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Original Article

miR-205 promotes apoptosis of hepatoma cells and enhances cisplatin drug sensitivity by inhibiting ERK1

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Abstract: As an important signal transduction molecule in extracellular signaling regulated kinase (ERK)/mitogen activated protein kinase (MAPK) pathway, ERK1 is related to invasion, metastasis and resistance of multiple tumors. MiR-205 is down-regulated in liver cancer tissues, and can target the 3'-UTR of ERK1. This study investigated the role of miR-205 in modulating ERK1 expression, liver cancer cell proliferation, apoptosis and cisplatin (DDP) resistance. Dual luciferase reporter gene assay confirmed targeted regulation between miR-205 and ERK1. Resistant cell lines Bel-7402/DDP and HepG2/DDP were generated to compare the expression of miR-205 and ERK1 against parental cells and normal human hepatocyte HL-7702 cells. Cell apoptosis was measured by flow cytometry to calculate IC-50. Cultured DDP resistant cells were divided into miR-NC and miR-205 groups for measuring ERK1 and p-EKR1 expression, followed by flow cytometry for apoptosis and EdU staining for proliferation. Targeted regulation existed between miR-205 and ERK1 mRNA. Bel-7402/DDP and HepG2/DDP cells had lower miR-205 expression than parental cells, plus higher mRNA and protein levels of ERK1. Under DPP concentrations equivalent to IC-50 of parental Bel-7402 and HepG2 cells, drug resistant cells showed significantly decreased apoptosis. Comparing to the miR-NC group, miR-205 mimic transfection into Bel-7402/DDP and HepG2/DDP cells showed remarkably decreased ERK1 and pERK1 protein expression, plus enhanced cell apoptosis and weakened proliferation. MiR-205 down-regulation is correlated with DDP drug resistance of liver cancer cells. Over-expression of miR-205 can induce liver cancer cell apoptosis and decrease DDP resistant via inhibiting ERK1 expression or its phosphorylation activity.

Keywords: MiR-205, ERK1, ERK/MAPK, liver cancer, DDP, drug resistance

Introduction

Hepatocellular carcinoma (HCC) is one of the frequent malignant tumors found in clinic, and its incidence and mortality are among the most prevalent cancers worldwide [1-4]. Hepatitis B virus (HBV) is an important pathogenic factor in HCC. China is a common epidemic region of HBV, having relatively higher incidence of HCC. Currently chemotherapy is the main treatment measurement for HCC. However, the occurrence of chemotherapy drug resistance largely impairs treatment efficiency and decreases survival rate.

Extracellular signaling regulated kinase 1 (ERK1) is an important protein in the ERK/mitogen activated protein kinase (MAPK) signal transduction pathway. Expressional or functional enhancement of ERK1 plays important roles in the onset, progression, metastasis and drug resistance of multiple tumors including colorectal carcinoma [5], prostate cancer [6] and breast cancer [7]. Various studies [8-11] showed that enhanced expression or function of ERK1 was correlated with onset, progression, invasion/metastasis and drug resistance of HCC, indicating that abnormal elevation of ERK1 was an oncogenic factor for HCC. MicroRNA (miR) is a group of endogenous non-coding small RNA molecules in eukaryotes, and can bind with the 3'-untranslated region (3'-UTR) of a target gene mRNA via complementary binding patterns, to regulate targeted gene expression via degrading mRNA or inhibiting mRNA translation, thus participating in the regulation of various biological processes such as cell survival, proliferation, apoptosis and migration. The role of abnormal expression or function of microRNAs thus has drawn increasing

research interests in tumor resistance [12, 13]. Various pieces of evidence [14-16] have shown significantly decreased miR-205 expression in HCC tumor tissues and cells. Moreover, the down-regulation of miR-205 is correlated with onset, progression, metastasis and prognosis of HCC, indicating the tumor suppressor role of miR-205 in HCC pathogenesis and progression. Bioinformatics analysis showed the existence of complementary binding sites between miR-205 and ERK1 mRNA, suggesting possibly targeted regulation between those two sequences. This study aimed to investigate the role of miR-205 in mediating ERK1 expression affecting proliferation, apoptosis and cisplatin (DDP) drug sensitivity of HCC cells.

