European Review for Medical and Pharmacological Sciences 2019; 23: 6478-6485

MiR-129 reduces CDDP resistance in gastric cancer cells by inhibiting MAPK3

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Abstract. – OBJECTIVE: Abnormal expression of mitogen-activated protein kinase 3 (MAPK3) is related to invasion, metastasis, and drug resistance of multiple tumor cells. MiR-129 expression is associated with gastric cancer. Bioinformatics analysis showed a targeting relation between miR-129 and MAPK3. This study investigated whether miR-129 plays a role in regulating MAPK3 expression, affecting proliferation, apoptosis, and cisplatin (CDDP) resistance of gastric cancer cells.

MATERIALS AND METHODS: The dual-luciferase reporter gene assay was used [illegible]sess the targeted regulation between [illegible] and MAPK3. The expression of miR-12[illegible]nd MAPK3 in CDDP-resistant cell line MGC[illegible]/CDDP and the parental MGC-803 cells was [illegible]sured. MGC-803/CDDP cells were cultured *vitro* and divided into miR-NC [illegible] and miR-129 mimic group. The expres[illegible]PK3 and p-MAPK3 protein were dete[illegible]by W[illegible]ern blot and the effect of CDDP tr[illegible]ent on [illegible] apoptosis and proliferation w[illegible]etected [illegible]tometry.

RESULTS: There w[illegible] tar[illegible]gulation relation between miR[illegible] and MA[illegible]RNA. MiR-129 expression [illegible]GC-803/CDD[illegible]ls was significantly lo[illegible] that in MGC[illegible]3 cells and the expr[illegible]n [illegible]PK3 mRNA and protein was sign[illegible]cantly h[illegible] than that in MGC-803 cells. [illegible]pared with [illegible]C group, the expression [illegible]MAPK3 and p-M[illegible] in MHC-803/CDDP [illegible] in mi[illegible]129 mimic transfection group was [illegible]ficantl[illegible]creased, with increased cell apo[illegible]is an[illegible]uced [illegible]ll proliferation.

C[illegible]S: The[illegible]reased expression of miR-12[illegible]e up-r[illegible]ation of MAPK3 are associated w[illegible]DP[illegible]istance in gastric cancer [illegible]verexp[illegible] of miR-129 inhibits MAPK3 exp[illegible]ion and [illegible] proliferation, it induces cell apo[illegible]is and reduces CDDP resistance.

[illegible]ords:
[illegible]iR-129, MAPK3, Gastric cancer, CDDP, Drug re[illegible]e.

[illegible]roduction

Gastric cancer (G[illegible]ne of the most common [illegible]nant tumors [illegible] digestive tract. Its i[illegible]ce rate is the fourth in common malignant [illegible]ors and the [illegible]rtality rate is the second in [illegible]mon malign[illegible]umors[1,2].

[illegible]itogen-activ[illegible]d protein kinase 3 (MAPK3), a[illegible]own [illegible]xtracellular signal-regulated kina[illegible], is an important signal transduction [illegible]ecule in the ERK/MAPK pathway. The ERK/MAPK pathway plays a crucial role [illegible]itting signals downstream and regu[illegible]ing [illegible]ological processes such as cell proliferation, apoptosis, and migration[3,4]. Studies[5-7] have shown that enhanced functional activity of MAPK3 plays a role in the development and progression of gastric cancer. MicroRNA is an endogenous non-coding small-molecule single-stranded RNA in eukaryotes with a length of about 22-25 nucleotides. It regulates the expression of a target gene by complementary binding to the 3'-UTR of the target gene mRNA, leading to degradation or inhibition of the translation of target genes, playing a crucial role in the regulation of various biological processes such as cell proliferation, apoptosis, migration, invasion, and drug resistance[8-11]. Research evidence shows that an abnormal expression of miR-129 is associated with the occurrence, progression, and metastasis of gastric cancer and may play a role as a tumor suppressor gene in gastric cancer[12-14]. Bioinformatics analysis showed that there is a targeted relation site between miR-129 and MAPK3 mRNA. This work investigated whether miR-129 plays a role in regulating MAPK3 expression and affecting gastric cancer cell proliferation, apoptosis, and cisplatin (CDDP) drug sensitivity.

