Possible Role of microRNA-122 in Modulating Multidrug Resistance of Hepatocellular Carcinoma

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Abstract Hepatocellular carcinoma (HCC) is a hyper-vascular primary liver cancer characterized by rapid progression, besides, resistance to traditional chemotherapeutic agents. It has been shown that micro-RNAs play critical roles in regulation of tumor cell sensitivity to drugs through modulating the expression of genes involved in drug transport. The present study investigated whether restoration of miR-122 in HCC cells could alter the cell cycle distribution and the expression of multidrug resistance (MDR)-related genes (ABCB1, ABCC1, ABCG2 and ABCF2). After overexpression of miR-122 in HepG2 cells treated or untreated with doxorubicin doses, total RNAs and protein extracts were isolated for application of QRT-PCR and western blotting techniques. Moreover, cell cycle distribution was monitored by flow cytometry. Our results revealed that, the over expression of miR-122 in HepG2 cells treated or untreated with doxorubicin could modulate the sensitivity of cells to chemotherapeutic drug through downregulation of MDR-related genes, ABCB1 and ABCF2. Interpretation of cell cycle distribution revealed that, the anti-proliferative effect of miR-122 is associated with the accumulation of cells in

G₀/G₁ phase. Moreover, treatment with miR-122 and doxorubicin resulted in high percentage of HCC cells in G₀/G₁ phase. Taken together, our findings revealed that, overexpression of miR-122 inhibited HCC cell growth by inducing cell cycle arrest and this arrest is associated with down-regulation of MDR-related genes.

Keywords Hepatocellular carcinoma · miRNA-122 · MDR · ABCs · Cell cycle

Introduction

Hepatocellular carcinoma (HCC) is the leading primary liver cancer. It is considered the third most common cause of cancer-related death worldwide [1, 2]. HCC develops as a result of chronic liver disease like cirrhosis due to hepatitis B virus (HBV) and/or HCV, non-alcoholic steatohepatitis, autoimmune hepatitis, iron overload syndromes, obesity, diabetes, alcohol abuse, smoking, oral contraceptive use and aflatoxin exposure [3–6]. The recent studies suggest that liver cancers arise either by the dedifferentiation of mature cells or by the maturation arrest of stem cells [7]. Unfortunately, clinical outcome of HCC is still poor due to the frequent occurrence of resistance to different chemotherapeutic drugs. This resistance that allows cancer cells to proliferate uncontrollably and to become more aggressive with a greater ability to metastasize to other organs. An important criteria in the field of chemoresistance is that a cancer cell may not only become resistant to one drug, but also to several drugs, resulting in a phenomenon that is known as multidrug resistance (MDR) [8, 9].

Alterations in drug efflux were reported as one of the primary causes of multidrug resistance (MDR) particularly;

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overexpression of ABC (ATP binding cassette) super family of transporters by tumor cells is closely linked to chemoresistance [10]. ABC transporters are transmembrane proteins that use ATP hydrolysis to transport drugs outside of the cells against a drug gradient. This super family is classified into seven distinct families (A–G) [11]. Three ABC proteins were mainly described for most MDR in humans: P-glycoprotein (P-gp or ABCB1), MDR associated protein 1 (MRP1 or ABCC1) and breast cancer resistance protein (ABCG2) [12]. ABCB1 is the prototypical efflux pump that is expressed also in normal tissues but its deregulation confers MDR phenotype [13], ABCC1 is expressed mainly in the less differentiated areas of tumors [14], and ABCG2 works as MDR protein [15]. The ABC transporter super family includes ABCF2 which lacks transmembrane domains so they cannot work as classical transporters; it is likely that this protein acts as a transcription factor in signaling pathways important in preventing resistance to anticancer drugs at metastatic sites and in endocrine pathway for breast cancer. Although detailed molecular mechanisms are still elusive, the role of ABCF2 in conferring drug resistance in cancer cells is still not fully understood [16]. It was postulated that ABCF2 may contribute to the chemoresistant cancer phenotype because it was overexpressed in chemoresistant ovarian carcinoma cells [17].

