Original Article

miR-205 Reverses MDR-1 Mediated Doxorubicin Resistance via *PTEN* in Human Liver Cancer HepG2 Cells

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Abstract

Objective: The aim of the recent study was to investigate the effects of *miR-205* on reversing Doxorubicin (DOX) resistance, as chemotherapeutic agents through up-regulation of *PTEN* in human liver cancer HepG2 cells.

Materials and Methods: In this experimental study, the drug resistance in liver cancer cells via drug efflux inhibition and enhancing apoptosis by the regulation of *PTEN* and multi-drug resistance/ P-glycoprotein (MDR/P-gp) expression was revealed. Using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, effect of DOX on cell proliferation was evaluated after *miR-205* transfection in HepG2 and HepG2/DOX cells. Activity of P-gp on drug efflux was measured by the Rhodamine 123 (Rho-123) assay. *PTEN* mRNA expression levels were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and flow cytometry was used to measure the apoptotic ratio of HepG2/DOX cells.

Results: *miR-205* overexpression considerably inhibited the HepG2/DOX cells viability (P<0.05). qRT-PCR results revealed that *PTEN* is a pivotal regulator in PI3K/Akt/P-gp axis. Overexpression *miR-205* resulted in up-regulation *PTEN* and ultimately down-regulation of P-gp. This inhibits drug resistance, proliferation and induces apoptosis in HepG2/DOX cells (P<0.05). Whilst, treatment with 10 μM of special inhibitors, including LY294002 (PI3K) or PD098059 (MAPK), increased Rho 123-associated MFI, treatment with 10 μM of SF1670 (*PTEN*) almost abolished the effect of miR-205 overexpression (P<0.05). Finally, we found that *miR-205* was down-regulated in HepG2/DOX cells, and its overexpression led to enhancing apoptosis with re-sensitization of HepG2/DOX cell lines to DOX through *PTEN*/PI3K/Akt/MDR1 pathway.

Conclusion: These findings may introduce *miR-205* as a predictive biomarker and a potential treatment target for liver cancer therapy during MDR.

Keywords: Drug Resistance, Liver Cancer, *miR-205*, P-Glycoprotein, *PTEN*

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Introduction

Liver cancer is one of the three most common causes of cancer-related mortality, all over the world. Considering high incidence of liver cancer, chemotherapy and prognosis of this malignancy are the primary focus of medical research (1). Although surgery and transplants applied as therapeutics for patients with early-stage of liver cancer, in many cases they are diagnosed when the disease has reached the stage beyond curative surgery (2). In this regard, cell lines that express differentiated hepatic functions have been used in liver studies, as an alternative to the cultured hepatocytes. HepG2 is a typical hepatoma cell line that survives well in various environments. Besides, it is widely applied for drug development and pharmaco-toxicological testing (3, 4).

Routinely, systemic chemotherapy serves a crucial role in the treatment of modalities for advanced liver cancer. Lamentably, systemic chemotherapy is usually inadequate because of the resistance of cancer cells to chemotherapeutic drugs, causing failure of this curative method (5). Doxorubicin (DOX) is a routinely anthracycline drugs applied as chemotherapeutic agents, particularly in patients with advanced or metastasis cancer, inducing apoptosis of these tumor cells. Mechanically, DOX represses topoisomerase II (Top II) and intercalates directly to DNA double-strand, finally resulting in the intervention of gene transcription (6, 7). Notwithstanding, DOX is extensively applied for treatment of cancers, drug-resistance largely restricted the clinical employment of DOX (8). In this case, resistance to a broad spectrum of chemotherapeutic agents in neoplasm is called multi-drug resistance (MDR) and it is the focal problem in chemotherapy (9-11). Many relevant mechanisms for MDR have been described and discussed, one of which overexpressed the plasma membrane drug efflux pumps P-glycoprotein (P-gp/ABCB1). It is encoded by *MDR1* gene and belongs to ATP-binding cassette (ABC). It is also involved in MDR (9, 12). Indeed, P-gp reduces the

intracellular accumulation of anti-cancer drugs including DOX to sub-therapeutic levels, thus decreasing or abolishing chemotherapy efficacy of this drug (13).

Among this, Akt/PKB is a pivotal element for protecting cells from undergoing programmed cell death. Thus, PKB-mediated survival-pathway is an attractive target for cancer chemotherapy. Loss of tumor suppressor gene "phosphate and tensin homolog deleted on chromosome 10" (*PTEN*) is common in cancer tumors, constitutively activating Akt (14). Phosphatase activity of *PTEN* is crucial in controlling the phosphatidylinositol-3 kinase (PI3K)/Akt signal transduction pathway. In this regard, down-regulation of *PTEN* in tumor cells may be associated with poor tumor prognosis and drug resistance (12). Indeed, inhibition of PI3K/Akt pathway can reverse MDR and sensitize tumor cells to these agents (12, 15).