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Materials and Methods

Main Reagents and Materials

Human normal gastric mucosal epithelial cells (RGM-1) were purchased from ScienCell (Invitrogen, Carlsbad, CA, USA), HEK293T cells and gastric cancer MGC-803 cells were purchased from Shanghai Meixuan Biotechnology Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), Opti-MEM reduced serum, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). TRNzol Universal total RNA extraction reagent was purchased from Beijing Tiangen Bio (Beijing, China), Lipofectamine 2000 transfection was purchased from Invitrogen (Carlsbad, CA, USA), PrimeScript Real-time (RT) Reagent Kit was purchased from TaKaRa (Dalian, China). MiR-129 mimic, miR-NC was designed and synthesized by Guangzhou Ribo Bio (Guangzhou, China); rabbit anti-human MAPK3, p-MAPK3, β-actin polyclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Goat anti-rabbit IgG (H+L) secondary antibody, CCK-8 reagent, and Annexin V/PI cell apo is were purchased from Thermo Fisher S (Waltham, MA, USA). The test kit was pur ed from Beijing Solarbio (Beijing, China); the l cell proliferation assay reagent was purcha from Jiangsu Biyuntian (Nanto China); t Dual-Glo Luciferase Assay Sy he pMIR carrier were purchased fro mega adison, WI, USA).

Cell Culture

RGM-1 and MG 3 cells cultured in a DMEM mediu taining 10% at 37°C with 5% CO_2. cells reached % confluence, cells co after 0.125% trypsin digestion a subcultured atio of 1:5 to 1:6. The cells og phase of cel h were selected for riment . This resea h was approved by th ics C ittee of our hospital.

Estab t of C Resistant Cell Model

blishi GC-803/CDDP drug-resistant model performed as follows: when MG 03 cells were in logarithmic growth phase, C d to SW480 cell culture medium inal concentration of 0.25 μg/mL. After cell wth was stable for 2 weeks, the CDDP con- on was increased to 0.5 μg/mL. After 2 wee culture, the CDDP treatment concentration gradually increased to 1.0 μg/mL, 2.0 μg/mL until the MGC-803 cells could maint growth at 2.0 μg/mL and repeate passag thereby establishing resistant M -803/CDDP cells against CDDP.

Dual Luciferase Activit Assay

The PCR product of full-length fragment of the MAPK e or th fragmen taining the mutant w igeste d ligated into the pMIR vector fo by sforma into bacteria. The c ct p were uenced and designate pMIR K3 , pMIR-MAPK3-M espectively. p APK3-WT (or pMIR MUT) was sfected into HEK29 ells iR-129 mimic (or miR-NC) using Lipofecta 000. Then, cells were pla 37°C 5% C culture incubator. A 48 h of incubation, the dual luciferase activ- was detected cording to the instructions of Dual-Glo Lu ase Assay System kit.

C ansfec and Grouping

M P cells were cultured *in vitro* and divided into two transfection groups: miR- C transfection group, miR-129 mimic trans- up, and cells were collected 72 h after ansfe ion.

QRT-PCR Detection of Gene Expression

The RNA was reversely transcribed into cDNA using the PrimeScript RT Reagent Kit, while the expression of the gene was detected by q-PCR using cDNA as a template. The reverse transcription reaction system included 0.5 μL of oligdT Primer (50 μM), 0.5 μL of Random 6 mers (100 μM), 0.5 μL of PrimeScript RT Enzyme Mix, 1.0 μg of RNA, 2 μL of 5×PrimeScript Buffer, and 20.0 μL of RNase Free H_2O. The reverse transcription reaction conditions were 37°C for 15 min and 85°C for 5 s. The qPCR reaction system was SYBR Fast qPCR Mix 10.0 μL, Forward Primer (10 μM) 0.8 μL, Reverse Primer (10 μM) 0.8 μL, cDNA 2.0 μL, RNase Free dH_2O 6.4 μL. Q-PCR reaction conditions were pre-denaturation 95°C, 10 min, denaturation at 95°C for 10 s, annealing at 60°C for 20 s, extension at 72°C for 15 s, cycle 40 cycles on Bio-Rad CFX96 Real-Time PCR Detection System. The primer sequences for miR-129 were as follows: Forward-5'-TGCGCCTTTTTG-CGGTCTGGG-3'; Reverse-5'-CCAGTG-CAGGGTCCGAGGTATT; MAPK3 was: Forward-5'-ACCTGCGACCTTAAGATTTGT-3'; Reverse-5'-GAAAAGCTTGGCCCAAGCC-3'.