Doxorubicin (Dox) is the most effective cytotoxic drug for HCC treatment that intercalates DNA and suppresses DNA replication through affecting predominantly S and G₂ phases of cell cycle. Doxorubicin can release reactive oxygen species (ROS) that lead to DNA damage, oxidative stress, and triggers apoptotic pathways of cell death [18]. Applications of doxorubicin cause development of MDR and gain P-gp expression in several tumors [19–21].

Numerous studies have referred to the importance of microRNAs (miRNAs) in the regulation of tumor cell sensitivity to drugs and modulating the expression of genes involved in drug transport. miRNAs control the stability and translation of targeted messenger RNAs (mRNAs) through complementary interaction with the 3' untranslated region (UTR) of target genes; one miRNA can interact with hundreds of genes [22, 23].

The liver-specific microRNA-122 (miR-122) is the most abundantly expressed miRNA in the liver, modulates hepatic lipid metabolism and it plays an important role in regulating hepatocyte development and differentiation [24, 25]. It plays a role as a potential tumour suppressor in two ways: suppressing hepatic cell growth by targeting cyclin G1 and enhancing apoptosis of hepatic cells by targeting BCL-w and ADAM17 involved in metastasis [26]. MiR-122 is commonly down-regulated in primary HCC. It has been shown that loss of miR-122 expression is greatly related to dysregulation of differentiation of

hepatocytes, poor prognosis and metastasis of liver cancer that is caused by development of MDR [27, 28].

In current study, we investigated the role of miR-122 restoration on regulation of MDR phenotype in HepG2 cells treated with either acute or chronic doxorubicin doses, or untreated.

Materials and Methods

Cell line and Cell Culture

Wild HepG2 cell line was purchased from ATCC (American Type Culture Collection). These cells were cultured and propagated in 75 cm² flasks in DMEM (Dulbecco's Modified Eagle Medium; Lonza, Belgium); supplemented with 10% Fetal Bovine Serum (Biocrom, Berlin), 1% Penicillin–streptomycin (Lonza, Belgium) and 4 mM L-glutamine (Lonza, Belgium) at 37 °C in 5% CO₂ incubator. HepG2 cell line was employed because it is derived from a hepatoblastoma that expresses undetectable levels of miR-122 and is more closely related to immature/undifferentiated hepatoblasts [8].

Acute and Chronic Doxorubicin Doses

Wild HepG2 cells were cultured in the presence of 1 μM DOX (Pfizer, USA) for 72 h (*Acute dose*). At the end of this period the cells were washed twice with sterile Dulbecco's phosphate buffered saline (DPBS) to remove the DOX and fresh DOX-free growth medium was added. Following a 3-day recovery period, 100 nM DOX was added to the cells for 2 weeks (*Chronic dose*) with washes and media changes every 3 days.

Cell Transfection with RNA Oligonucleotides

HepG2 cells were transfected with 100 nM of miScript miR-122 mimics or miScript Inhibitor Negative Control (Qiagen, Valencia, CA, USA) using 1 μL of HiperFect transfection reagent (Qiagen, Germany). At 24 h after transfection, transfection media were replaced by fresh media. At the same time point, treated cells were harvested for microRNA isolation and detection. Protein extracts and total RNA were isolated at 72 h after transfection for further analyses.

Quantitative Real-Time PCR Analysis for miRNA and mRNAs Expression

For microRNA, miScript miRNA PCR system (miRneasy mini kit for miRNA extraction, miScript RT II for miRNA reverse transcription, 10× miR-122a-1 miScript Primer

Assay and miScript SYBR Green PCR kit for PCR amplification) (Qiagen, Valencia, CA, USA) was used according to the manufacturer's protocol. The Cycling conditions for real-time PCR were as follow: 95 °C for 15 min, 94 °C for 15 s, 55 °C for 30 s, and then 70 °C for 30 s, the number of cycles were 40 cycles. Fluorescence measurements were performed with real-time PCR (MiniOpticon Real-Time PCR System, Bio-Rad, France).