Developing inhibitors of ABC transporters-based MDR is one of the solutions for MDR in carcinomas in the recent decades (16). However, with regard to the undesired side-effects and toxicity issues, these drugs are failed in the clinic (17). Therefore, combined therapy with some chemo-sensitizers agents is desirable to improve the anti-cancer effect to overcome DOX-resistance.

So far, several negative associations have identified between gene expression and MDR in human hepatoma cells (18). In the framework of modulate direct posttranscriptional regulation of gene expression, it is widely identified that microRNAs (miRNAs, also known as "miRs") are a class of short endogenous non-protein-coding single-stranded RNAs, which by binding to partially complementary sites within the 3′-untranslated region (3′-UTR) of target mRNAs, through Watson-Crick base pairing postulate, result in translational suppression or mRNA deadenylation and degradation (19). Growing evidence has illustrated that miRNAs have critical role in various biological processes such as cell survival, proliferation, differentiation, migration, invasion, sensitivity to chemotherapy and so on (20, 21). Recent studies have revealed that many specific miRNAs are capable of modulating MDR in cancers (22). miRNAs can function either as a promoter or suppressor to regulate MDR in cancers (23). Among this, *miR-205* is one of the best-studied conserved miRNAs, located in chromosome 1q32.2 and expressed in squamous epithelium-derived tissues as well as the mammary gland progenitor cells (24). *miR-205* targets *PTEN* and leucine-rich repeat protein phosphatase 2 to increase Akt pathway and drive malignant phenotypes in non-small cell lung (NSCL) tumors (25). Previously findings revealed that *miR-205* improves the chemosensitivity of breast cancer cells to TAC (docetaxel, doxorubicin plus cyclophosphamide) chemotherapy by repressing both VEGFA and FGF2, leading to apoptosis (20). Therefore, better understanding the role of miRNA during MDR chemotherapy may provide new avenues for liver cancer diagnostic and treatment. In this study, miRNA targeting MDR-associated molecules has been illustrated, as potentially an effective factor in cancer therapy.

Overall, the aim of the recent study was to investigate the effects of *miR-205* on reversing DOX resistance, as chemotherapeutic agents through up-regulation of *PTEN* in human liver cancer HepG2 cells. For this purpose, we examined the pivotal role of *miR-205* on P-gp, as a member of ABC transporters through the *PTEN*/PI3K/Akt/MDR1/ABCB1/P-gp signaling pathway.

Materials and Methods

The multidrug resistance in liver cancer cells through drug efflux inhibition and apoptosis enhancement by the regulation of *PTEN* and MDR/P-gp expression was evaluated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Effect of DOX on cell proliferation was tested after *miR-205* transfection in HepG2 and HepG2/DOX cells. P-gp activity on drug efflux was measured by Rhodamine 123 (Rho-123) assay. *PTEN* mRNA expression levels were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and flow cytometry was utilized to calculate the apoptotic ratio of HepG2/DOX cells.

This study was performed at the Shaanxi Provincial Cancer Hospital under Ethical Committee permission (SXNo.20190728).

Cell culture and cell transfection

In this experimental study, the human liver cancer cell line HepG2 and DOX-resistant cell line HepG2/DOX was obtained from National Cell Bank of China. All cells were cultured in DMEM basic medium with 0.33% sodium bicarbonate (Gibco, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco, USA), streptomycin (100 µg/ml), penicillin G (100 IU/ml; both from Sigma Aldrich, USA), and incubated at 37˚C in a humidified (vol/vol) atmosphere of 5% CO_2. HepG2/DOX cells were seeded and cultured in the presence of 0.5 µM of DOX or DOX-free medium for two weeks before investigations. Sequence of the miR-205 mimic was 5′-UCCUUCAUUCCACCGGAGUCUG-3′. Cells (3×10^5 cells/2 ml/well) were seeded in six-well microplates at 70% confluence. After 48 hours, the *miR-205* mimic or the negative control was transfected into the cells using Lipofectamine™ 2000 (Life Technologies, USA), at a final concentration of 50 nM, according to the manufacturer's protocols. In brief, the *miR-205* mimic or the negative control was mixed with 100 µl Lipofectamine 2000 in 12 ml culture medium and divided into per well of six-well microplates. Twenty-four hours after transfection, the

media was replaced with fresh medium and the cells were incubated for an additional 24 hours.

