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Effect of circ MTHFD2 on resistance to pemetrexed in gastric cancer through regulating expression of miR-124

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Abstract. – OBJECTIVE: The aim of this study was to explore the effect of circ MTHFD2 on resistance to pemetrexed (MTA) in gastric cancer by regulating the expression of micro ribonucleic acid (miR)-124.

MATERIALS AND METHODS: Human gastric cancer MGC-803 cells were induced by MTA at an increasing concentration. MGC-803/MTA resistant cell model was successfully established after 5 months. The half-maximal inhibitory concentration (IC50) of MTA on the two kinds of cells was detected via cell counting kit-8 (CCK-8) assay. Differentially expressed circular RNAs (circRNAs) were screened using circRNA microarray analysis. Meanwhile, the target miRNAs of circRNAs were predicted using bioinformatics tool and verified via luciferase reporter assay, respectively. In MGC-803 cells, the effects of overexpression and knockdown of circ MTHFD2 on the expression of miR-124 were detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Finally, the effects of circ MTHFD2 on the protein expressions of FDZ5 and multidrug resistance gene-1 (MDR-1) were detected via Western blotting.

RESULTS: MGC-803/MTA resistant cell lines were successfully constructed via persistent drug exposure to MTA at an increasing concentration for 5 months. Compared with parental cells, MGC-803/MTA resistant cells showed significantly enhanced drug resistance. Subsequently, differentially expressed circRNAs were screened using circRNA microarray analysis. A total of 673 circRNAs were screened out based on Fold Change >3 and adjusted p-value. The results of qRT-PCR showed that the expression levels of all differentially expressed circRNAs were significantly changed when compared with MGC-803/MTA resistant cells. Compared with MGC-803/MTA resistant cells, the drug resistance of cells increased significantly in circ MTHFD2 overexpression group. However, it markedly decreased in circ MTHFD2 knockdown group. According to the results of bioinformatics and luciferase reporter assay, circ MTHFD2 could target bind to miR-124. In MGC-803/MTA cells, miR-124 could remarkably increase the resistance of MGC-803/MTA cells to MTA. Western blotting results revealed that overexpression of circ MTHFD2 significantly increased the protein expressions of FDZ5 and MDR-1. However, miR-124 mimics reversed the inhibitory effect of circ MTHFD2 on FDZ5 and MDR-1.

CONCLUSIONS: Circ MTHFD2 directly binds to miR-124 through the molecular sponge effect. This may induce increased protein expression of MDR-1, ultimately enhancing the drug resistance of MGC-803/MTA cells.

Key Words:
Gastric cancer, Circ MTHFD2, MiR-124, Drug resistance mechanism.

Abbreviations

IC50: half maximal inhibitory concentration; CCK-8: cell counting kit-8; circRNAs: circular ribonucleic acids; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; MDR-1: multidrug resistance gene-1; MTA: multitargeted antifolate; FBS: fetal bovine serum; RPMI-1640: Roswell Park Memorial Institute-1640; EP: Eppendorf; cDNA: complementary deoxyribose nucleic acid; RIPA: radioimmunoprecipitation assay; PMSF: phenylmethylsulfonyl fluoride; BCA: bicinchoninic acid; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PVDF: polyvinylidene difluoride; TBST: Tris Buffered Saline and Tween-20; ECL: enhanced chemiluminescence; SPSS: Statistical Product and Service Solutions.

Introduction

The fatality rate of gastric cancer ranks 5th in the world, making it an important public health problem[1]. The prognosis and survival rate of gas-

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tric cancer patients are highly dependent on the diagnostic time[2]. Most of gastric cancer patients have already been in the progressive stage when diagnosed. At that time, the survival rate of these patients is relatively low due to the poor efficacy of chemotherapy drugs[3]. Although a large number of new chemotherapy drugs have been applied in clinical practice, the resistance to chemotherapy drugs is still the main cause of chemotherapy failure[4]. Several reports[5-7] have shown that the resistance mechanism of tumor cells to chemotherapy drugs mainly includes: (1) enhanced drug efflux capacity, (2) reduced drug intake or increased drug metabolic capacity, (3) inhibited apoptosis due to the altered regulatory mechanism of the cell cycle, and (4) enhanced DNA repair capacity. Meanwhile, other mechanisms can also lead to the drug resistance of tumor cells, including gene mutation, chromosomal translocation, and epigenetic modification.

Circular ribonucleic acids (circRNAs) have long been regarded as an unexpected by-product. Recent studies have shown that they play important roles in a variety of biological processes. Currently, it has been demonstrated that circRNAs mainly possess three biological functions: (1) miRNA sponge, (2) regulator of RNA-binding proteins, and (3) encoding of polypeptides[8]. Therefore, the differential expression of circRNAs may affect the biological processes of various diseases, such as a malignant tumor. Due to the unique cyclic structure of circRNAs, they exhibit higher resistance to ribonuclease. Therefore, circRNAs are more stable in tissues, serum, saliva, and urine. With the development of high-throughput sequencing technique, increasingly more abnormally expressed circRNAs have been discovered in solid tumors[9]. Moreover, the number of circRNA molecules is far larger than that of miRNA molecules. Therefore, the chance of screening sensitive and specific biomarkers for tumor resistance from circRNAs is higher.