Total RNA from cells was extracted using Qiazol (Qiagen, Germany) following the manufacturer's instruction. ABCB1, ABCC1, ABCG2 and ABCF2 gene expression levels were estimated using QRT-PCR (MiniOpticon Real-Time PCR System, Bio-Rad, France) and β -actin was used as a house-keeping gene. The primer sequence for β -actin was as follow: (F) 5'-CCTTCCTGGGCATGGAGTCCT-3', (R) 5'-GGAGCAATGATCTTGATCTTC-3'. The following primers were used, ABCB1 (F) 5'-AGACATG ACCAGGTA-TGCCTAT-3', ABCB1 (R) 5'-AGCCTA TCTCCTGTGCGATTA-3'; ABCC1 (F) 5'-CATTGAGCT CGTCTTGTCTG-3', ABCC1 (R) 5'-GGATTAGGG TCGTGGATGGTT-3'; ABCG2 (F) 5'-GCGACCTGCCA ATTTCAAATG-3', ABCG2 (R) 5'-GACCCTGTTAAT CCGTTCGTTT-3'; ABCF2 (F) 5'-TGGAGCAGGGAAGT CAACTCTT-3', ABCF2 (R) 5'-TTTTCGGATCATGCCA TCTG-3'. The gene copy numbers were normalized to 100,000 copies of housekeeping beta-actin gene. The RT and subsequent PCR cycling conditions were as follow: 50 °C for 10 min, 95 °C for 5 min, 95 °C for 10 s, and then 60 °C for 30 s, the number of cycles were 40 cycles. BioRad MiniOpticon™ Real-Time PCR cyler was used for quantitative estimation.

Western blot Analysis

After preparation of cell lysates by adding Radio Immuno Precipitation Assay buffer (RIPA buffer) (Abcam, UK) to the cells for 30 min on ice, protein extracts (supernatants) were collected by centrifugation of cell lysates at 12,500 rpm for 10 min at 4 °C. Total protein concentrations were measured using Bradford assay [29]. Proteins extracted from cell lysates were immunoblotted with antibodies following published protocol [30]. Briefly; extracted proteins were diluted using 6× solubilization buffer consisting of 375 mM Tris-HCl, 9% SDS, 50% glycerol, 0.03% Bromophenol blue, and 10% (v/v) 2-mercaptoethanol and were then heated (70 °C for 10 min). Equal amounts (40 μ g) of total proteins were loaded per each lane, and proteins were separated by SDS-PAGE on 7% polyacrylamide gels (Serva Electrophoresis, Germany). The resolved proteins were transferred to 0.45- μ m nitrocellulose membranes (Serva Electrophoresis, Germany). Membranes were subsequently blocked with

5% skimmed milk in Tris-buffered saline with Tween 20 (TBST) buffer consisting of Tris, 125 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature followed by incubation with rabbit polyclonal Ab to ABCB1 (Abcam, UK), diluted at 1:1000, or mouse monoclonal β -actin (loading control, diluted at 1:2000, Abcam) overnight at 4 °C. Excess antibodies were removed by extensive washing in TBST, and blots were then reprobed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG H&L (1/10,000 dilution, Abcam) for ABCB1 or rabbit anti-mouse IgG (1:10,000) for β -actin. Membranes were then washed extensively with TBST and treated with enhanced chemiluminescence (ECL) reagent (Optiblot™, Abcam, UK). Light-sensitive films (Fuji, Japan) were used for X-ray film exposure.