Materials and methods

Major reagents and materials

Normal human hepatocyte cell line HL-7702, HCC cell line Bel-7402/DDP, HepG2 cell and HEK293T cells were purchased from Procell (China). DMEM culture medium was purchased from Lonza (US). Serum-free Opti-MEM and fetal bovine serum (FBS) were purchased from Gibco (US). Trizol and Lipofectamine 2000 transfection reagent were purchased from Thermo (US). QuantiTech SYBR Green RT-PCR Kit was purchased from Qiagen (Germany). MiR-205 mimic, miR-205 inhibitor and miR-NC were purchased from RioBio (China). Rabbit anti-human ERK1 and p-ERK1 polyclonal antibody were purchased from Abcam (US). Rabbit anti-human β-actin polyclonal antibody was purchased from Abnova (US). Goat anti-rabbit HRP conjugated secondary antibody was purchased from Sangon Biotech (China). BeyoECL Plus developing reagent, Annexin V/PI cell apoptosis test kit and BCA protein quantification kit were purchased from Beyotime (China). Luciferase activity assay test kits, Dual-Glo Luciferase Assay System and pGL3 plasmid were purchased from Promega (US). EdU Flow Cytometry Kit and DDP were purchased from Sigma (US). Cell Counting Kit-8 was purchased from MedChemExpress (US). FORMA 3131 cell incubator was purchased from Thermo (US).

Cell culture

HL-7702, Bel-740 and HepG2 cells were incubated in RPMI 1640 medium containing 10% FBS, and were kept in a 37°C incubator containing 5% CO_2. RGM-1 cells were kept in DMEM/F12 medium containing 10% FBS, within a 37°C incubator containing 5% CO_2. Cells were passed at 1:4 ratios, and the cells in log-growth phase were used for further experiments.

Generation of DDP drug resistant cell model

For generating a DDP resistant cell model, Bel-7402 and HepG2 cells in log-growth phase were given DDP into their culture medium reaching a final concentration at 0.1 μg/mL. After 2-weeks of stable growth, the DDP concentration was gradually elevated to 0.2 μg/mL for another 2 more weeks maintaining incubation. Following this pattern, DDP treatment concentration was gradually increased to 0.4 μg/mL, 0.8 μg/mL and 1.6 μg/mL, until reaching stable growth of Bel-7402 and HepG2 cells at 1.6 μg/mL DDP for repeated passaging. The DDP drug resistant HCC cell lines were thus generated and named as Bel-7402/DDP and HpeG2/DDP.

Bel-7402, HepG2, Bel-7402/DDP and HepG2/DDP cells were inoculated into 96-well plates at 10^4 cells per well density. After 24 h of attached growth, the cell culture was mixed with 0, 0.1, 1, 10, 100 and 1000 μg/mL DDP, with 6 parallel replicates at each concentration. After 48 h of incubation, 10 μL CCK-8 solution was added into each well for 4 h reaction, and absorbance values at 450 nm wavelength (A450) were measured from each well. Inhibition rate was calculated at = (1-A450 of drug treatment group)/A450 of control group ×100%. SPSS software was used to calculate the concentration of drugs for inhibiting 50% cell growth (IC_{50}). Resistance index (RI) - IC_{50} of drug resistant cell/IC_{50} of parental cells.

Flow cytometry for measuring cell proliferation

EdU Flow Cytometry Kit was used to test cell proliferation. In brief, RPMI 1640 medium containing 10% FBS was used to re-suspend cells. After 2 h rinsing in 10 μM EdU at 37°C, cells were inoculated in a culture plate for 48 h of continuous incubation. Cells were digested by trypsin and were collected in PBS for centrifugation and rinsing. Cells were fixed in paraformaldehyde, and were rinsed with PBS for centrifugation. Next, 100 μL permeable buffer was added for cell rupture, and 500 μL reaction test

buffer was added for 30 min of dark incubation at room temperature. Three mL permeable buffer was added for centrifugation and washing, and cells were re-suspended in 500 μL wash buffer. Cell proliferation was measured by FC500MCL flow cytometry apparatus.