Western Blot

The radioimmunoprecipitation assay (RIPA) buffer lysate was added to the cell pellet to extract the total protein from the cells. After quantitative determination of the mass concentration by bicinchoninic acid (BCA) method, 40 μg of the sample was separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (12% separation gel, 5% concentrated gel; 45V, 150 min), transferred to a polyvinylidene difluoride (PVDF) membrane (300 mA, 100 min), and blocked with 5% skim milk powder at room temperature. Then, it was incubated with the primary antibody at 4°C overnight (the dilution ratios of MAPK3, p-MAPK3, and β-actin primary antibodies were 1:2000, 1:1000, 1:8000). After washing the membrane 3 times with Phosphate-Buffered Saline and Tween-20 (PBST), horseradish peroxidase (HRP)-conjugated secondary antibody was added and incubated with the membrane for 60 min at room temperature (diluted by 1:5000). Next, the membrane was washed 3 times with PBST, the chemiluminescence solution was added, and it was incubated under the dark for 2-3 min. After that, the membrane was exposed and developed.

Detection of Apoptosis

The two transfected cells collected above were inoculated into a 6-well plate. When the cells reached 50% confluence, 2.0 μg/mL of CDPP was added for treatment. After 48 h of action, cells were collected by trypsin digestion and washed by phosphate-buffered saline (PBS). Then, 100 μL Annexin V Binding Buffer was added to suspend the cells followed by addition of 10 μL Annexin V-FITC and 5 μL Propidium Iodide (PI) staining. After incubation for 15 min at room temperature, 400 μL Annexin V Binding Buffer was added, and cell apoptosis was detected by Beckman Coulter FC500 MCL flow cytometry.

Flow Cytometry Detection of Cell Proliferation

Two transfected cells were collected by trypsin. After the digestion was terminated, the two transfected cells were resuspended in RPMI 1640 medium containing 10% FBS and incubated with 10 μM of EdU at 37°C for 2 h. Then, the cells were seeded in culture. The plate was cultured for 48 h and treated with 2.0 μg/mL of CDDP. Cells were collected by trypsinization, washed once with PBS, fixed with paraformaldehyde, washed once with PBS, and 100 μL permeabilized. The membrane was ruptured, 500 μL of the reaction solution was added, and the mixture was incubated at room temperature for 30 min under the dark. Cells were washed by centrifugation with 3 mL of permeabilized solution and were resuspended with 500 μL of wash buffer, while the cell proliferation was detected by flow cytometry.

Statistical Analysis

Statistical analysis of the data was performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were expressed as mean ± standard deviation (SD). The comparison between the two groups was measured by the Student's t-test. The comparison between the measurement data of multiple groups was the first-way analysis of variance (ANOVA). Then, Bonferroni's post-hoc analysis was used. $p < 0.05$ was considered statistically significant.

Results

A Targeted Regulatory Rrelationship Between miR-129 and MAPK3 mRNA

Bioinformatics analysis of the Targetscan revealed a complementary binding site between miR-129 and the 3'-UTR of MAPK3 mRNA (Figure 1A). Dual luciferase gene reporter assays showed that the transfection of miR-129 mimic significantly reduced the relative luciferase activity in pMIR-MAPK3-WT transfected HEK293T cells; however, miR-NC or miR-129 mimic did not have a significant effect on the relative luciferase activity in HEK293T cells transfected with pMIR-MAPK3-MUT (Figure 1B), indicating a targeted regulatory relation between miR-129 and MAPK3 mRNA.

High Resistance of MGC-803/CDDP Cells

The results of CCK-8 assay showed that the same dose of CDDP could significantly inhibit the activity of MGC-803/CDDP cells, while the same dose of CDPP inhibited the proliferation of MGC-803/CDDP cells markedly. The IC50 of SW480 cells was 1.21 ± 0.11 μg/mL, the IC50 of resistant SW480/CDDP cells was 11.67 ± 1.23 μg/mL, and the resistance index of SW480/CDDP cells was 9.64 (Table I).