Flow Cytometric Analysis of Cell Cycle Distribution

Cell cycle distribution was analyzed by the DNA content using propidium iodide (PI) staining method. HepG2 cells were grown at a density of 1×10^6 cells/ml in six-well plate and then transfected with miR-122 mimic in the absence or presence of 0.5 μ M doxorubicin. Following transfection, the cells were treated with doxorubicin for 14 h. Cells were then washed with DPBS, trypsinized using 0.05% trypsin-EDTA, fixed in 70% ethanol in DPBS and stored at 4 °C overnight. Cells were incubated with 50 μ g/ml PI (Thermo Scientific) containing 8 μ g/ml RNase A in the dark at 37 °C for 30 min. Cells were then analyzed by Backman coulter FC-500 (USA), 10,000 events were acquired. The percentage of cells in G₀/G₁, S and G₂/M phases of cell cycle was calculated using KALUZA software and Multicycle AV for windows version 284.

Statistical Analysis

A Student's *t* test, in addition to one-way ANOVA, was used to compare individual data points among each group. The data were represented as the mean $\pm$ SE of mean. A *P* value of <0.05 was set as the criteria for statistical significance and highly significant when *P* < 0.01.

Results

Morphological Appearance of Wild HepG2 Versus Doxorubicin Resistant HepG2 Monolayers

HepG2 cells treated with chronic dose of doxorubicin appeared morphological change compared with control wild HepG2 (Fig. 1). Cells revealed signs of toxicity, such as cell rounding and shrinkage.

Fig. 1 Morphological appearance of wild HepG2 (a) versus doxorubicin resistant HepG2 (b) monolayers

Fig. 2 Copy numbers of miR-122 in cells treated with inhibitor negative control (NC) and miR-122 mimic

Expression of miR-122 in HCC Cells After miR-122 Mimic and Inhibitor Negative Control Transfection

To validate dynamic expression of miR-122 after transfection in wild and doxorubicin treated HepG2 cells, we isolated the microRNA at 24 h after transfection and the quantity of miR-122 was measured. Our data showed that there was a dynamic change in miR-122 expression at 24 h after transfection as compared to inhibitor negative control, with a fold change of 218 (Fig. 2).

ABC Genes Expression Levels

ABCB1 Gene Expression Levels

To test whether treatment of HepG2 cells with miR-122 mimics and/or acute/chronic doxorubicin doses could affect expression of ABCB1 gene, we estimated ABCB1 gene expression by QRT-PCR. As shown in Fig. 3, there was statistical significant decrease of ABCB1 expression level in wild HepG2 cells transfected with miR-122 mimics (miR-122 II) as compared to inhibitor negative control (NC II). On contrary, statistical significant increases of ABCB1 expression levels were detected in HepG2 cells treated with acute high doxorubicin dose + miR-122 mimics.

Fig. 3 ABCB1 gene expression levels. Dox I; cells treated with chronic doxorubicin dose, NCI; cells treated with chronic doxorubicin dose then transfected with inhibitor negative control, miR-122 I; cells treated with chronic doxorubicin dose then transfected with miR-122 mimics, U; wild HepG2 cells, NCII; Wild HepG2 cells transfected with inhibitor negative control, Dox II; HepG2 cells treated with acute high doxorubicin dose, miR-122 II; HepG2 cells transfected with miR-122 mimics and D + M II; HepG2 cells treated with acute high doxorubicin dose and transfected with miR-122 mimics. Data was presented as mean $\pm$ SEM. * $P < 0.05$

ABCC1 Gene Expression Levels

As detected by QRT-PCR and demonstrated in Fig. 4, there were no significant changes in ABCC1 expression levels of HepG2 cells treated with miR-122 mimics and/or doxorubicin, as compared to inhibitor negative control,

Fig. 4 ABCC1 gene expression levels. Dox I; cells treated with chronic doxorubicin dose, NCI; cells treated with chronic doxorubicin dose then transfected with inhibitor negative control, miR-122 I; cells treated with chronic doxorubicin dose then transfected with miR-122 mimics, U; wild HepG2 cells, NCII; Wild HepG2 cells transfected with inhibitor negative control, Dox II; HepG2 cells treated with acute high doxorubicin dose, miR-122 II; HepG2 cells transfected with miR-122 mimics and D + M II; HepG2 cells treated with acute high doxorubicin dose and transfected with miR-122 mimics. Data was presented as mean $\pm$ SEM. * $P < 0.05$