RNA extraction, cDNA synthesis and quantitative reverse transcription polymerase chain reaction

Briefly, 2×10^6 cells/2 ml/well was seeded onto six-well plates for 24 hours. According to the manufacturer's instructions, total RNA was extracted from the cells using a TRIzol® reagent (Invitrogen Life Technologies, USA). Complementary DNA (cDNA) was synthesized at 37˚C for 15 minutes and 95˚C for 10 minutes using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, USA). next, quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (TaKaRa Bio, Japan) and specific primers in the Roche Light Cycler® 96 System (Roche, Germany). PCR amplification was performed as follow: 5 minutes of primary denaturation at 95˚C, 40 cycles of 95˚C for 45 seconds, 57˚C for 30 seconds and 72˚C for 60 seconds, followed by a final incubation at 72˚C for 5 minutes.
The applied primers include:

miR-205-

F: 5′-GCTCCTTCATTCCACCGG-3′
R: 5′-CAGTGCAGGGTCCGAGGT-3′

MDR-1-

F: 5′-CCCATCATTGCAATAGCAGG-3′
R: 5′-TGTTCAAACTTCTGCTCCTGA-3′

PTEN-

F: 5′-GGACGAACTGGTGTA-3′
R: 5′-CCTCTGACTGGGAATAG-3′

β-actin-

F: 5′-AAGCTGTGTTACGTGGCCCT-3′
R: 5′-GGGCAGCTCGTAGCTCTTCT-3′

All experiments were carried out in triplicates. Raw data were analyzed with the comparative Ct method using *β-actin* as an internal control gene.

Biological analysis by MTT assay

The effect of DOX on cell viability in liver cancer cell lines growth was evaluated by MTT assay. HepG2 and HepG2/DOX cells were seeded onto 96-well plates (1×10^4 cells/200 µl/well) and incubated overnight and transfected with *miR-205* at 37˚C. Upon 24 hours incubation, both cell lines were treated with diverse concentrations of DOX for 48 hours. Subsequently, the cells were treated with 10% (5 mg/ml) 20 µl MTT (Sigma Aldrich, USA) was added to each well for another 4 hours. Next, 200 µl dimethyl sulfoxide (DMSO) was added to each well for dissolving formazan product. Optical density was measured at 490 nm using a Benchmark Plus microplate spectrometer (Bio-Rad laboratories, USA). The fold reversal (FR) of MDR was determined by dividing IC_{50} value of the cells treated with the indicated anticancer drug in the absence of a modulator by IC_{50} value of the cells treated with the same anticancer drug in the presence of a modulator. Absorbance values were normalized, allocating the value of the cells in medium without drug to 1.0 and the value of the no-cell control to 0.

Rho 123 efflux assay

To visualize the intracellular accumulation of P-gp and its level, the substrate (Rho 123) was applied. 1×10^6 cells/2 ml/well was seeded onto six-well plates. After 24 hours incubation, the cells were treated with serum-free medium containing 3.4 µM Rho 123 in the presence or absence of *miR-205* and they were incubated for 2 hours at 37˚C. Verapamil was used as positive control for P-gp inhibition. The intracellular mean fluorescence intensity (MFI) associated with intracellular Rho 123 content was determined using FP-6200 fluorometer (Jasco Co., Japan).

Flow-cytometric analysis

Flow-cytometry was used to analyze the apoptotic rate of cells. The HepG2/DOX Cells (1.5×10^6 cells/2 ml/well) were seeded onto 6-well microplate after 70% confluence, incubated overnight and transfected with *miR-205* at 37˚C. The cells were then centrifuged at 1500 × g for 5 minutes at RT. The cell pellets were suspended in 10 µl of fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI) solution for 10 minutes at dark in RT and then analyzed using a fluorescence-activated cell sorting (FACS) flowcytometer (BD LSR; Becton-Dickinson, USA) and Cell Quest software (Becton-Dickinson, USA).

Statistical data analysis

All experiments from at least three independent repeated tests were performed and analyzed using analysis of variance (ANOVA). Bonferroni's and Sidak's tests were used to ascertain the statically significant differences between the groups. Data analysis was performed with GraphPad Prism v6 software (USA) to examine significant differences. The results are expressed as the means ± standard deviation (SD), and $P<0.05$ was considered statistically significant.

Results

Down-regulation of *miR-205* resulted in viability enhancement of HepG2/DOX cells in response to DOX compared to parental cells

To examine the possible role of *miR-205* in the HepG2/DOX cell, qRT-PCR analysis was firstly applied. The result of these investigations demonstrated that expression level of *miR-205* was higher in the HepG2 cells compared to the HepG2/DOX cells, following transfection of *miR-205* (Fig.1), which revealed that *miR-205* may be inversely correlated with DOX resistance in liver tumor cells. Next, we assessed the effect of DOX on cell viability using the MTT assay. As shown in (Fig.1), DOX (concentration range from 0.01 to 10 µM) revealed lower cytotoxicity towards the drug resistant cells compared to the parental cells causing enhancement of the viability of HepG2/DOX cell lines. The IC_{50} values for DOX were 0.21 µM in

HepG2 and 2.84 μM in HepG2/DOX cells, showing 13.52-fold resistance to DOX, compared to the HepG2 cells.