Abnormal Expression of MiR-129 and MAPK3 in MGC-803/CDDP Cells

The results of qRT-PCR showed that compared with human normal gastric mucosal epithelial

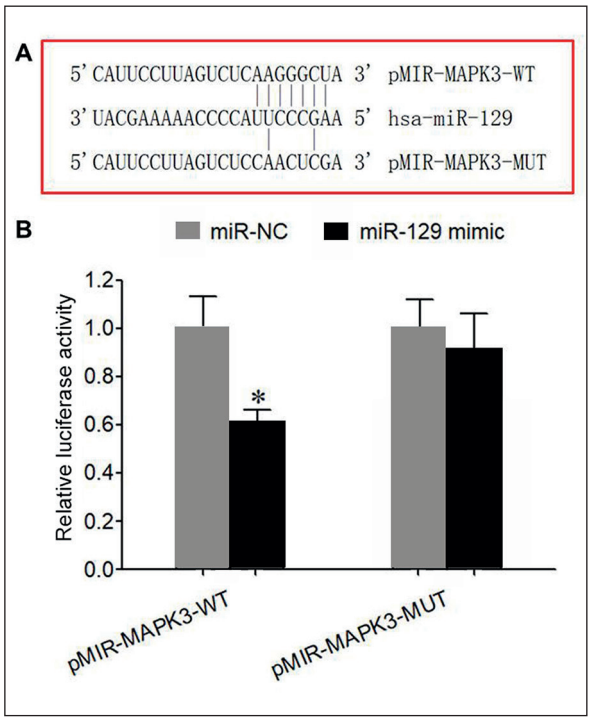

Figure 1. Targeted regulatory relation between miR-129 and MAPK3 mRNA. **A,** Schematic diagram of the interaction site between miR-129 and the 3'-UTR of mRNA; **B,** Dual luciferase gene reporter assay. * $p < 0.05$ compared with miR-NC.

RGM-1 cells, the expression of miR-129 in gastric cancer MGC-803, MGC-803/CDDP cells were significantly decreased, while the expression of MAPK3 mRNA was higher. The expression of miR-129 in drug-resistant MGC-803/CDDP cells was lower than that in the parental MGC-803 cells, and the expression of MAPK3 mRNA was higher (Figure 2A). Western blot analysis showed that the expression of MAPK3 protein in MGC-803/CDDP cells was significantly higher than that in MGC-803 cells (Figure 2B).

Overexpression of MiR-129 Expression Enhances Drug Sensitivity in MGC-803/CDDP Cells

The results of qRT-PCR showed that compared with the miR-NC group, miR-129 mimic transfection significantly upregulated the expression of miR-129 in MGC-803/CDDP cells, while the expression of MAPK3 was significantly decreased (Figure 3A). Western blot analysis showed that transfection of miR-129 mimic significantly decreased the expression of MAPK3 and p-MAPK3 in MGC-803/CDDP cells (Figure 3B). Flow cytometry analysis showed that the transfection of miR-129 mimic significantly increased MGC-803/CDDP cell apoptosis (Figure 3C), while it significantly attenuated cell proliferation (Figure 3D).

Discussion

Chemotherapy is an important approach in the treatment of gastric cancer; however, drug resistance is one of the important factors which limit the chemotherapeutic effect of gastric cancer and affect the survival and prognosis of patients[15,16]. Therefore, studying the drug resistance mechanism of gastric cancer and the abnormally altered signaling molecules in the process of drug resistance is of great significance for improving the therapeutic effect, prognosis, as well as survival rate.

The ERK/MAPK signal transduction pathway is widely expressed in various tissues and cells, and it can regulate various biological processes such as cell proliferation, cycle, apoptosis, migration, and invasion[17,18]. MAPK3 is a silk/threonine

Table Effect of CDDP on the proliferation of MGC-803 and MGC-803/CDDP cells.

Concentration (μg/ml)	MGC-803 Cell proliferation (%)	MGC-803/CDDP Cell proliferation (%)
0	100 ± 7.65	100 ± 9.61
1	86.31 ± 5.97	99.22 ± 8.59
2	59.29 ± 3.81	95.63 ± 9.18
4	18.33 ± 1.56	83.51 ± 7.63
8	2.36 ± 0.21	76.29 ± 6.64
16	1.59 ± 0.91	63.75 ± 6.61
32	1.82 ± 0.78	39.55 ± 2.86
64	0.82 ± 0.09	10.31 ± 1.98