In the present work, differentially expressed circRNAs were compared between MGC-803 cells and MGC-803/MTA cells using the high-throughput screening technique. The results found that circ MTHFD2 (methylenetetrahydrofolate dehydrogenase/cyclohydrolase), as a mitochondrial precursor, might play an important role in the drug resistance of gastric cancer cells. Meanwhile, miRNAs regulated by circ MTHFD2 and its downstream mechanism were explored. Our findings might help to provide a valuable biomarker for pemetrexed (MTA) resistance and a new drug target for overcoming the drug resistance of gastric cancer.

Materials and Methods

Cell Culture and Construction of Drug-Resistant Cell Lines

Human gastric cancer MGC-803 cells were purchased from Shanghai SX Biotechnology Co., Ltd. (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone; South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C.

MGC-803 cells in the logarithmic growth phase were induced by MGC-803 (0.5 mg/L) to construct the MGC-803 resistant cell model. The culture medium was replaced every 72 h and the dead cells were removed. When the cells became stable, cell passage continued and the drug concentration increased by 20%. After repeated drug induction for 5 months, the cells could stably survive in the medium containing multitargeted antifolate (MTA) (5 mg/L), which were defined as MGC-803/MTA cell lines[10].

Detection of Resistance of MTA-803 Cells and MGC-803/MTA Cells to MTA Via Cell Counting Kit-8 (CCK-8) Assay

MTA-803 cells and MGC-803/MTA cells in the logarithmic growth phase were uniformly inoculated into 96-well plates (1×10^4/well). MTA with different diluted concentrations was added into the medium. 6 replicate wells were set in each concentration gradient. The cells were cultured in an incubator for 72 h. Subsequently, 20 μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) and 170 μL of culture medium were added, followed by incubation in the dark at 37°C for 2 h. Next, the cells were shaken on a micro-vibrator for 3 min. Absorbance at the wavelength of 450 nm was finally detected using a microplate reader. Half maximal inhibitory concentration (IC50) of MTA on MTA-803 cells and MGC-803/MTA cells was calculated as follows: resistance Index = IC50 of resistant cells/ IC50 of parental cells.

Screening of Differentially Expressed CircRNAs Via CircRNA Microarray Analysis

Total RNA was extracted from MTA-803 cells and MGC-803/MTA cells and quantified using

the NanoDrop kit (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of extracted RNA was evaluated using Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). 100 ng of total RNA was prepared into cRNA using the Affymetrix 3' IVT Express kit, followed by hybridization on an Affymetrix Primeview Human array at 45°C for 16 h according to the instructions of GeneChip 3' Array (Affymetrix, Santa Clara, CA, USA). The array was washed and stained in the Affymetrix FS-450 fluid station, followed by scanning on the Affymetrix GeneChip scanner according to the manufacturer's program. Next, the raw data of. CEL file were imported into Partek Genomics Suite 6.6 software. The probe set was normalized using the Robust Multiarray Average method. Finally, differentially expressed genes were determined using one-way analysis of variance (ANOVA) and the *p*-value was corrected using FDR[10].

Prediction of Target MiRNAs of CircRNAs

Target miRNAs of circRNAs were predicted using the bioinformatics websites: http://www.circbase.org/, http://circnet.mbc.nctu.edu.tw/, and http://www.targetscan.org/.

Luciferase Reporter Gene Assay

In this experiment, wild-type and mutant-type circ MTHFD2 were amplified and cloned into psi-CHECK™-2 luciferase plasmid (Promega, Madison, WI, USA) to generate wild-type and mutant-type reporter genes, respectively. The cells were first cultured in 24-well plates. Subsequently, miR-124 mimics or miR-con and wild-type or mutant-type plasmids were co-transfected into MGC-803 cells, respectively. Luciferase activity was determined at 48 h after transfection using Dual-Luciferase reporter reagent (Promega, Madison, WI, USA).

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

The gene expression level was detected *via* qRT-PCR. Tissue samples in the cryopreserved tube were taken, drained and ground with liquid nitrogen in 5 mL tubes. After complete homogenization using a tissue homogenizer, the liquid was transferred into a clean imported 1.5 mL Eppendorf (EP) tube. After incubation at room temperature for 5-10 min for complete lysis, they were centrifuged at 1,200 rpm for 5 min. Then, the precipitate was discarded. Chloroform and Trigol were added (200 μL of chloroform/mL Trigol) to prepare the chloroform/Trigol reagent, followed by shaking and mixing evenly. After incubation at room temperature for 15 min, they were centrifuged at 12,000 rpm and 4°C for 15 min. Next, the supernatant was aspirated into another centrifuge tube, and added with isopropanol (0.7-1-fold volume of the supernatant). Then, the tubes were placed at room temperature for 10-30 min and centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and RNAs were precipitated to the bottom of tube. 75% ethanol (1 mL of 75% ethanol/mL Trizol) was then added into the centrifuge tube, and gently shaken to suspend the precipitates, followed by centrifugation at 12,000 rpm and 4°C for 5 min. The supernatant was discarded as far as possible. The precipitates were blown dry on a super clean bench for 10-20 min and dissolved using 10-50 μL of diethyl pyrocarbonate-treated ddH_2O. The concentration of extracted RNA was detected using OneDrop micro-spectrophotometer. RT reaction was performed as follows, including 4.5 μL of RNase-free ddH_2O, 2 μL of 5×RT reaction buffer, 0.5 μL of random primers, 0.5 μL of Oligo dT, 0.5 μL of reverse transcriptase and 2 μL of RNAs. The cDNA samples were divided into three pieces (diluted at 1:20), and 3 μL of complementary deoxyribose nucleic acid (cDNA) was taken for PCR amplification. The amplification level of the target gene was verified *via* 5% agarose gel electrophoresis. LabWorks 4.0 image acquisition and analysis software was used for quantification and data processing. To obtain reliable data, this experiment was repeated for three times. Changes in the relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta Ct}$ method. Primers used in this study were synthesized by Shanghai Generay Biotechnology Co., Ltd. Primer sequences were shown in Table I.