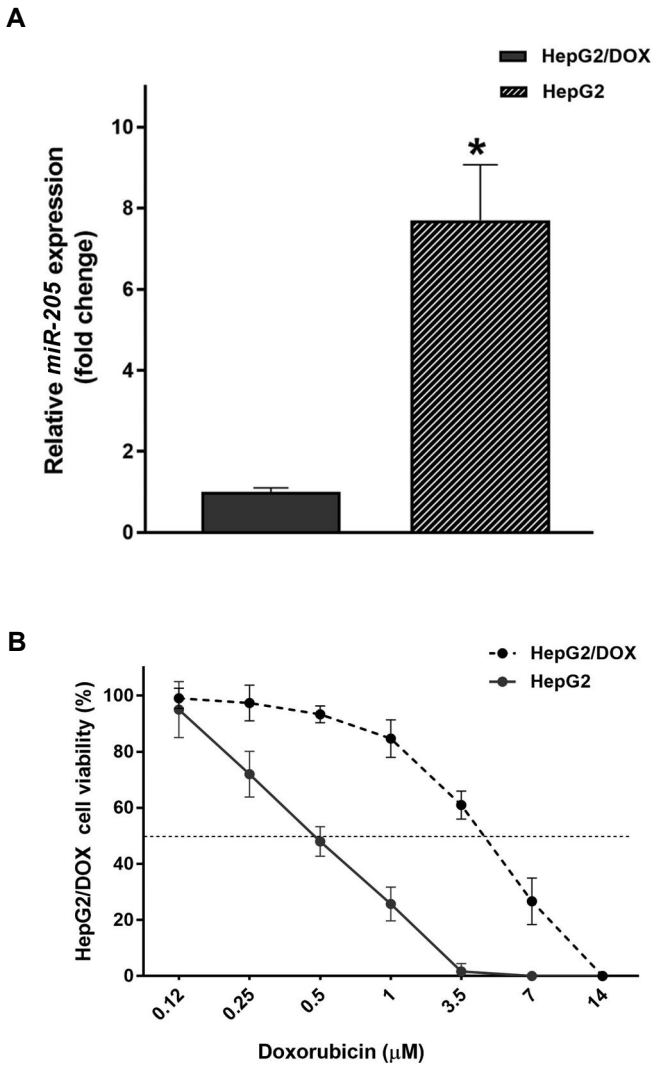

Fig.1: Expression levels of *miR-205* and sensitivity of HepG2 and HepG2/DOX cells to Doxorubicin (DOX). **A.** Expression levels of *miR-205* in HepG2 and HepG2/DOX cells. **B.** Cells were treated with versatile concentrations of DOX. After 48 hours of incubation, viability rates of the cells were measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each point represents the mean ± SD (n=3 independent experiments, *; P<0.05 compared to HepG2/DOX cells).

Overexpression of *miR-205* resulted in inhibiting viability and inducing apoptosis of HepG2/DOX cells

To ascertain the correlation between *miR-205* and HepG2/DOX cell lines, the effect of up-regulation of *miR-205* on the DOX-resistance cells was analyzed. The treated HepG2 cells with DOX and DOX+*miR-205* exhibited equal cytotoxicity (Fig.2A). In contrast, HepG2/DOX cells transfected with DOX+*miR-205* showed statically significant less survival rates than DOX group (Fig.2B). These results showed that overexpression of *miR-205* can sensitize the HepG2/DOX cells to DOX.

Flow-cytometric analysis was conducted to assess the impact of HepG2/DOX cells by Annexin V–FITC and PI staining, and apoptotic cells were detected 48 hours after *miR-205* transfection. The lower right quadrant indicates pro-apoptotic cell rate. This revealed that combination of *miR-205* with DOX enhanced migration of the HepG2/DOX cells into apoptotic regions compared to the DOX group (Fig.2C).