Dual luciferase activity assay

Full length or mutant forms of the 3'-UTR of the ERK1 gene sequence was amplified by PCR, and was digested by dual restriction enzymes for ligating into pGL3 plasmids. After transforming competent bacteria, plasmids with the correct sequence insertion were screened by sequencing and were named as pGL3-ERK1-WT and pGL3-ERK1-MUT. Lipo200 was used to co-transfect pGL3-ERK1-WT (or pGL3-ERK1-MUT) and miR-205 mimic (or miR-NC or miR-205 inhibitor) into HEK293T cells. Cells were kept in a 37°C incubator with 5% CO_2. After 48 of continuous incubation, a Dual-Glo Luciferase Assay System kit was used to measure the activity of dual luciferase.

Cell transfection and grouping

Cultured Bel-7402/DDP and HepG2/DDP cells were divided into a miR-NC transfection group and a miR-205 mimic transfection group. In brief, 100 μL serum-free Opti-MEM medium was used to dilute 10 μL Lipo2000, 50 nmolL miR-NC and 50 nmoL miR-205 mimic. After 5 min at room temperature incubation, Lipo2000 was gently mixed with miR-NC and miR-205 mimic for 20 min at room temperature. The transfection mixture was added into culture medium for gentle mixture and 72 h of incubation. Cells were then collected.

Cells from the four treatment groups were inoculated into 6-well plates. When reaching 50% confluence, 1.6 μg/mL DDP was added for treatment. After 48 h of continuous incubation, cell apoptosis was measured by flow cytometry as described below.

Cells from the four treatment groups were collected by trypsin digestion. After 2 h of incubation in 10 μM EdU, cells were kept for 48 h using 1.6 μg/mL DDP as described in previous sections. EdU cell proliferation test kit was used for measuring cell proliferation potency.

qRT-PCR for measuring gene expression

Trizol was used to extract cellular RNA. Gene expression was measured by one-step qRT-PCR using QuantiTest SYBR Green RT-PCR Kit. In a 20 μL qRT-PCR reaction system, we added 10.0 μL 2XQuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL forward and reverse primer (0.5 μm/L), 2 μg template RNA, 0.5 μL QuantiTect RT Mix, and ddH_2O up to 20.0 μL. Reaction conditions of qRT-PCR were: 45°C for 5 min, and 94°C for 30 s, followed by 40 cycles each consisting of 95°C for 5 s and 60°C for 30 s. Gene expression was measured on CFX96 real-time fluorescent quantitative PCR cycler (Bio-Rad, US).

Western blot

One hundred μL RIPA lysis buffer was added into each well with 1×10^6 cells for protein extraction. BCA approach was used to measure quality and quantity of protein, and 40 μg samples were loaded for separation (45 V, 150 min) using SDS-PAGE (12% separating gel and 4% condensing gel). Proteins were then transferred to PVDF membrane (250 mA, 100 min). The membrane was then blocked using 5% defat milk powder at room temperature. Primary antibody (ERK1 at 1:500, p-ERK1 at 1:1000, β-actin at 1:6000) was added for 4°C overnight incubation. On the next day, the membrane was washed with PBST three times. HRP conjugated secondary antibody (1:10000 dilution) was added for 60 min at room temperature, followed by three PBST rinses. BeyoECL Plus developing reagent was added for 2~3 min in dark incubation. The film was then exposed for scanning and data storage.

Cell apoptosis assay

Cells were collected and digested by trypsin. After PBS centrifugation rinsing, cells were re-suspended in 100 μL Annexin V-FITC and 5 μL PI were sequentially added. After 15 min room temperature incubation, 400 μL Annexin V Binding Buffer was added. Cell apoptosis was measured using FC500MCL flow cytometry (Beckman Coulter, US).