Western Blotting

An appropriate amount of radioimmunoprecipitation assay (RIPA) lysis buffer was prepared, and protease inhibitor phenylmethylsulfonyl fluoride (PMSF; RIPA: PMSF = 100:1; Beyotime, Shanghai, China) was added and mixed evenly. The cells were digested with tyrisin, collected, and added with lysis buffer. Subsequently, the lysate was collected and transferred into an EP tube, followed by centrifugation at 14000 rpm and 4°C for 30 min. Next, protein supernatant was collected and subjected to a heating bath at 95°C for 10 min for protein denaturation. Extracted protein samples were placed in a refrigerator at –80°C for later

Table I. Primer sequences.

Name	Primer sequences (5'--3')
Circ MTHFD2	5'-TTCCACGAACGTGUCTCGCTT-3'
	5'-CAGCGACACGTTCGGAGAATT-3'
MiR-124	5'- TTCACAGCGGACCTTGA-3'
	5'-GAACATGTCTGCGTATCTC-3'
U6	5'- CGCTTCGGCAGCACATATACTA-3'
	5'- CGCTTCACGAATTTGCGTGTCA-3'

use. The concentration of extracted protein was quantified using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Next, protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under the constant pressure of 80 V for 2.5 h, and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) using a semi-dry transfer method. Then, the PVDF membranes were immersed in Tris Buffered Saline and Tween-20 (TBST) containing 5% skim milk powder and shaken slowly for 1 h on a shaking table. After incubation with primary antibodies diluted with 5% skim milk powder, the membranes were rinsed with TBST for 3 times (10 min/time). On the next day, the membranes were incubated with corresponding secondary antibody at room temperature for 2 h, followed by rinsing again with TBST twice, and TBS once (10 min/time). Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) reagent and exposed in a darkroom. The relative expression of protein was analyzed using Image-Pro Plus v6 (Media Cybernetics, Silver Spring, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as mean ± standard deviation. The Student's *t*-test was performed to compare the difference between two groups. $p<0.05$ was considered statistically significant.

Results

Gastric Cancer MGC-803/MTA Resistant Cell Lines

MGC-803/MTA resistant cell lines were successfully constructed *via* persistent drug exposure to MTA at an increasing concentration for 5 months. Compared with parental cells (MGC-803 cells), MGC-803/MTA resistant cells showed significantly enhanced drug resistance. This indicated that gastric cancer MGC-803/MTA resistant cell lines were successfully constructed. The proliferation inhibitory curves of MGC-803 and MGC-803/MTA cells by MTA were shown in Figure 1.

Differentially Expressed CircRNAs Screened Via CircRNA Microarray Analysis

To explore the important role of circRNAs in MGC-803/MTA resistant cell lines, differentially expressed circRNAs were screened *via* circRNA microarray analysis. A total of 673 circRNAs were screened out based on Fold Change >3 and adjusted *p*-value. Among 321 differentially expressed circRNAs, 173 circRNAs were significantly up-regulated, while 138 circRNAs were down-regulated. The difference in the expression of 44 circRNAs was greater than 6-fold, including 31 up-regulated circRNAs and 13 down-regulated circRNAs. Furthermore, the expression of circ MTHFD2 (hsa-circR0003936) exhibited the greatest difference (Figure 2).

Difference in CircRNA Expression in MGC-803 and MGC-803/MTA Cells Detected Via QRT-PCR

Previous works have found that circRNAs may play important roles in tumor resistance. To further verify the reliability of high-throughput screening results, 5 significantly up-regulated circRNAs and 5 down-regulated circRNAs were selected and verified *via* low-throughput qRT-PCR. The results revealed that compared with MGC-803 cells, the expression levels of 10 differentially expressed cir-

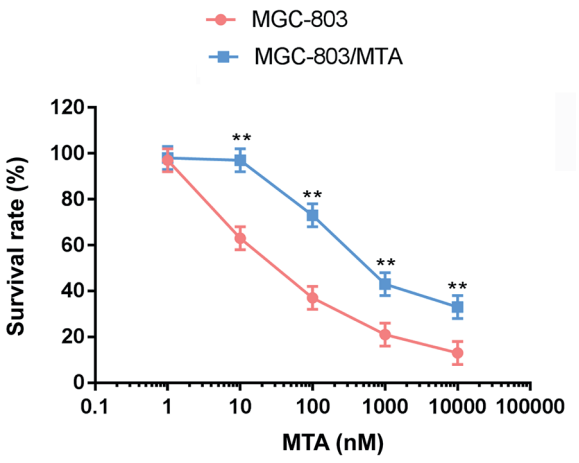

Figure 1. Effect of MTA on survival rate of MGC-803 and MGC-803/MTA cells.