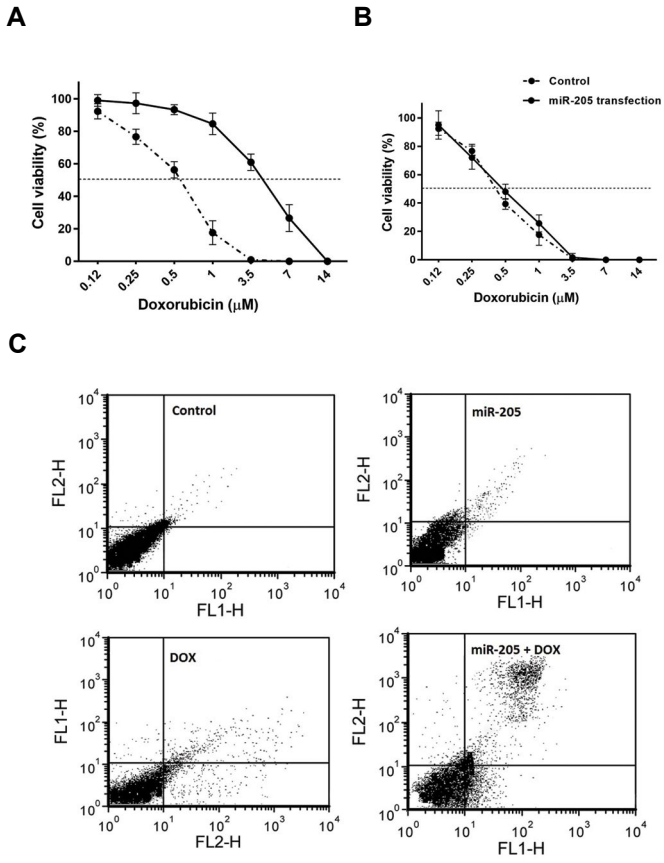

Fig.2: Effects of *miR-205* transfection on Doxorubicin (DOX) cytotoxicity and apoptotic analysis of HepG2/DOX cells. Upon culture for 48 hours after transfection with the *miR-205*, the **A.** HepG2/DOX cells and **B.** HepG2 cells were incubated with various concentrations of DOX for 48 hours and cell viability was defined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **C.** Then, cells were stained with Annexin V–FITC and PI 48 hours after transfection with *miR-205*. Control, *miR-205*, DOX 1 μM and *miR-205* plus DOX 1 μM. Each point represents mean ± SD (n=3 independent experiments, *; P<0.05 compared to control).

Overexpression of *miR-205* alleviated P-gp activity in HepG2 cell line

P-gp activity was determined using intracellular Rho 123 accumulation assay. Verapamil was used as a positive control agent. Figure 3 obviously demonstrates that *miR-205* enhanced Rho 123 accumulation in the HepG2/DOX cells after 2 hours, while the HepG2 cells did not show such enhancement when transfected with *miR-205*. Indeed, transfection of *miR-205* in the HepG2/DOX cells with the Rho 123 resulted in enhancement in the MFI (P<0.05). This effect is comparable with that observed in the HepG2/DOX cell lines treated with verapamil. Nevertheless, no accumulation of substrate was recognized in parental HepG2 cells over the same period. Therefore, it was proposed that activity of P-gp was inhibited via *miR-205* in HepG2/DOX cells.

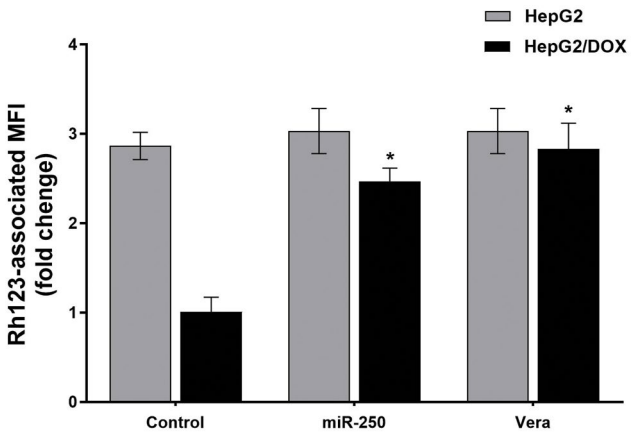

Fig.3: Effect of *miR-205* on the intracellular accumulation of Rho 123. Upon culture for 48 hours after treatment, the cells were incubated with 5 μM of Rho 123 in the presence or absence of *miR-205* and 10 μM of verapamil for 2 hours and then, Rho 123-associated mean fluorescence intensity (MFI) was measured. Each point depicts the mean ± SD from four tests. *; P<0.05 compared to control.

Overexpression of *miR-205* reversed resistance through down-regulation of MDR-1 in HepG2 cell line

Herein, we extended our work to a HepG2/DOX cell to evaluate whether the observed effects were cancer cell type-specific or they might be more generally applicable. PCR analyses showed while the HepG2/DOX cells expressed significant amounts of MDR-1, no MDR-1 was detectable in the parental HepG2 cells. These data robustly showed that the HepG2/DOX cell line was phenotypically MDR and this MDR phenotype was correlated to up-regulation of P-gp (Fig.4). We found out that *miR-205* down-regulated P-gp in the mRNA levels in HepG2/DOX cells. This can describe the elevated intracellular accumulation of DOX and cytotoxicity in the *miR-205*-treated HepG2/DOX cells.

Inhibition of *PTEN* abolished *mi*R-*205* effects on DOX-mediated resistance in HepG2 cell line

We speculated that *miR-205* may play crucial role in the inhibition of MDR-1 expression by down-regulating PI3K/Akt signaling pathway activity. Therefore, to further scrutinize the fundamental molecular mechanisms, we investigated the MDR-reversing effect of *miR-205* in the presence of special inhibitors of *PTEN* (SF1670) and PI3K (LY294002). As shown in Table 1, compared to the control group, DOX cytotoxicity was heightened in the *miR-205*-treated group. The IC_{50} values (μM) were diminished from 2.84 ± 0.37 to 0.32 ± 0.09, 0.29 ± 0.08, 0.38 ± 0.08, and 1.99 ± 0.31 in control (DOX), *miR-205*+DOX, *miR-205*+LY294002 10 μM+DOX, *miR-205*+SF1670 10 μM+DOX, respectively. However, *miR-205* effect was significantly attenuated by SF1670 treatment. In addition, results revealed that FR in *miR-205*+SF1670 10 μM+DOX was significantly different from *miR-205*+DOX. After transfection with *miR-205*, cultured for 48 hours. The effect of SF1670 and LY294002 on the *miR-205*-induced intracellular Rho 123 related MFI accumulation was observed in HepG2/DOX cells. Indeed, treatment with 10 μM LY294002 enhanced Rho 123-correlated MFI and treatment with 10 μM SF1670 almost abrogated the action of *miR-205* overexpression (Fig.5A).

