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

Mei Li, M.D.1, Zhubin Li, M.D.1, Juanrong Song, M.D.1, Xu Li, M.D.2, Pengtao Zhai, M.D.1, Xudong Mu, M.D.1, Hakai Qiu, M.D.1, Le Yao, M.D.3*

1. Department of Minimally Invasive Intervention, Shaanxi Provincial Cancer Hospital, Xi’an, Shaanxi, China
2. Department of Oncology, Shaanxi Provincial Cancer Hospital, Xi’an, Shaanxi, China
3. Department of Infectious Diseases, The First Hospital of Yulin, Yulin, Shaanxi, China

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

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 MDRI 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 chemosensitizers 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/PI3KAkt/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% CO2. 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′-UCCUUAUUCCACGGAGUCUG-3′. Cells (3×10⁵ cells/2 ml/well) were seeded in six-well microplates at 70% confluency. 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 follows: 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’-CAGTGACGGGTCCGAGGT-3’

**MDR-1-**
- F: 5’-CCCATCATTGCAATAGCCCAGG-3’
- R: 5’-TGTTCAAACCTCTGCTCTGTA-3’

**PTEN-**
- F: 5’-GGACGAACTGGTGTA-3’
- R: 5’-AGGCTGTGGATGCGGCCCT-3’

**β-actin-**
- F: 5’-AAGCTGTGTTACGTGGCCCT-3’
- R: 5’-GGGCAGCTGCTAGCTCTCTC-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 transcribed 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 the 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 transcribed 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.

**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).

**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.
miR-205 Reverses MDR-1 Mediated DOX Resistance

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 miR-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).

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).](image)

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, Bel-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 chemoresistance 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 conducted 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.

References

1. Chai Z, Yin X, Chen J, Shi J, Sun J, Liu C, et al. MicroRNA-101 modulates cisplatin chemoresistance in liver cancer cells via the DNA-PKcs signaling pathway. Oncol Lett. 2019; 18(4): 3655-3663.
2. Pan C, Wang X, Shi K, Zheng Y, Li J, Chen Y, et al. MiR-122 reverses the doxorubicin-resistance in hepatocellular carcinoma cells through regulating the tumor metabolism. PLoS One. 2016; 11(5): e0152090.
3. Donato MT, Tolosa L,Gomez-Lechon MJ. Culture and functional characterization of human hepatoma HepG2 cells. Methods Mol Biol. 2015; 1250: 77-93.
4. Harjumäki R, Nugroho RWN, Zhang X, Lou YR, Ylipertula M, Valle-Delgado JJ, et al. Quantified forces between HepG2 hepatocellular carcinoma cells and human serum albumin. Biomaterials. 2015; 36: 164-173.
21. Li J, Hu K, Gong G, Zhu D, Wang Y, Liu H, et al. Upregulation of
20. Hu Y, Qiu Y, Yagüe E, Ji W, Liu J, Zhang J. miRNA-205 targets
19. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres
18. Wang J, Chan JYW, Fong CC, Tzang CH, Fung KP, Yang M. Trans-
17. Longley DB, Johnston PG. Molecular mechanisms of drug resist-
16. Joshi P, Vishwakarma RA, Bharate SB. Natural alkaloids as P-gp
15. Nanayakkara AK, Follit CA, Chen G, Williams NS, Vogel PD, Wise
14. Oki E, Baba H, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, et al. Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer. Int J Cancer. 2005; 117(3): 376-380.
13. Yousfi B, Samadi N, Ahmadi Y, Akt and p53R2, partners that dictate the progression and invasiveness of cancer. DNA Repair (Amst). 2014; 22: 24-29.
12. Joshi P, Vishwakarma RA, Bharate SB. Natural alkaloids as P-gp inhibitors for multidrug resistance reversal in cancer. Eur J Med Chem. 2017; 138: 273-292.
11. Zhang J, Gu Y, Chen B. Mechanisms of drug resistance in acute myeloid leukemia. Onco Targets Ther. 2019; 12: 1937-1945.
10. Yousfi B, Azimi A, Majidinia M, Shelfei-irnejad V, Babalzadeh R, Baradaran B, et al. Balaglitazone enhances the expression and function of ABCB1/MDR1 in human cancer cells. New J Sci. 2014; 2014: 9(1): 967.
9. Katayama K, Noguchi K, Sugimoto Y. Regulations of P-glycoprotein/ABCB1/MDR1 in human cancer cells. New J Sci. 2014; 2014: 119(1): 213-220.
8. Xu F, Wang F, Yang T, Sheng Y, Zhong T, Chen Y. Differential drug resistance acquisition to doxorubicin and paclitaxel in breast cancer cells. Cancer Cell Int. 2014; 14(1): 142.
7. Nitiss JL. Targeting DNA topoisomerase II-targeting drugs. Int J Cancer. 2008; 118(1): 118-122.
6. Lage H, Aki-Sener E, Yalcin I. High antineoplastic activity of new heterocyclic compounds in cancer cells with resistance against classical DNA topoisomerase II-targeting drugs. Jpn J Clin Oncol. 2017; 48(2): 103-114.
5. Ikeda M, Moritance C, Ueno M, Okusaka T, Ishi H, Funuse J. Chemotherapy for hepatocellular carcinoma: current status and future perspectives. Jpn J Clin Oncol. 2017; 48(2): 103-114.
4. Lage H, Aki-Sener E, Yalcin I. High antineoplastic activity of new heterocyclic compounds in cancer cells with resistance against classical DNA topoisomerase II-targeting drugs. Jpn J Clin Oncol. 2017; 48(2): 103-114.
3. Li et al.