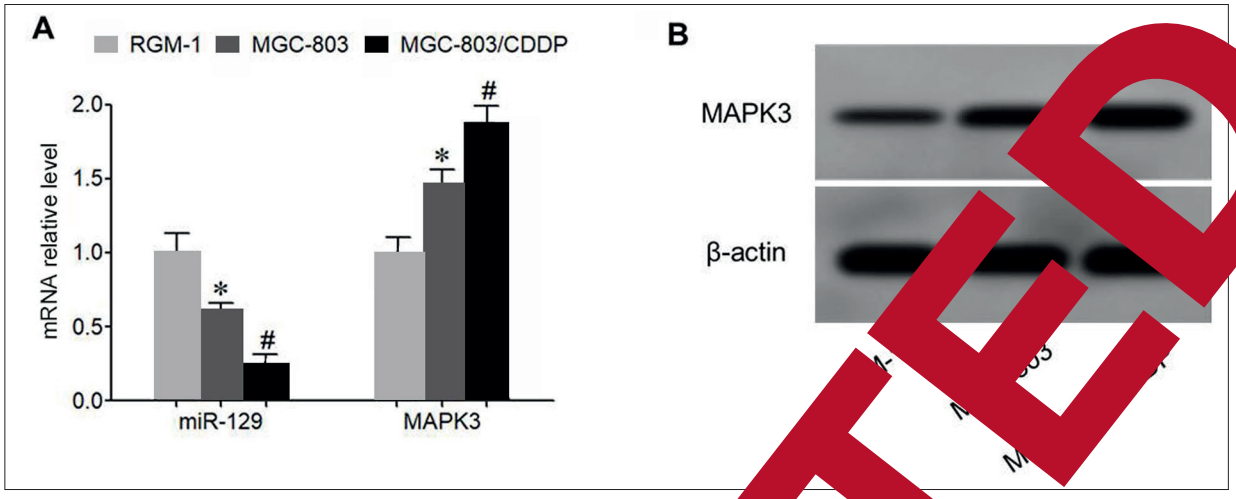

Figure 2. Abnormal expression of miR-129 and MAPK3 in drug-resistant MGC-803/CDDP cells. **A,** QRT-PCR was used to detect the expression of miR-129 and MAPK3 mRNA; **B,** Western blot analysis of ERK1 protein expression. * represents $p < 0.05$ compared with RGM-1 cells; # represents $p < 0.05$ compared to MGC-803 cells.

kinase. After receiving the upstream cascade signal, MAPK3 can phosphorylate the cytoplasmic protein which regulates multiple nuclear transcription factors such as c-fos and c-Jun, which are involved in the regulation of cell proliferation and apoptosis[19,20]. The expression or functional activity of MAPK3 is associated with the occurrence, progression, metastasis, and drug resistance of