Table 1: Cytotoxicity effect of DOX and reversing MDR in HepG2/DOX cells role of miR-205 and co-exposure with LY294002 and SF1670 as selective inhibitors

Treatment	HepG2/DOX	
	IC_{50} (μM)	FR
Control (DOX)	4.54 ± 0.57	-
miR-205+DOX	0.52 ± 0.8[a]	8.65
miR-205+LY294002 10 μM+DOX	0.49 ± 0.08[a, b]	9.26
miR-205+SF1670 10 μM+DOX	3.69 ± 0.31	1.23

MTT reduction activity was used to determine cell viability. Each value represents the mean ± SD of three independent tests. The fold reversal (FR) of MDR was distinguished by dividing the IC_{50} of control to IC_{50} of each test. Statically significant different from control at [a]; P<0.05, No significant difference from miR-205+DOX group [b]; P>0.05.

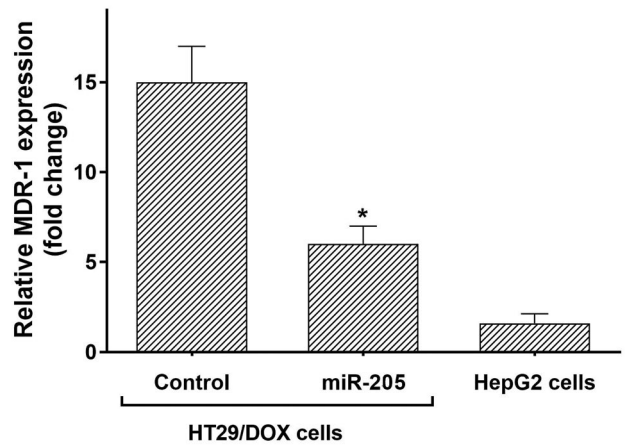

Fig.4: Effect of *miR-205* on the MDR-1 expression in HepG2 and HepG2/DOX cells. *miR-205* down-regulated MDR-1 in HepG2/DOX cells. Each point represents the mean ± SD from four experiments (n=3, *; P<0.05 compared to control).

In this regard, our findings clearly demonstrated that *miR-205* in HepG2/DOX cells, 48 hours after transfection, attenuated *MDR-1* mRNA. Besides, SF1670 suppressed the effects of *miR-205* (Fig.5B, C). Further examinations showed that in the presence of 10 μM SF1670, eliminated *miR-205* effects on P-gp expression was noted. However, no similar effect with 10 μM ofLY294002 was noticed. Overall, these results illustrated that *miR-205*-induced down-regulation of P-gp was correlated as *PTEN*-dependence in HepG2/DOX cells.

Up-regulation of *PTEN* by *miR-205* overexpression resulted in down-regulation of MDR-1 in HepG2 cell line

PTEN induces cell death and MDR reversal effect by suppression of PI3K/Akt pathway. Finally, this

mechanism resulted in the down-regulation of P-gp and enhancement of drug accumulation in cells. In order to investigate correlation between *miR-205* and *PTEN*, *PTEN* expression was assessed in HepG2/DOX cell lines (Fig.5C).