Statistical analysis

SPSS 18.0 was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Student t-test was used for comparing measurement data between two groups. One-way analysis of variance (ANOVA) was used to compare measurement data from multiple groups, followed by

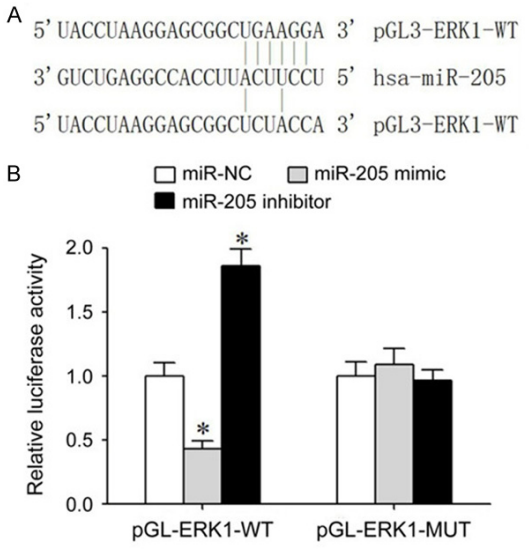

Figure 1. Targeted regulation between miR-205 and ERK1 mRNA. A. Illustration of the functional site between miR-205 and 3'-UTR of ERK1 mRNA. B. Dual luciferase gene reporter assay. *, $P<0.05$ compared to miR-NC group.

Bonferroni test in between-group comparison. Statistical significance was defined when $P<0.05$.

Results

Targeted regulatory relationship between miR-205 and ERK1 mRNA

Bioinformatics analysis showed the existence of complementary binding sites between miR-205 and 3'-UTR of ERK1 mRNA (**Figure 1A**). Dual luciferase gene reporter assay showed that transfection of miR-205 mimic remarkably depressed relative luciferase activity of HEK-293T cells transfected with pGL3-ERK1-WT, and miR-205 inhibitor transfection was remarkably elevated relative to luciferase activity of HEK293T cells transfected with pGL3-ERK1-WT. Furthermore, transfection of miR-205 mimic or miR-205 inhibitor had no significant effect on the relative luciferase activity of HEK293T cells transfected with pGL3-ERK1-MUT (**Figure 1B**), indicating that miR-205 could target 3'-UTR of ERK1 mRNA to suppress its gene expression.

Relative strong apoptotic resistance of drug resistant HCC cells

Bel-7402 cells presented IC_{50} at 1.34±0.09 μg/mL, whilst drug resistant cells Bel-7402/DDP showed IC_{50} at 12.75±0.81 μg/mL. BGC823/DDP cells had a RI of 9.513. HepG2 cells had IC_{50} value at 1.89±0.15 μg/mL, whilst HepG2/DDP cells had IC_{50} at 21.37±1.93 μg/mL, making the RI 11.31.

Under treatment of 1.34 μg/mL DDP, Bel-7402/DDP cells presented relatively higher apoptotic rate reaching 31.6±3.2%, whilst Bel-7402/DDP cells had an apoptotic rate only at 4.2±0.7% (**Figure 2A**). Under 1.89 μg/mL DDP treatment, HepG2 cells presented an apoptotic rate as high as 22.6±2.5%, whilst HepG2/DDP cells had an apoptotic rate only at 3.3±0.6% (**Figure 2B**).

Down-regulation of miR-205 and up-regulation of ERK1 in drug resistant HCC cells

qRT-PCR results showed that compared to HL-7702 cells, HCC cells presented remarkably depressed miR-205 expression, which was higher than drug resistant HCC cells (**Figure 3A**). qRT-PCR results also showed that compared to HL-7702 cells, HCC cells had remarkably decreased ERK1 mRNA expression, which was even higher in drug resistant cells (**Figure 3B**). Western blot results showed that compared to HL-7702 cells, HCC cells had significantly elevated ERK1 protein expression, which was even up-regulated in drug resistant cells (**Figure 3C**).