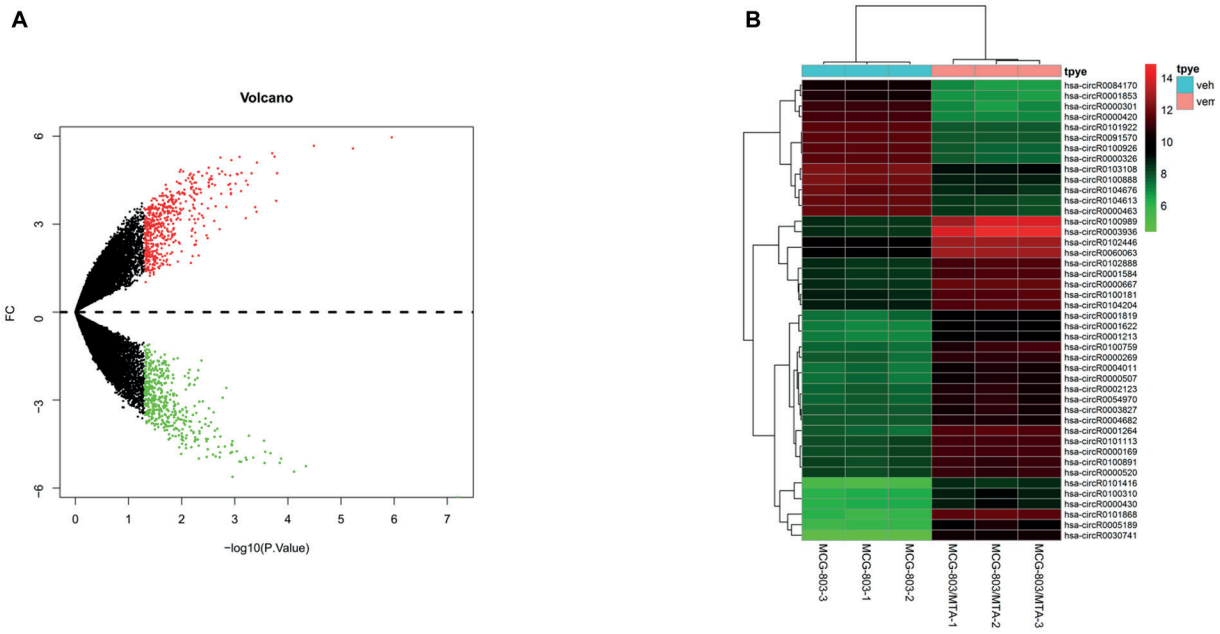

Figure 2. Differentially expressed circRNAs screened *via* circRNA microarray analysis.

cRNAs were significantly changed, showing statistically significant differences ($p<0.01$). Meanwhile, the expression of circ MTHFD2 exerted the greatest difference. Therefore, it was selected for further functional verification (Figure 3).

Effect of Knockdown or Overexpression of Circ MTHFD2 on Survival Rate of MGC-803/MTA Cells

QRT-PCR results revealed that compared with MGC-803 cells, the expression of circ MTHFD2 in MGC-803/MTA cells increased or decreased remarkably by knockdown or overexpression of circ MTHFD2, respectively ($p<0.01$) (Figure 4A).

Compared with MGC-803/MTA cells, the survival rate of cells in circ MTHFD2 overexpression group increased significantly ($p<0.01$). However, it decreased remarkably in cells of circ MTHFD2 knockdown group ($p<0.01$). The above results indicated that the expression of circ MTHFD2 could markedly affect the survival rate of MGC-803/MTA cells (Figure 4B).

Prediction and Verification of Target Genes for Circ MTHFD2

The most classical mechanism of action of circRNA is the molecular sponge of miRNA. In this study, target miRNAs of circRNA were predicted using the bioinformatics websites: http://www.circbase.org/, http://circnet.mbc.nctu.edu.tw/ and

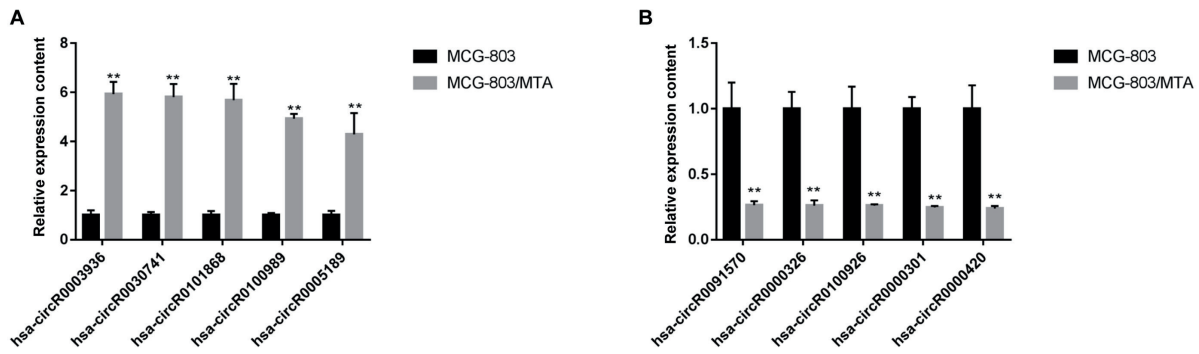

Figure 3. Difference in circRNA expression in MGC-803 and MGC-803/MTA cells detected *via* qRT-PCR. $^{**}p<0.01$.