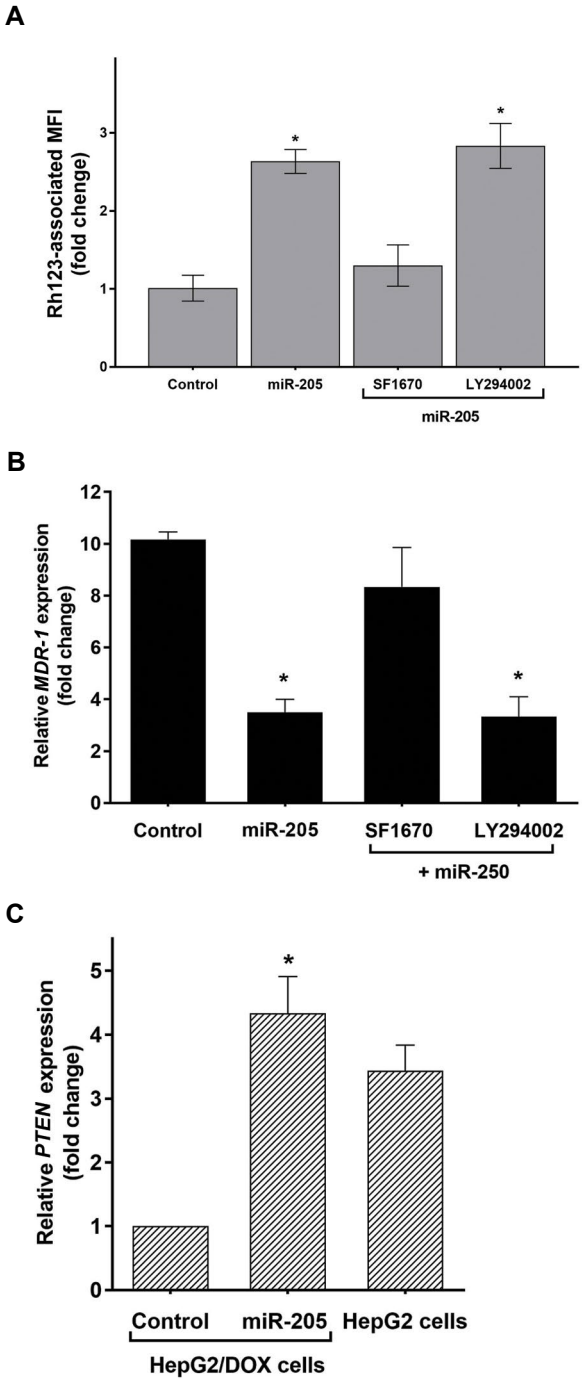

Fig.5: *PTEN* inhibition abolished *miR-205* effects on MDR. **A.** Intracellular Rho 123 accumulation in HepG2/DOX cells was affected by *miR-205* and specific inhibitors. Treatment with 10 µM LY294002 increased Rho 123-associated MFI and treatment with 10 µM SF1670 almost abolished the effect of miR-205 overexpression. **B.** *MDR-1* mRNA in HepG2/DOX cells 48 hours after transfection. SF1670 suppress the effects of *miR-205*. **C.** *miR-205* up-regulated *PTEN* levels in HepG2/DOX cells. The data represent mean ± SD (n=3, *; P<0.05 compared to non-transfected HepG2/DOX cells).

Discussion

Herein, we showed that *miR-205* overexpression considerably inhibited drug resistance, viability and proliferation of HepG2/DOX cells. Furthermore, our data revealed that significant down-regulation of P-gp expression with *PTEN,* was through *miR-205,* which inhibits MDR in HepG2/DOX cells. In fact, *miR-205* re-sensitized human liver cancer HepG2/DOX cells to DOX by targeting *PTEN*/PI3K/Akt/MDR1/P-gp signal transduction pathway.

Cancer resistance to chemotherapeutic agents led to the manifested MDR due to the overexpression of ABC transporters, including P-gp. It is a crucial unresolved impede that hinders the chemotherapy of liver cancer. Hence, it is crucial to identify unique strategies to enhance the effectiveness of DOX for curative purposes. Recent researches have revealed that miRNAs play a key role in the MDR of liver cancers and they could modulate MDR by regulating P-gp (22, 26). In this regard, many miRNAs have been found to regulate drug resistant genes including *ABCG2*, *Bcl-2*, *MDR1* and *PTEN* (27).

MicroRNAs have emerged as pivotal regulators of cellular pathways, which result in altered gene expressions (28). *miR-205* is correlated with cancer development (29), as an epithelial-specific miRNA and it has been demonstrated to modulate chemo-sensitivity and chemo-resistance in a diverse population of cancer cells through behaving as tumor suppressor (ts-miRNAs) or oncogenes (onco-miRNAs) depending on the function and mechanism of their targets (30, 31). *miR-205* conducts conflicting functions in human cancers (32). In this regard, Bhatnagar and colleagues (33) demonstrated that in prostate cancer cell lines *miR-31* and *miR-205* are down-regulated leading to the resistance to chemotherapy-induced apoptosis of these cells. Additionally, Chaudhary and co-workers (34) demonstrated that *miR-205* overexpression re-sensitized gemcitabine (GEM)-resistant pancreatic cancer cells to GEM. Thus, it acts as a tumor suppressor. Shao and colleagues (31) revealed that *miR-205-5p* expression was down-regulated in all hepatic cancer cell line, whilst *miR-205-5p* expression was up-regulated by 5-fluorouracil (5-Fu) treatment in Bel-7402 (Bel) cells. With referring to the previous studies, our data revealed that *miR-205* was significantly down-regulated in HepG2/DOX cells, compared to the parental HepG2 liver cancer cells. Indeed, overexpression of *miR-205* in HepG2/DOX cells restored DOX sensitivity and significantly declined viability of HepG2/DOX cells. Indeed, we sought to clarify whether or not combination of *miR-205* with DOX enhanced apoptosis of HepG2/DOX cells compared to HepG2 cells. In this regard, we received desirable response from these DOX-resistant cells. In fact, DOX was particularly concentrated in the nuclei when it combined use with *miR-205* or verapamil. On the other hand, DOX accumulation in the cytoplasm happens when it administrated as a single drug in HepG2/DOX cells. In addition, *miR-205* could improve the drug-transport function of MDR1 assessed

by Rho 123. This showed that *miR-205* had the capability to improve DOX-mediated apoptosis.