Over-expression of miR-205 can induce HCC cell apoptosis and suppress DDP resistance

qRT-PCR results showed that compared to the miR-NC group, miR-205 mimic transfection effectively suppressed ERK1 mRNA expression in Bel-7402/DDP and HepG2/DDP cells (**Figure 4A**). Western blot results showed that transfection of miR-205 mimic can significantly decrease expression of ERK1 and p-ERK1 protein in Bel-7402/DDP and HepG2/DDP cells (**Figure 4B**). Flow cytometry results showed that transfection of miR-205 mimic could remarkably elevate apoptosis of Bel-7402/DDP and HepG2/DDP cells (**Figure 4C**), whilst cell proliferation potency was remarkably depressed (**Figure 4D**).

Discussion

The ERK/MAPK signal transduction pathway is widely expressed in various tissues and cells, and can regulate multiple biological processes

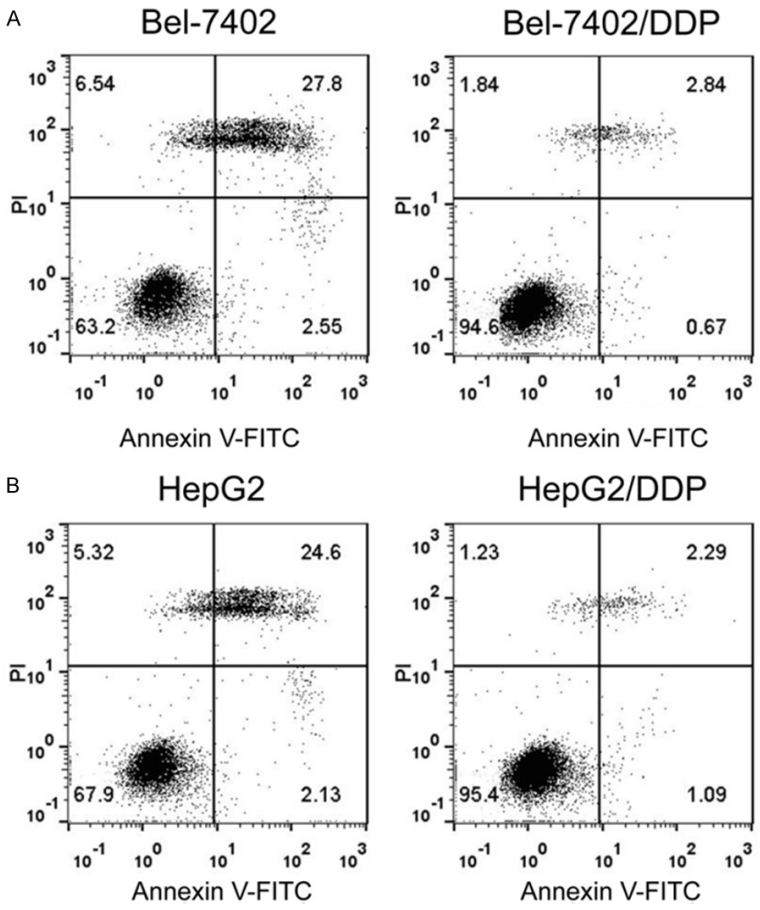

Figure 2. Strong apoptotic resistance of drug resistant HCC cells. A. Flow cytometry measuring the apoptotic rate of Bel-7402 and Bel-7402/DDP cells. B. Flow cytometry for cell apoptosis of HepG2 and HepG2/DDP cells. *, $P<0.05$ comparing between two groups.