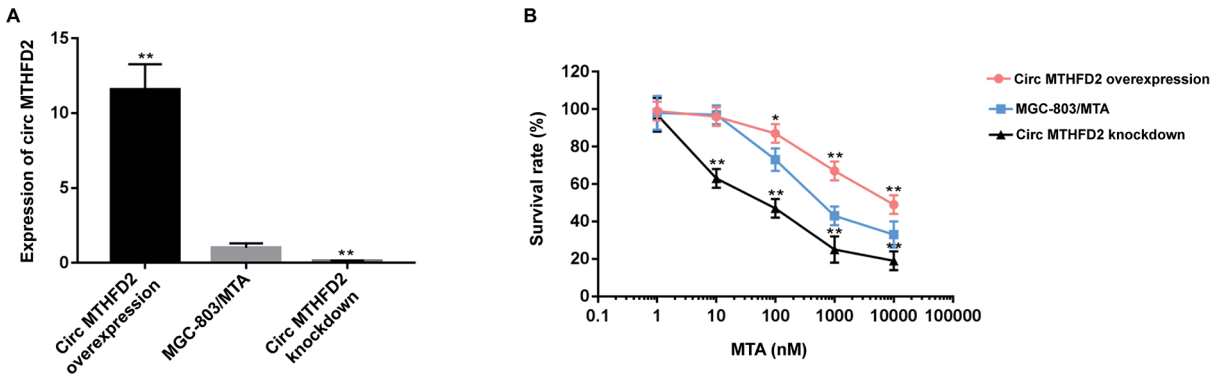

Figure 4. Effect of knockdown or overexpression of circ MTHFD2 on survival rate of MGC-803/MTA cells. **A,** Expression of circ MTHFD2 in MGC-803/MTA cells with knockdown or overexpression of circ MTHFD2 detected *via* qRT-PCR. **B,** Effect of knockdown or overexpression of circ MTHFD2 on survival rate of MGC-803/MTA cells.

http://www.targetscan.org/. As a result, a total of 21 potential miRNAs of circ MTHFD2 were obtained (Figure 5A). Among them, miR-124 was found closely related to the drug resistance of tumor cells, which was then selected for verification. Predicted binding sites for miR-124 and circ MTHFD2 were shown in Figure 5B. Luciferase reporter gene assay was performed to verify the direct binding relation between miR-124 and circ MTHFD2. In MGC-803 /MTA cells transfected with wild-type circ MTHFD2, the fluorescence density in miR-124 mimics group was significantly lower than that of miR-con group, (p<0.01) (Figure 5C). In addition, the results of qRT-PCR showed that transfection of si-circ MTHFD2 in MGC-803/MTA cells could markedly reduce the expression of miR-124 (p<0.01). The above findings suggested that circ MTHFD2 could directly bind to miR-124 to exert the molecular sponge effect.

Effect of MiR-124 Expression on Drug Resistance of MGC-803/MTA Cells

Compared with miR-con and MGC-803/MTA cells, miR-124 mimics transfection significantly up-regulated the expression of miR-124 in MGC-803/MTA cells (p<0.01) (Figure 6A). Compared with MGC-803/MTA cells, the survival rate of cells in miR-124 mimic group increased significantly (p<0.01) (Figure 6B).

Effects of Circ MTHFD2 on Protein Expressions of Thymidylate Synthase (TS), ATP-Binding Cassette Transporter C11 (ABCC11) and RCF1 Through Binding to MiR-124

Compared with MGC-803/MTA cells, MGC-803 cells showed an evidently lower protein expression of miR-124 (p<0.01) (Figure 7A). In addition, Western blotting results manifested that compared with MGC-803/MTA cells, MGC-803 cells exhibited significantly lower protein expressions of thymidylate synthase (TS) and ABCC11, as well as significantly higher protein expression of RCF1. Moreover, compared with MGC-803/MTA cells, the protein expressions of TS and ABCC11 were remarkably declined, while RCF1 was remarkably up-regulated in cells overexpressing circ MTHFD2 (Figure 7B). However, the protein expressions of TS, ABCC11 and respiratory supercomplex factor 1, mitochondrial (RCF1) showed the opposite trends in cells with circ MTHFD2 knockdown (Figure 7C). MiR-124 mimic could significantly reverse increased protein expressions of TS and ABCC11, and decreased protein expression of RCF1 induced by circ MTHFD2.