Fig. 5 ABCG2 gene expression levels. Dox I; cells treated with chronic doxorubicin dose, NCI; cells treated with chronic doxorubicin dose then transfected with inhibitor negative control, miR-122 I; cells treated with chronic doxorubicin dose then transfected with miR-122 mimics, U; wild HepG2 cells, NCII; Wild HepG2 cells transfected with inhibitor negative control, Dox II; HepG2 cells treated with acute high doxorubicin dose, miR-122 II; HepG2 cells transfected with miR-122 mimics and D + M II; HepG2 cells treated with acute high doxorubicin dose and transfected with miR-122 mimics. Data was presented as mean $\pm$ SEM. * $P < 0.05$

except for cells that were treated with chronic doxorubicin dose; Dox I.

ABCG2 Gene Expression Levels

Wild HepG2 cells transfected with miR-122 mimic (miR-122 II) displayed statistical significant increase in ABCG2 expression level, as compared to inhibitor negative control (NC II) (Fig. 5).

ABCF2 Gene Expression Levels

ABCF2 gene was expected as predictive target for miR-122 by targetscan, miRwalk and miRanda bioinformatic programs. Where miR-122 binds to 3' untranslated region of ABCF2 gene with P value = 0.024. To study the effect of treatments with miR-122 mimics and/or acute/chronic doxorubicin doses on expression levels of ABCF2 gene, we

Fig. 6 ABCF2 gene expression levels. Dox I; cells treated with chronic doxorubicin dose, NCI; cells treated with chronic doxorubicin dose then transfected with inhibitor negative control, miR-122 I; cells treated with chronic doxorubicin dose then transfected with miR-122 mimics, U; wild HepG2 cells, NCII; Wild HepG2 cells transfected with inhibitor negative control, Dox II; HepG2 cells treated with acute high doxorubicin dose, miR-122 II; HepG2 cells transfected with miR-122 mimics and D + M II; HepG2 cells treated with acute high doxorubicin dose and transfected with miR-122 mimics. Data was presented as mean $\pm$ SEM. * $P < 0.05$ and ** $P < 0.01$

detected ABCF2 gene expression by QRT-PCR. As shown in Fig. 6, cells treated with chronic doxorubicin dose then transfected with miR-122 mimics (miR-122 I) showed statistical significant decrease as compared to inhibitor negative control (NC I). While, wild HepG2 cells transfected with miR-122 mimics (miR-122 II) displayed statistical significant increase as compared to inhibitor negative control (NC II). There were also statistical significant increases in cells subjected to acute and chronic doxorubicin doses (Dox II and Dox I; respectively) as compared to their control (U). High statistical significant increase was observed in D + M II as compared to inhibitor negative control.

Expression Level of ABCB1 by Western Blotting

As shown in Fig. 7, HepG2 cells transfected with miR-122 mimic (miR-122II) showed reduction in ABCB1 protein expression levels, as compared to inhibitor negative control (NCII).

Cell Cycle Distribution

Cells distribution was monitored through the different phases of the cell cycle by flow cytometry analysis. An accumulation of cells in G₀/G₁ phase in cases of restoration of miR-122 $\pm$ doxorubicin was recorded. However, cells treated with doxorubicin revealed an increase in G₂/M phase (Fig. 8; Table 1).

Discussion

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, that arises either by the dedifferentiation of mature cells or by the maturation arrest of stem