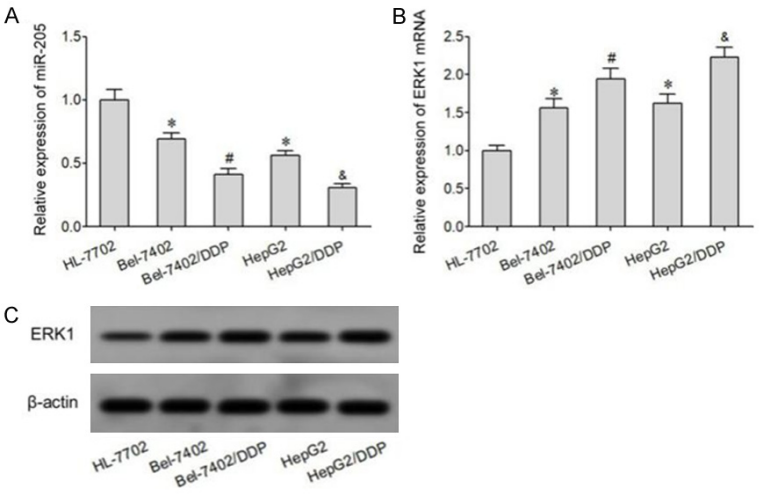

Figure 3. MiR-205 down-regulation and ERK1 up-regulation in drug resistant HCC cells. A. qRT-PCR for miR-205 expression; B. qRT-PCR ERK1 mRNA expression; C. Western blot for ERK1 protein expression. *, $P<0.05$ compared to HL-7702 cells; #, $P<0.05$ compared to Bel-7402 cells; *, $P<0.05$ compared to HepG2 cells.

including cell proliferation, cell cycle, apoptosis, migration and invasion [17, 18]. The over-excitation of the ERK/AMPK signaling pathway causes abnormal proliferation, apoptosis and differentiation deficits of cells, and is closely related with the onset, progression and metastasis of multiple tumors including oral cavity carcinoma, esophagus cancer and lung cancer [19-21]. The ERK/MAPK signal transduction pathway mainly consists of the small G proteins Ras, Raf kinase, MEK and ERK. The ERK/MAPK signal transduction pathway fits the classical three step enzymatic cascade reaction, and has similar activation models under stimuli by various factors. The MAPK signaling pathway family mainly consists of four transducing pathways: ERK, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), and ERK5/big MAP kinase 1 (BMK1). Among those the ERK induced MAPK pathway is believed to be the classical MAPK signal transduction pathway [17, 18, 22]. As an important downstream signal transducing molecule of the ERK/MAPK pathway, ERK1 plays crucial roles in activating the ERK/MAPK pathway for downstream signal transducing [23, 24]. MiR-205 is a microRNA molecule that is frequently studied. Current knowledge revealed the tumor suppressor gene role of miR-205 in multiple cancers, as down-regulation of miR-205 plays important roles in onset and progression of multiple tumors including cervical carcinoma, endometrial carcinoma and breast cancer [25-27]. Multiple studies [14-16] demonstrated the

