurrent GC. To date, the molecular mechanism of the drug resistance have not been fully elucidated. Previous studies established numerous molecular models for cisplatin resistance, including low efficiency in drug transportation, increased DNA damage repair response and suppression of cell cycle inhibitors and apoptosis signaling (4,5).

Epigenetic regulation is important in the pathogenesis and progression of GC, and recent evidence has indicated a central role of microRNAs in the regulation of drug resistance in GC (6). A number of studies have demonstrated that various microRNAs have altered expression profiles in GC, and that they are involved in GC carcinogenesis. For example, miR-532-5p targets runt-related transcription factor 3 in GC, thereby serving an oncogenic role (12); and miR-429, by targeting ZEB proteins, is able to regulate the invasiveness of GC cells (13). Additionally, a recent study by Zhao *et al* (13) revealed that miR-181a acts to sensitize cells to cisplatin treatment. In the present study, miR-25 was shown to be significantly upregulated in the established cisplatin-resistant GC cell line SGC-7901/DDP compared with the cisplatin-sensitive parental cell line SGC-7901. Overexpression of miR-25 in the parental GC cell line led to acquisition of the cisplatin-resistance phenotype, whereas inhibition of miR-25 in the cisplatin-resistant cell line resensitized cells to cisplatin and increased cisplatin-induced cell death. Previous studies have identified the oncogenic potential of miR-25 in GC: Gong *et al* (7), Zhao *et al* (9) and Li *et al* (10) revealed that miR-25 promoted proliferation and cell invasiveness by inhibiting various tumor suppressors. The results of the present study support the findings of these previous studies, and demonstrate the involvement of miR-25 in the cisplatin responsiveness of GC cells, thereby expanding current knowledge on the effects of miR-25.

In the present study, FOXO3a was identified as a novel functional target of miR-25. FOXO3a is a critical transcriptional factor in the processes of autophagy, cell cycle progression and apoptosis (14,15), and the functioning of FOXO3a is associated with GC. A series of genes critical for regulating cell survival are under the control of FOXO3a. For example, p27Kip1, which is a negative regulator of the cell cycle that inhibits cell cycle progression, is a well-documented transcriptional target of FOXO3a (16,17). p27Kip1 is able to prevent the activation of cyclin-CDK complexes (18,19), which are essential for mitotic cell cycle transition. The present study revealed that the expression of p27Kip1 was also decreased when miR-25 was overexpressed, which is consistent with the cell cycle data obtained from the flow cytometric analysis; inhibition of miR-25 in cisplatin-resistant GC cells induced a significant G_0/G_1 cell cycle arrest, and p27Kip1 may be involved in this process.

Previous clinical evidence has demonstrated that high expression levels of FOXO3a were commonly observed in less aggressive types of GC, and were associated with a good prognosis in patients with GC (20,21). These findings are corroborated by *in vitro* data from the present study, which indicated low FOXO3a expression levels in cells with the cisplatin-resistance phenotype. Notably, cell cycle regulation by FOXO3a may not be the only process that contributes to cisplatin resistance; FOXO3a regulates a network of genes that is crucial in numerous cellular processes, including apoptosis, cell cycle regulation and autophagy (14). The additive effects of these FOXO3a-regulated processes may also promote the drug-resistance phenotype. Thus, the present study revealed the essential role of FOXO3a in the cisplatin resistance of GC cells, and this conclusion is in accord with previous findings.

As multiple different genes may be targeted by a single microRNA, FOXO3a may not be the only target of miR-25, and it is possible that other mechanisms may underlie miR-25-induced cisplatin resistance. Recent studies demonstrated that a number of tumor suppressive genes, including F-box and WD repeat domain-containing 7, large tumor suppressor kinase 2, and transducer of ERBB2-1, can be targeted by miR-25 in GC (7,8,10). Thus, these proteins are possibly involved in the acquisition of cisplatin resistance. However, the findings of the present study reinforce the importance of miR-25 in the development and progression of GC.

In conclusion, the present findings demonstrated that miR-25 is upregulated in cisplatin-resistant GC cells, and represses FOXO3a expression to promote cell cycle progression. The inhibition of miR-25 resulted in cell cycle arrest and enhanced chemotherapeutic sensitivity of GC cells to cisplatin, which may be a novel approach to reversing drug resistance in clinical practice.

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