Fig. 7 ABCB1 protein expression levels by western blotting. Dox I; cells treated with chronic doxorubicin dose, NC I; cells treated with chronic doxorubicin dose then transfected with inhibitor negative control, miR-122 I; cells treated with chronic doxorubicin dose then transfected with miR-122 mimics, U; wild HepG2 cells, NC II; Wild HepG2 cells transfected with inhibitor negative control, Dox II; HepG2 cells treated with acute high doxorubicin dose, miR-122 II; HepG2 cells transfected with miR-122 mimics and D + M II; HepG2 cells treated with acute high doxorubicin dose and transfected with miR-122 mimics

cells [7]. Applications of doxorubicin in HCC treatment cause alterations in of ABC (ATP binding cassette) super family of transporters and consequently development of multidrug resistance (MDR). This can be attributed to the uncontrollable cell proliferation and greater ability to metastasize to other organs [8, 9]. Numerous studies have figured out the importance of microRNAs (miRNAs) in the modulation of tumor cell sensitivity to drugs and regulating the expression of genes involved in drug transport [22, 23]. Loss of miR-122 (liver-specific miRNA) expression is closely related to dysregulation of differentiation of hepatocytes, poor prognosis and metastasis of liver cancer that is a result of development of MDR [27, 28].

In the present study, statistical significant increase in ABCB1 mRNA expression was detected in HepG2 cells treated with acute high doxorubicin dose as compared to control cells. This is in line with previous reports, demonstrating an up-regulation of ABCB1-mRNA in HepG2 and Huh-7 cells as well as in patients with HCC after treatment with doxorubicin [31–35]. Other study [36], used human breast cancer cell line MCF-7 treated with 1 μ M doxorubicin was in the same line with our study. Our statistical significant increase in ABCB1 mRNA expression was undetectable with continuous treatment of HepG2 cells with chronic doxorubicin dose, as compared to control cells. Our finding was similar to another recent study that used tamoxifen resistant human breast cancer cell line MCF7. ABCB1 expression was detected in early phases of generating of tamoxifen resistant MCF7 cells or also after short term incubation of control MCF7 cells with tamoxifen [37]. Similarly, Feifei et al. [38] found that ABCB1 induction was an early event in MCF7 cell line treated with doxorubicin. We can interpret our results as suggested by

Fig. 8 Analysis of cell cycle in HepG2 cells after treatment. **a** Cell cycle analysis of HepG2 cells transfected with miR-122 in the absence or presence of 0.5 μ M doxorubicin. Following transfection, the cells were incubated with doxorubicin for 14 h before fixing with ethanol and staining with PI. U; untreated wild HepG2 cells, NC; Wild HepG2 cells transfected with inhibitor negative control, Dox; HepG2 cells treated with 0.5 μ M doxorubicin, miR-122/Dox; HepG2 cells transfected with miR-122 mimic then treated with 0.5 μ M doxorubicin, and miR-122; HepG2 cells transfected with miR-122 mimics. **b** Graphical representation of cell cycle distributions showing the percentage of cells in different phases in cell cycle

researcher working with tamoxifen resistant human breast cancer cell line [37], where the overexpression of ABCB1 transporter may be important in early phases of resistance until cells evolve other compensatory mechanism(s) to withstand chemotherapy. Doxorubicin was suggested to regulate the transcription of ABCB1 in short term manner, through a transient activation of nuclear factor-kappa B (NF- κ B) that leads to the upregulation of ABCB1 expression [38].

Our investigation indicated that transfection of wild HepG2 cells with miR-122 mimics resulted in statistical significant decrease in ABCB1 mRNA expression level as compared to the control. Our data was confirmed with another study used human HCC cell line Hep3B infected with adenoviral vector carries miR-122 (Ad-miR 122) [35]. Although, in current study, the overexpression of miR-122 reduces ABCB1 mRNA level, there is no attempt to verify binding of miR-122 on ABCB1 3' UTR, according to our knowledge and as stated by Kenneth. [39]. However, MiR-122 may target the 3' UTR of ABCB1 gene, or alternatively, miR-122 may affect the expression of ABCB1 indirectly, by targeting its positive regulators. Consequently, further investigations are recommended to elucidate possible bindings of miR-122 with the 3' UTR of ABCB1 or other of its positive regulators.

Referring to what is discussed above; it's accepted to record statistical significant increase in ABCB1 mRNA level in cells treated with both acute high dose of doxorubicin and miR-122 mimics (D + M II), as compared to control cells. This increase still less than what observed in cells treated with acute high dose of doxorubicin only.