Evidence revealed that *PTEN* negatively regulates cell cycle progression and cell survival by dephosphorylating PIP3 to PIP2 followed by inhibition of the Akt/PKB pathway (35). Inhibiting the PI3K/Akt axis with a specific inhibitor (LY294002) or Akt siRNA could retrieve drug sensitivity with down-regulating expression of P-gp and MRP1. Although the accurate mechanisms that underlie the role of PI3K/Akt pathway activation remain unclear. These results clearly revealed that the PI3K/Akt pathway played a major role in the pathogenesis of MDR (36). So far, several studies have showed that *PTEN* is a direct target of *miR-205*. In this regard, a study was conducted on two different cell lines of human ovarian cancer. They demonstrated that inhibition of *miR-205-5p* in C13K cells led to overexpression of *PTEN* and following decrease in its downstream targets including p-AKT. This was resulted in cisplatin sensitiveness in these cells. In contrast, overexpression of *miR-205-5p* in OV2008 cells led to down-regulation of *PTEN* and following enhancement of p-AKT level, which resulted in cisplatin resistance (37). To validate whether *PTEN* was direct targets of *miR-205*, we demonstrated for the first time a positive regulation of *PTEN* exert by *miR-205* in liver cancer HepG2 cells. Moreover, we showed that *PTEN* expression was significantly lower in HepG2/DOX cells. In fact, *miR-205* led to overexpression of *PTEN* in HepG2/DOX cell lines. Furthermore, FR results revealed when *PTEN* is inhibited compared to the other groups, *miR-205* could not reverse MDR in HepG2/DOX cells. Furthermore, to confirm the participation of *PTEN* or *PI3K* in this pathway and to elucidate its fundamental molecular mechanism, we investigated the MDR-reversing effect of *miR-205*. The investigation was performed in the presence of special inhibitors for *PTEN* (SF1670) and PI3K (LY294002). Desirable results of this investigation illustrated crucial role of *miR-205* involvement in this pathway.

MDR reversal is significantly important in the progress of cancer treatment (38). Recently, novel therapeutic approaches focused on affected miRNAs in modulating MDR by modifying the expression of ABC transporters including P-gp in cancer cells (39). Studies revealed that up-regulation of P-gp in tumors can be either intrinsic or acquired following chemotherapy depending on the tissue of origin (13). It is reported that many anti-cancer drugs, such as DOX and paclitaxel, are substrates of P-gp (40). Up-regulation of P-gp in tumor cells elevated drug resistance, meaning that inhibiting the P-gp was shown to reverse drug resistance. These indicate defined the classical MDR associated role of P-gp. In this regard, Zheng et al. (40) demonstrated that *miR-34a* overexpression significantly enhanced the lethal effect of DOX on HepG2 cells by down-regulating MDR1/P-gp. To our knowledge, there is no similar study about the role of *miR-205* on P-gp expression in liver cancer cells, until recently. In this regard, we illustrated that *miR-205* is able to hinder the efflux of Rho 123 in HepG2/DOX cell lines. Furthermore, transfection of *miR-205* in HepG2/DOX cells with the Rho 123 resulted in enhancement of the MFI. In fact, our data showed that transfecting resistant cells with *miR-205* could reverse MDR by increasing *PTEN* expression, inhibiting the P-gp activity and enhancing the intracellular accumulation of DOX, in HepG2/DOX cells. Additionally, verapamil, a known P-gp inhibitor, enhanced the Rho 123 accumulation in HepG2/DOX cells, as a positive control for validation of our results. With this perspective, the results showed that *miR-205* significantly diminished activity of mRNA/protein expression levels of P-gp in HepG2/DOX cell lines. Furthermore, inhibition of P-gp with a specific inhibitor significantly enhanced the growth inhibition rate of the HepG2/DOX cell lines rather than the control group. Hence, *miR-205* re-sensitized HepG2/DOX cells to DOX.

Conclusion

The results of this study revealed that *miR-205* had a key role in improving drug resistance in liver cancer cells via inhibiting drug efflux and enhancing apoptosis via up-regulation of *PTEN* and down-regulation of MDR1/P-gp expression. The data obtained from this study revealed that transfection of *miR-205* in HepG2/DOX cells along with DOX could inhibit MDR, cell proliferation and promoted apoptosis. These are through the regulation in *miR-205*/*PTEN*/PI3K/Akt/MDR1/P-gp pathway and attenuated expression of P-gp. Notwithstanding, these results may provide an axillary step to reach into the potential role of *miR-205* in the progression of therapeutic target for liver cancer treatment during MDR.

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Authors' Contributions

Z.L., M.L.; Participated in study design and performance of experiments. J.S., X.L.; Contributed extensively in interpretation of the data and conclusion. X.M.; Prepared materials and solutions. P.Z., F.Q., L.Y.; Performed revision and statistical analysis, L.Y; Designed the study. All authors performed editing and approved the final version of this paper for submission.

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