Discussion

CircRNAs are a kind of circular, closed-loop, and non-coding RNA molecules with high content and stability. They are difficult to be metabolized by nucleases in mammalian cells. With the advancement of new-generation sequencing and bioinformatics techniques, the number and biological functions of circRNAs have been further clarified. Recent studies have found that the expression levels of some circRNAs are abnormal in a variety of solid tumors, such as colon cancer, gastric cancer, and esophageal cancer. For example, Zhang et al[11] have pointed out that compared with healthy people, the expression level of hsa_circ_0014130 is evidently up-regulated in patients with non-small cell lung cancer (NS-

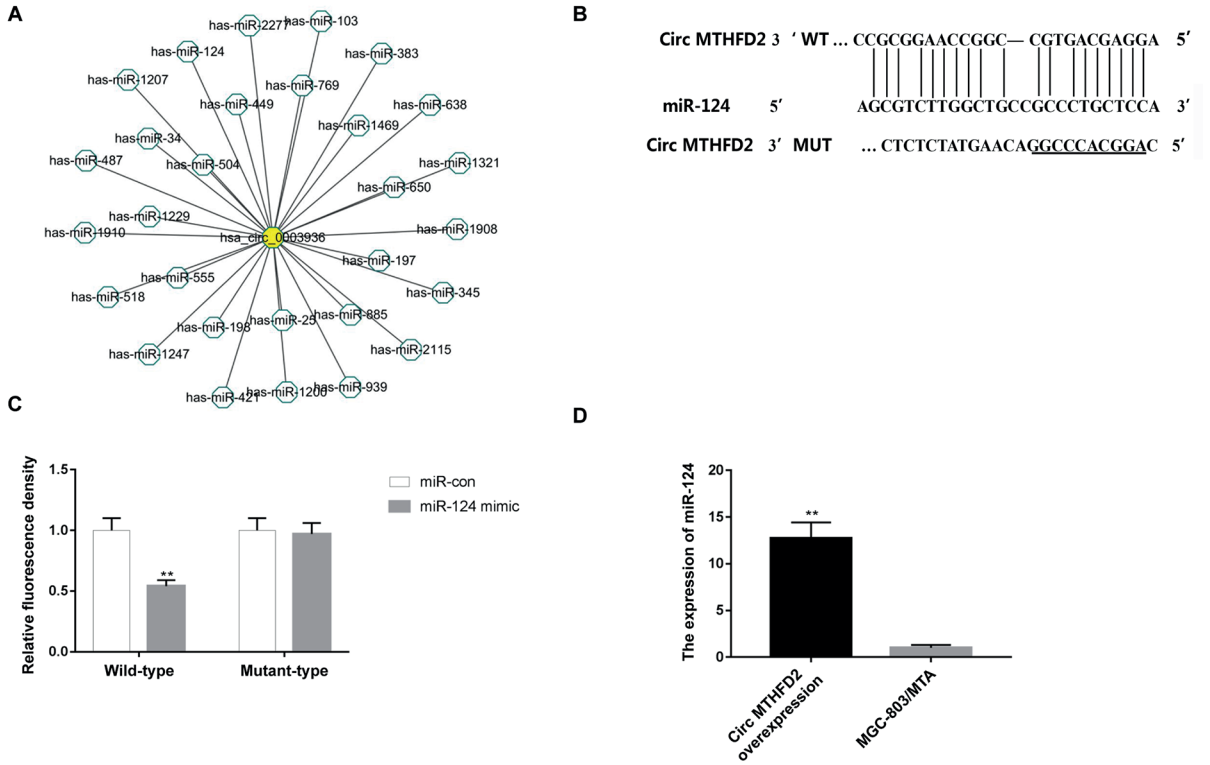

Figure 5. Circ MTHFD2 targets miR-124. **A,** Potential target miRNAs of circ MTHFD2 predicted by bioinformatics software. **B,** Predicted binding sites for circ MTHFD2 and miR-124. **C,** Direct binding between circ MTHFD2 and miR-124 verified *via* luciferase reporter assay: wild-type: transfection of wild-type circR0030741, and mutant-type: transfection of mutant-type circR0030741. **D,** Effect of transfection of si-circR0030741 on the expression of miR-124. $^{**}p<0.01$

CLC). Meanwhile, hsa_circ_0014130 expression shows associations with TNM stage and lymph node metastasis of NSCLC. Bioinformatics analysis has also indicated that hsa_circ_0014130 is involved in the pathogenesis of NSCLC. In addition, a large number of studies have demonstrated that circRNAs play important roles in the sensitivity of tumors to radiotherapy and chemotherapy. For example, Shang et al[12] have manifested that the expression of circPAN3 is abnormal in adriamycin-resistant patients with acute myeloid leukemia when compared with that in leukemia patients with good response to adriamycin. *In vitro* experiments have suggested that circPAN3 regulates the drug resistance of tumor cells to adriamycin in acute myeloid leukemia by regulating the miR-153-5p/miR-173-5p-XIAP axis. Besides, Su et al[13] analyzed differentially expressed circRNAs between radiotherapy-resistant cell lines and normal cell lines. They have found that there are 74 differentially expressed circRNAs, confirming the important role of circRNAs in radiotherapy sensitivity.