Our finding confirmed a statistical significant decrease in ABCC1 gene expression when HepG2 cells treated with chronic dose of doxorubicin only, as compared to control. One recent study has implicated the same result for ABCC1 expression that appeared mainly in MCF-7 cells treated with low doses of doxorubicin and subsided in case of treatment with acute high dose. Only ABCB1 was overexpressed in MCF-7 treated with acute high doxorubicin dose [36]. Two other studies didn't observe any significant increase or even showed negative expression of ABCC1 in doxorubicin-resistant and—sensitive metastatic

(B)

Cell cycle distribution

Table 1 The percentage of cells in each phase of cell cycle as represented in Fig. 8

	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
U	71	29	0.00
NC	57.50	38	4.54
Dox	65	24.40	10.60
miR-122/Dox	85	6.04	9
miR-122	74.50	18.80	6.70

human breast cancer cell lines (MDA-MB-231) [40], and in Hep3B cells treated with doxorubicin [35].

On the other hand, doxorubicin led to enhanced expression of mRNA of ABCC1 compared to control cells in human HCC cell lines Huh-7 and HepG2 [33, 34].

ABCC1 is expressed mainly in the less differentiated areas of tumor and was found to be increased in subpopulation of stem cells within hepatocellular tumors [14]. It was reported that the transport of doxorubicin is mediated by ABCC1 with glutathione (GSH) dependent mechanism [41, 42]. Reactive oxygen species that are released by doxorubicin treatment for long time may aid in increasing the damage of the less differentiated cells in HCC, and ABCC1 expression level has reduced. Consequently, we could attribute the reduced ABCC1 levels to the reduced GSH levels in the cells under oxidative stress due to long term chronic exposure to doxorubicin.

Data presented in this study clearly showed that, HepG2 cells transfected with miR-122 mimics displayed a statistical significant increase in ABCG2 gene expression level as compared to control. So, miR-122 may likely indirectly regulate ABCG2 gene but not via ABCG2 3' UTR. Our study also referred above to statistical significant decrease in ABCB1 expression level in HepG2 cells transfected with miR-122 mimics.

Our results were in line with another study focused on the expression linkage between ABCB1 and ABCG2 in doxorubicin-resistant lung cancer cell subline SK-MES-1/DX1000. They found an inverse relation between ABCB1 and ABCG2 expression in the absence of doxorubicin [43]. Similarly, Cisternino et al. [44] found that defective ABCB1 was associated with increased ABCG2 mRNA at the mouse blood–brain barrier. It has been proposed that an agent can suppress one or more mechanisms of drug resistance while enhancing others and that one protein from one drug resistance gene can compensate for the activity of the protein from another [45].

On the other hand, positive correlations between ABCG2 and ABCB1 expression have been found [46, 47].

As far as our knowledge, there are very limited studies about ABCF2 expression in cancers and its role in drug resistance. Our results recorded statistical significant increase of ABCF2 expression level in HepG2 cells treated

with high acute doxorubicin dose (Dox II), as compared to untreated control cells (U). Likewise, but with slight decrease, cells treated with chronic doxorubicin dose (Dox I) showed significant increase in ABCF2 level. ABCF2 may act as drug resistant gene that is activated by doses of doxorubicin as illustrated in ABCB1 induction.

ABCF2 gene was predicted as target gene of miR-122, where miR-122 binds to 3' UTR of ABCF2 gene. So, we must expect statistical significant decrease in doxorubicin-induced ABCF2 gene expression after transfecting cells with miR-122 mimics. This expectation was confirmed with our results of cells treated with chronic doxorubicin dose then transfected with miR-122 mimics (miR-122 I); showed significant reduction in ABCF2 expression level, as compared to control cells. The precise mechanisms and pathways, by which miR-122 participates in the down-regulation of ABCF2 gene expression, require further investigations.