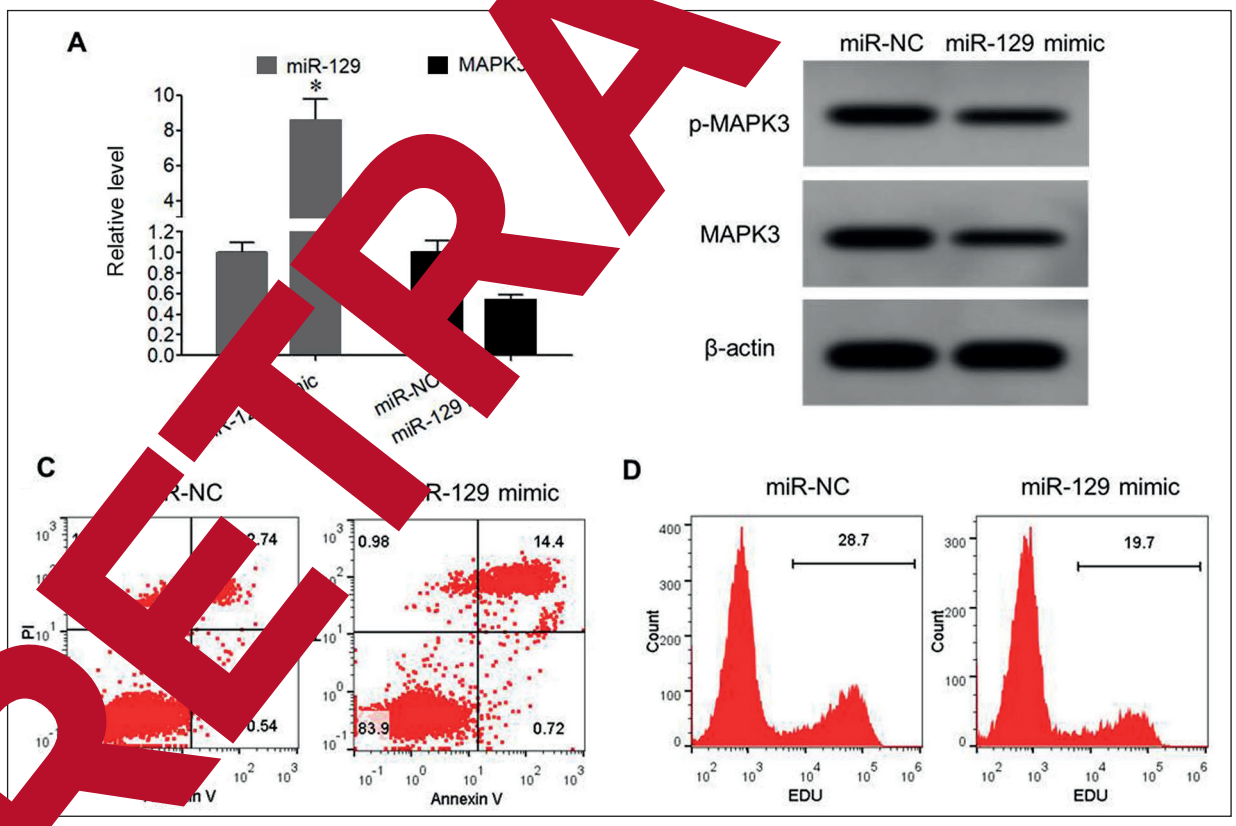

Figure 3. Overexpression of miR-129 expression enhances drug sensitivity in MGC-803/CDDP cells. **A,** QRT-PCR was used to detect the expression of miR-129 and MAPK3 mRNA; **B,** Western blot analysis of MAPK3, p-MAPK3 protein expression; **C,** Flow detection of apoptosis; **D,** Flow detection of cell proliferation. * represents $p < 0.05$ compared to miR-NC group.

various tumors such as liver cancer, thyroid cancer, and lung cancer. Reports[5-7] have shown that the enhanced functional activity of MAPK3 plays a role in the development and progression of gastric cancer. Several studies[21-22] have shown that the expression of miR-129 is decreased in the occurrence, progression, and resistance of various tumors such as prostate cancer, gastric cancer, and intestinal cancer. Research evidence[12-14] demonstrates that an abnormal expression of miR-129 is associated with the occurrence, progression, and metastasis of gastric cancer. Also, it may play a role as a tumor suppressor gene in gastric cancer. This work investigated whether miR-129 plays a role in regulating MAPK3 expression and affects gastric cancer cell proliferation, apoptosis, and CDDP drug sensitivity.

In this study, the dual luciferase gene reporter assay showed that transfection of miR-129 mimic significantly reduced the relative luciferase activity in pMIR-MAPK3-WT transfected HEK293T cells, whereas it did not have a significant effect on the relative luciferase activity in HEK293T cells transfected with pKIR-MAPK3-MUT, indicating that there is a targeted regulation r[illegible]n between miR-129 and MAPK3. Results [illegible] 8 assay showed that the proliferation acti[illegible] of MGC-803/CDDP cells was significantly h[illegible] than that of the parental MGC-803 cells u[illegible] the same dose of CDDP treatment, indicati[illegible] that gastric cancer cells resist[illegible]DP were successfully established. Th[illegible]sults [illegible]ene and protein assay showed that [illegible]express[illegible]of miR-129 in drug-resistant M[illegible]3/CD[illegible] significantly lower th[illegible] th[illegible]ital MGC-803 cells, while the [illegible]pressio[illegible]APK3 was significantly incr[illegible]. Compared [illegible] normal gastric mucosa[illegible]l cells, the ex[illegible]ssion of miR-129 and [illegible]PK[illegible]ug-resistant and parental cells [illegible]as abnormal[illegible]sults showed that decreased [illegible]pression of mi[illegible]and increased expres[illegible]of MAPK3 were no[illegible] only related to gastri[illegible]ncer, [illegible]lso related to the drug resistan[illegible]astri[illegible]ncer ce[illegible]. In the research of the relatio[illegible]n miR[illegible]and gastric cancer, Ji-[illegible] et al[illegible]ed th[illegible]e expression of miR-129 [illegible]nifica[illegible] in tumor tissues than that in a[illegible]ent tiss[illegible]and compared with healthy con[illegible] the expression of miR-129 in peripheral bl[illegible]s was abnormally decreased. Liu [illegible] showed that the expression of miR-129 was [illegible]ificantly decreased in tumor tissues of gastric c[illegible]patients compared with adjacent tissues, and t[illegible]expression of miR-129 was decreased and related to the tumor tissue size, lymph node metastasis, and patient prognosis. Wang et [illegible] that the expression of miR-129 wa[illegible]normal tumor tissues of gastric cancer pa[illegible]s compared with adjacent tissues. In this st[illegible] expression of miR-129 was reduced in gastri[illegible]r, which was consistent with the res[illegible]of Jian[illegible]. Liu et al[13], and Wang et al[23].