MTA is a folate antagonist for multiple enzymes, targeting pyrimidine and purine biosynthesis. The anticancer activity of MTA is mainly realized by inhibiting DNA synthesis by suppressing TS, ultimately inducing apoptosis. However, a high concentration of MTA can inhibit dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyl transferase (GARFT)[14]. MTA can also inhibit the activity of multiple proteins; therefore, it is considered as a multi-target drug[15,16]. The physiological process of MTA in the cells is mainly the following: MTA enters the cells through reduced folate carrier (RFC) and is glutamate by folylpolyglutamate synthase (FPGS), forming glutamate MTA. The inhibitory activity of glutamate MTA on thymidylate synthase (TS) is 100 times that of MTA, making it the most active folate drug. Currently, the reason for acquired drug resistance of tumor cells to MTA has not been fully understood. Current researches on the mechanism of drug resistance have shown that the reasons for acquired drug resistance to MTA may include drug transport into cells, decreased glutamate MTA, and increased protein expressions of DHFR and GARFT. Ozasa et al[17] have demonstrated that compared with parental

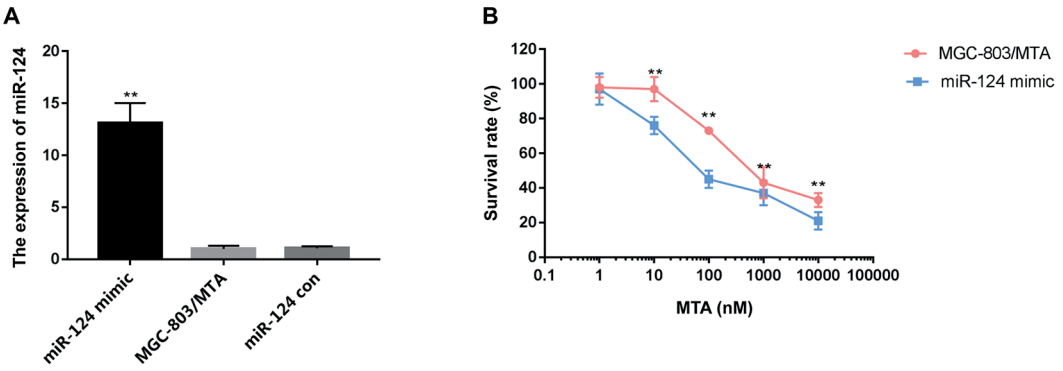

Figure 6. Effect of miR-124 expression on drug resistance of MGC-803/MTA cells. **A,** Effect of miR-124 mimic on miR-124 expression in MGC-803/MTA cells. **B,** Effect of miR-124 on survival rate of MGC-803/MTA cells.

PC6 cells, drug-resistant PC6 cells shows markedly increased expression of TS and decreased expression of RCF. The knockdown of TS by siRNA transfection in drug-resistant PC6 cells can significantly enhance the cytotoxicity of MTA on PC6 cells. These results suggest the important role of TS in the mechanism of MTA resistance. In addition, Uemura et al[18] have illustrated that MTA-resistant cells often have cross-resistance to methotrexate, which is the substrate for ABCC11. ABCC11 is an important drug efflux pump that plays an important role in drug metabolism. Therefore, it is inferred that ABCC11 plays a major role in the MTA resistance. Subsequent works have shown that the knockdown of ABCC11 protein expression using ABCC11 siRNA transfection in 13 kinds of cell lines can significantly weaken the drug resistance of cells to MTA.

In the present study, MGC-803/MTA resistant cell lines were successfully constructed *via* persistent drug exposure to MTA at an increasing concentration for 5 months. Compared with parental cells, MGC-803/MTA resistant cells showed significantly enhanced drug resistance. Differentially expressed circRNAs were screened *via* circRNA microarray analysis. A total of 673 circRNAs were screened out based on Fold Change >3 and adjusted *p*-value. To further verify the reliability of high-throughput screening results, qRT-PCR was performed. It was found that the expression levels of 10 differentially expressed circRNAs were significantly changed when compared with MGC-803 cells ($p<0.01$). The expression of circ MTHFD2 had the greatest difference, which was then selected for further functional verification. Subsequently, cell lines with overexpression and knockdown of circ MTHFD2 were constructed successfully, respectively. CCK-8 assay results showed that compared with MGC-803 /MTA, the drug resistance increased significantly in circ MTHFD2 overexpression group, while decreased remarkably in circ MTHFD2 knockdown group. These findings indicated that circ MTHFD2 was associated with the drug resistance of MGC-803/MTA cells.

To further explore the mechanism of acquired drug-resistance of gastric cancer MGC-803 cells to MTA, the miRNA targets of circ MTHFD2 were predicted using bioinformatics tool and verified *via* the luciferase reporter gene assay. It was found that circ MTHFD2 could targeted bind to miR-124. To further investigate the role of miR-124 in MGC-803/MTA cells, miR-124 mimics was transfected into MGC-803/MTA cells. The results of CCK-8 assay revealed that miR-124 mimics could remarkably enhance the drug resistance of MGC-803/MTA cells to MTA. Western blotting results also demonstrated that overexpression of circ MTHFD2 significantly increased the protein expressions of TS and ABCC11, whereas decreased RCF1. Furthermore, miR-124 mimic reversed increased protein expressions of TS and ABCC11, as well as decreased protein expression of RCF1 induced by circ MTHFD2.