We observed also statistical significant increase in ABCF2 gene expression level in wild HepG2 cells transfected with miR-122 mimics (miR-122 II), instead of expected downregulation of its expression. Referring to the statistical significant decrease in ABCB1 expression level in the same treatment and to what mentioned above about overexpression of ABCB1 transporter that may be important in early phases of resistance until cells evolve other compensatory mechanism(s) to withstand chemotherapy. These results suggest that a decrease in ABCB1 level by miR-122 lead to an increase in ABCF2 level; as compensatory mechanism in MDR.

Noteworthy, the expression levels of ABCF2 gene in cells treated with miR-122 mimics (miR-122 II) was lower than both cells treated with acute doxorubicin alone (Dox II) and cells treated with acute doxorubicin dose and miR-122 mimics (D + M II); with the latter having the lower expression levels than the former. This finding may confirm the role played by miR-122 in decreasing the ABCF2 gene expression levels.

The analysis of cell cycle distribution revealed an increase in the G₀/G₁-phase population (74.50%) that is corresponding to decrease in the G₂/M phase population (6.70%) in cells transfected with miR-122 mimics. Other studies that used HepG2 cells transfected with miR-122 mimics or cyclin G1 small interfering RNAs (siRNA) assured the same results and stated that, restoration of miR-122 expression in HCC cells limits their tumorigenic properties, specifically growth, invasion, proliferation, migration and clonogenic survival. Cyclin G1 is a gene target of miR-122 [48–50]. The decrease of cells in the G₂/M phase was as a response to cyclin G1 knockdown [51].

Cyclin G1 is activated in response to DNA damage that is mediated by doxorubicin, leading to increase of cell population in G₂/M phase [51]. So that, in our study there was an increase in G₂/M phase (10.60%) in case of

treatment of cells with doxorubicin only as compared to the control cells (U).

Our results showed an increase in G₀/G₁-phase population (85%) and an increase in G₂/M phase (9%) in HepG2 cells transfected with miR-122 mimics then treated with doxorubicin (miR-122/Dox) as compared to inhibitor negative control (NC). Depending on the above discussed for cell cycle distribution analysis; we can explain our results in case of treatment of HepG2 cells with miR-122 mimics then with doxorubicin. MiR-122 restoration in HepG2 cells has increased the percentage of cells in G₀/G₁-phase (85%) while decreasing the percentage of cells in G₂/M phase through targeting cyclin G1 gene. Then treatment with doxorubicin, DNA damaging agent, leading to reactivation of cyclin G1 and increased the accumulation of cells in G₂/M phase (9%).

Another study, that used HCC-Hep3B cells infected with Ad-miR-122 and doxorubicin, recorded an inhibition of HCC cell growth by an induction of G₂/M phase arrest. This arrest was associated with reduction in, cyclin B1, the key regulatory factor of G₂/M transition [35].

Finally, our results indicated that the ectopic expression of miR-122 in HepG2 cells treated or untreated with doxorubicin; resulted in reduced expression of MDR-related genes including ABCB1 and ABCF2. Inhibition of HCC cell growth by inducing G₀/G₁ phase arrest was also noticed with miR-122 restoration. Moreover, treatment with miR-122 mimics and doxorubicin resulted in high accumulation of HCC cells in G₀/G₁ phase. Interestingly, the chronic exposure to doxorubicin in low doses resulted in reduction of ABCB1 and ABCC1 levels; as compared with acute high doxorubicin treatments. Nevertheless, this decrease reached significance only in case of ABCC1.

In conclusion, transfection of miR-122 mimics into cultured HepG2 cells induces cell-cycle arrest and sensitizes these cells to doxorubicin by modulating the expression of multidrug resistance genes. The regulation of MDR-related gene expression by miR-122 may be direct or indirect through the down regulation of transcription factors involved in regulation of MDR gene expression. Our experiment indicates that clinical sector may benefit from considering lower doses of doxorubicin, in order to reduce risk of potential side effects, as well as decrease risk of developing drug resistance. Therefore, combination of miR-122 restoration and low chronic doses of doxorubicin might has important implications for the development of tailored treatment strategies in patients with highly resistant hepatocellular carcinoma.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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