Further studies[24] sh[illegible]d that [illegible] transfe[illegible] of miR-129 mimic s[illegible]nificantl[illegible]uced the expression of MAPK3[illegible]M[illegible]3 in dr[illegible]esistant MGC-803/C[illegible]DP c[illegible]le it si[illegible]ficantly increased the [illegible]ptosis o[illegible]-re[illegible]nt cells, and decreas[illegible]ell proliferati[illegible]y, as well as CDDP [illegible]. In the work [illegible] the relation between [illegible]R-12[illegible] the biological effects of gastric cancer cells, [illegible]t al[22] showed that the tra[illegible]n of miR-129 [illegible]c in gastric cancer S[illegible]901 cells can significantly inhibit cell pro-[illegible]ation and colo[illegible]y formation, and weaken cells [illegible]ratory and i[illegible]ive ability. The transfection [illegible]iR-129 inhi[illegible]r can significantly promote c[illegible]liferatio[illegible]nhance cell migration and invasi[illegible]. anti-cancer effect of miR-129 is achieved by inhibition of IL-8 gene expression. Liu et al[13] found that there is a targeted regulation [illegible]iR-129 and ADAM9 in gastric cancer [illegible]KN[illegible] and SGC-7901 cells. Overexpression of miR-129 can inhibit the proliferation of gastric cancer MKN45 and SGC-7901 cells by inhibiting the expression of ADAM9, attenuate colony formation and cell invasion. Lu et al[25] found that the expression of miR-129 was significantly decreased in drug-resistant gastric cancer tissues and tumor cells. An elevated expression of miR-129 in drug-resistant gastric cancer cells promoted cell apoptosis and decreased CDDP drugs resistance, while down-regulating the expression of miR-129 can reduce the drug sensitivity of gastric cancer resistant cells to CDDP, while miR-129 affects the drug sensitivity by targeting the expression of P-gp. Ma et al[26] revealed that miR-129 exerts a tumor suppressor effect on the proliferation and migration of gastric cancer cells by targeting WWP1. Wang et al[23] observed that there is a targeting relation between miR-129 and BDKRB2 in gastric cancer BGC-823 cells. Increasing the expression of miR-129 can inhibit the expression of BDKRB2 and weaken the migration ability of cells. Wu et al[27] detected that the methylation level of the promoter region of miR-129 gene was significantly increased in relation to the drug resistance of gastric cancer cells. Overexpression of miR-129 could reduce the drug resistance of

gastric cancer SGC7901 cells by targeting the expression of ABCB1, ABCC5, and ABCG1. This study shows that miR-129 has a tumor-suppressing effect by promoting cell apoptosis, inhibiting cell proliferation, and reducing cell resistance, which is consistent with the above results. This work combines the targeted regulatory relation between miR-129 and MAPK3, revealing that miR-129 plays a role in targeting the inhibition of MAPK3 expression, promoting gastric cancer cell apoptosis, and reducing CDDP resistance, which have not been reported in the previous studies. However, whether miR-129 is related to the drug resistance of gastric cancer patients by regulating MAPK3 is still unclear. Further research is needed to confirm this.

Conclusions

We found that the decreased expression of miR-129 and the up-regulation of MAPK3 are associated with CDDP resistance in gastric cancer cells. The overexpression of miR-129 inhibits MAPK3 expression and cell proliferation induces a[p]o-sis and reduces CDDP resistance.

Conflict of Interest

The Authors declare that they have n[o] f interests.

Acknowledgements

This research was support[ed] b[y] City [S]ocial Development Fund (No. [2]01701[...]).

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