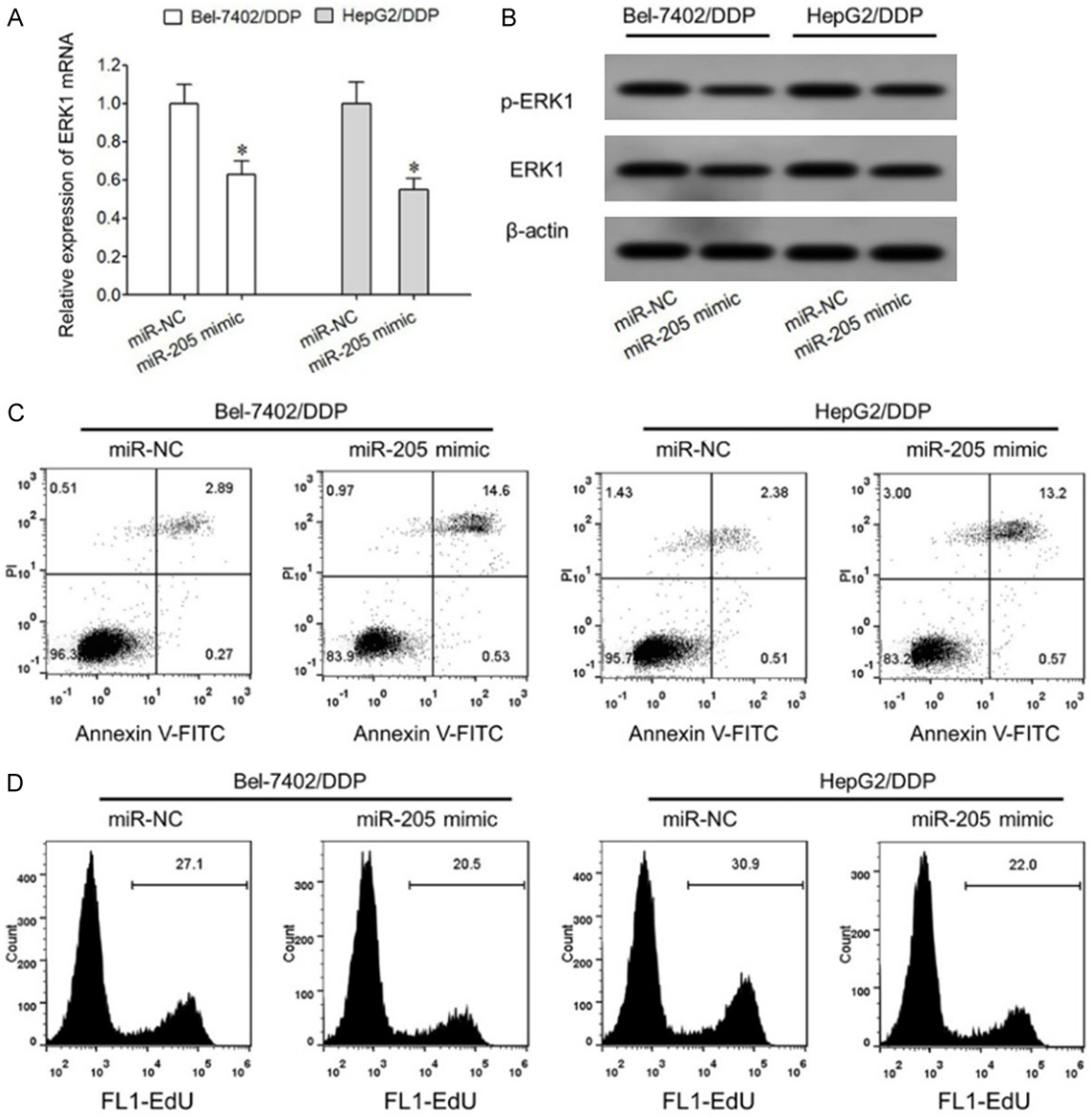

Figure 4. Over-expression of miR-205 induces HCC cell apoptosis and depresses DDP resistance. A. qRT-PCR of ERK1 mRNA expression. B. Western blot of protein expression. C. Flow cytometry of cell apoptosis. D. Flow cytometry of cell proliferation. *, $P<0.05$ compared to miR-NC group.

prominent down-regulation of miR-205 in tumor tissues and cells of HCC patients, and the involvement of miR-205 down-regulation in the onset, progression, metastasis and prognosis of HCC, indicating the tumor suppressor gene role of miR-205 in HCC pathogenesis and progression. Bioinformatics analysis showed the existence of complementary binding sites between miR-205 and ERK1 mRNA, suggesting possibly targeted regulation between these two sequences. This study thus investigated if miR-205 plays a role in modulating ERK1 expression, and in affecting HCC cell proliferation, apoptosis and DDP drug resistance.