However, there are also many limits in the present study. For example, current reports have shown that tumor resistance is the result of the combined effect of microenvironment in tumor cells. However, in this research, we only performed experiments *in vitro*, and there were no further *in vivo* experiments. In addition, we revealed that circ MTHFD2 affected the expression of miR-124 in cells and altered the protein expressions of TS, ABCC11, and RCF, ultimately resulting in drug resistance of gastric cancer cells. However, the effects of miR-

124 on TS, ABCC11, and RCF proteins still remained unclear. These will be explored in the future studies.

Conclusions

We showed that circ MTHFD2 directly binds to miR-124 through the molecular sponge effect, increasing the protein expressions of TS and ABCC11, and decreasing in the protein expression of RCF1. Ultimately, this may enhance the drug resistance of MGC-803/MTA cells. Our findings may provide a valuable biomarker for MTA resistance and a new drug target for overcoming the drug resistance of gastric cancer.

Conflict of Interests

The Authors declare that they have no conflict of interests.

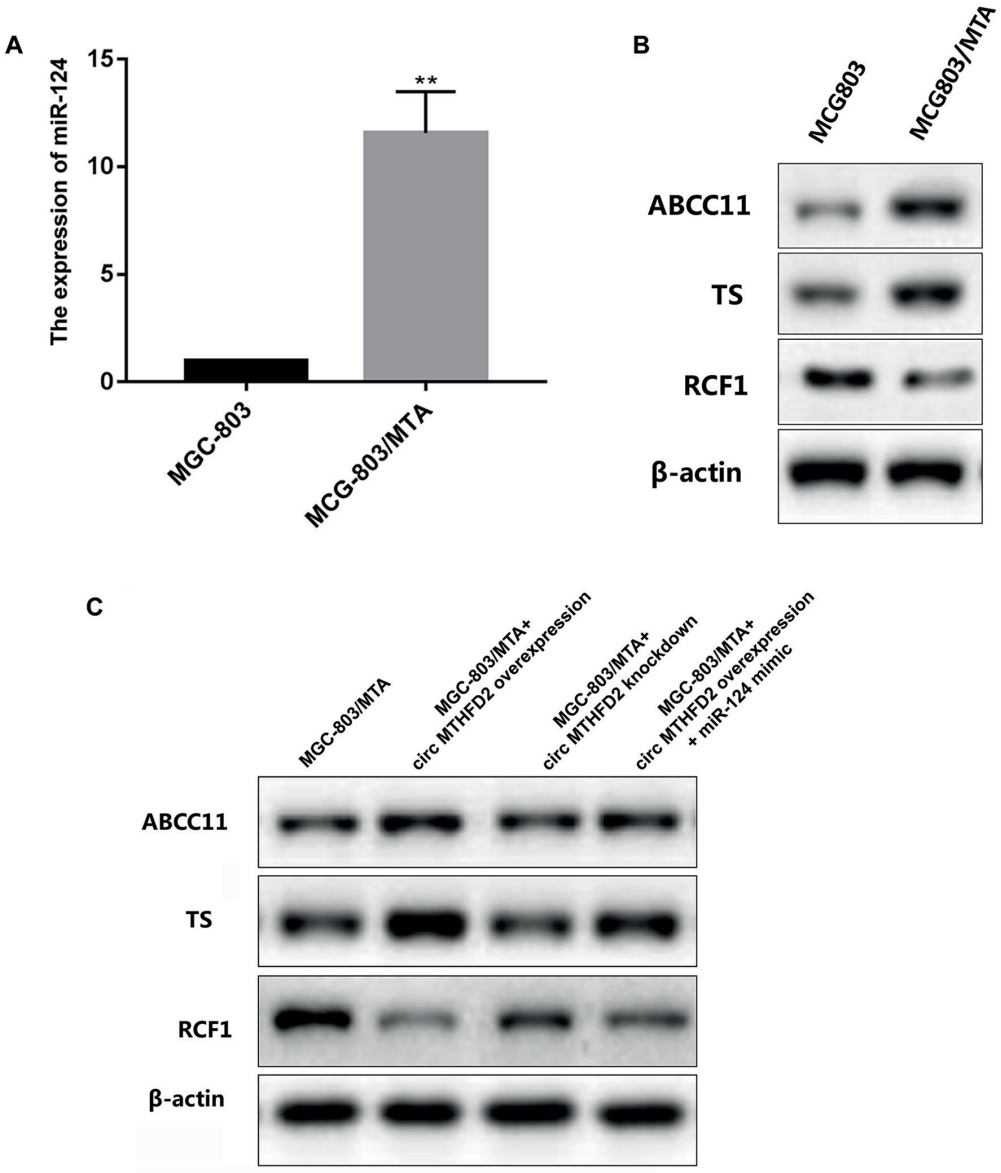

Figure 7. Effects of circ MTHFD2 on protein expressions of TS, ABCC11 and RCF1 through binding to miR-124. **A,** Difference of miR-124 expression between MGC-803 cells and MGC-803/MTA cells detected *via* qRT-PCR. **B,** Protein expressions of TS, ABCC11 and RCF1 in MGC-803 cells and MGC-803/MTA cells detected *via* Western blotting. **C,** Protein expressions of TS, ABCC11 and RCF1 in cells with circ MTHFD2 overexpression, circ MTHFD2 overexpression + miR-124 mimic and circ MTHFD2 knockdown.

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