Dual luciferase gene reporter assay showed that transfection of miR-205 mimic remarkably decreased relative luciferase activity of HEK293T cells transfected with pGL3-ERK1-WT, and miR-205 inhibitor transfection effectively elevated relative luciferase activity in HEK293T cells transfected with pGL3-ERK1-WT, confirming the targeted regulation between miR-205 and ERK1. Based on CCK-8 assay, we

calculated IC_{50} of parental and drug resistant cell lines against DDP. Results showed that drug resistant HCC cells had significantly higher IC_{50} of drug resistant HCC cells than parental cell line, along with higher apoptotic resistance, suggesting successful generation of DDP resistant HCC cells. Comparison of parental and drug resistant HCC cells showed that resistant cells had significantly decreased miR-205 expression than parent cells, whilst ERK1 mRNA and protein expression was significantly higher. Results showed that miR-205 down-regulation was correlated with ERK1 over-expression, and the abnormal expression of both genes is related to malignant biological features and drug resistance of HCC cells. In a correlation study between miR-205 and HCC, Lu et al found that HCC patients had significantly decreased miR-205 expression in tumor tissues compared to tumor adjacent tissues [14], suggesting the involvement of miR-205 expression level in tumor metastasis, patient survival and prognosis. Moreover, abnormal suppression of miR-205 expression was also found in the HCC cell line HepG2. Zhang et al found that comparing to tumor adjacent tissues, HCC patients had abnormally decreased miR-205 expression in tumor tissues, and such down-regulation was caused by the targeted inhibition by lncRNA LINC00673 [28]. Zhang et al also found that compared to tumor adjacent tissues, HCC tissues had abnormally decreased miR-205 expression, and such down-regulation was caused by HBVX protein induced hypermethylation of the miR-205 gene promoter [29]. All these studies suggested the tumor suppressor role of miR-205 in HCC, and the involvement of miR-205 down-regulation in HCC, as similar with our observation.

Currently few studies have been performed regarding the relationship between miR-205 and drug resistance of HCC. This study thus further explored if miR-205 can modulate DDP resistance of HCC cells via modulating ERK1. Test results showed that miR-205 mimic transfection can significantly decrease protein expression of ERK1 and p-ERK1 in drug resistant HCC cells, and remarkably enhanced apoptosis of resistant cancer cells that originally could maintain stable growth in DDP, plus prominent inhibition on cell proliferation and enhanced DDP drug sensitivity. In a study for the role of miR-205 in modulating biological effects of HCC cells, Zhang et al showed that compared to normal human hepatocyte THLE-3 and L02, HCC cell lines HCCLM3, MHCC97L and HepG2 had remarkably decreased miR-205 expression, and the proliferation, clonal formation and invasion potency of those tumor cells were enhanced after suppressing miR-205 expression using inhibitors [28]. Lu et al showed the over-expression of miR-205 in HCC cells remarkably inhibited epithelial-mesenchymal transition (EMT) process of HCC cells and weakened the migration or invasion potency of HCC cells via targeted inhibition on SEM4C gene expression [14]. Zhang et al showed that up-regulation of miR-205 expression in HCC cells can antagonize the pro-proliferation function of HBVX protein on HCC cells, exerting its tumor suppressor gene role [29]. Zhao et al found that compared to tumor adjacent tissues, miR-205 expression was remarkably depressed in HCC tissues, and lower miR-205 expression indicated worse prognosis [16]. A study on biological effects showed that miR-205 down-regulation was correlated with enhanced stem properties of liver cancer stem cells: the over-expression of miR-205 can suppress stem cell properties of HCC cells and weaken the stem cell clonal formation potency of HCC cells via targeting PLCβ1 gene expression. This study combined the targeted regulation between miR-205 and ERK1, and revealed the targeted inhibition on ERK1 expression by miR-205 to suppress ERK1 phosphorylation activity, and to induce HCC cell apoptosis and to suppress DDP resistance, all of which have not been reported before and are hence a novelty of this study. However, whether miR-205 regulation on ERK1 expression is related with drug resistance of HCC patients is not clear yet, and requires further assays for substantiation to fulfill the weakness of this study.

Conclusion

miR-205 down-regulation is correlated with DDP resistance of HCC cells. Over-expression of miR-305 can induce HCC cell apoptosis and decrease its DDP drug resistance via targeting inhibition on ERK1 expression and suppression of ERK1 phosphorylation activity.

Disclosure of conflict of interest

None.

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