RESEARCH ARTICLE

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Possible Use of miR-223-3p as a Prognostic Marker in Transarterial Chemoembolization Treatment of Hepatocellular Carcinoma Patients

Heba E. Abdelaziz[1], Nadia El-Guendy[1], Enas M Radwan[2], Sabry K Sharawi[1], Motawa E El Houseini[1], Abdel-Hady A Abdel-Wahab[1]*

Abstract

Introduction and Objectives: Transcatheter chemoembolization (TACE) is the recommended therapy for intermediate stage hepatocellular carcinoma patients. Unfortunately, one of the main reasons for its failure is the emergence of multidrug resistance (MDR). Therefore, this study explored the possibility of using MDR-related miRNA as a response biomarker in HCC patients treated with doxorubicin drug-eluting bead TACE (DEB-TACE). **Patients and Methods:** Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to evaluate the expression level of 14 MDR-related miRNAs in doxorubicin-resistant HepG2 cells (HepG2/Dox) developed by single-dose of doxorubicin mimicking the situation of liver cells surviving TACE. The sera level of miR-223-3p, which was the most significantly downregulated in the HepG2 cells, was determined in 60 primary HCC patients undergoing TACE. Restoring miR-223-3p in HepG2/Dox cell line was achieved by its mimic transfection. Cell sensitivity was measured by SRB assay. Cell apoptosis and doxorubicin uptake were assessed by flow cytometry. The expression of miR-223-3p target protein, P-glycoprotein, was evaluated using qRT-PCR and western blotting. **Results:** We detected a significant downregulation of circulating miR-223-3p in patients non-responders to TACE treatment compared with responders. The expression of miR-223-3p was markedly decreased in resistant HepG2/Dox cells compared to the parental control. In addition, the expression of miR-223-3p was found to be inversely correlated with P-glycoprotein expression thus confirming the role of miR-223-3p in MDR. Furthermore, overexpression of miR-223-3p suppressed P-glycoprotein which promoted cellular uptake of doxorubicin and increased apoptosis. **Conclusions:** Our data suggest a potential role for miR-233-3p as a prognostic as well as a therapeutic target for HCC.

Keywords: microRNA- DEB-TACE- Doxorubicin Drug resistance- P-glycoprotein- Hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) has the highest incidence and mortality in Africa and East Asia (McGlynn et al., 2015). Despite the major advancements made in HCC treatment, management options are limited and rely on the patient's stage. Moreover, the prognosis of liver cancer remains poor as HCC patients have a high recurrence rate after surgery or chemotherapy, and the 5-year survival rate is only about 20% (Society, 2022). Treatment with familiar cytotoxic chemotherapeutic agents such as cisplatin, doxorubicin, and 5-FU results in limited impact because of systemic toxicity, poor efficacy, and intrinsic or acquired cross-resistance of tumors to chemotherapy (Beaugrand et al., 2005). For patients in middle or advanced stage HCC, transcatheter arterial chemoembolization (TACE), is considered the recommended therapy used to diminish tumor load (Lopez et al., 2006). Doxorubicin, which is the first-line agent for chemoembolization has been shown to induce drug resistance, resulting in a high recurrence rate (Bach et al., 2017). As a result, TACE has a poor long-term outcome with a partial response in only 15–55% of patients, and the reported five-year survival rates range from 1–8% (Kong et al., 2018). Therefore, it is of utmost importance to explore new markers that can predict response to treatment and might provide a tool for patient selection and prioritization.

One of the most common mechanisms of doxorubicin resistance is the overexpression of adenosine triphosphate-binding cassette (ABC) transporters (Germann, 1996). Approximately 13 out of 49 known ABC in humans are directly relevant to chemoresistance. They mediate the efflux of chemotherapeutic agents across the cellular

*[1]Department of Cancer Biology, National Cancer Institute, Cairo University, Cairo, Egypt. [2]Department of Clinical Pathology, National Cancer Institute, Cairo University, 1 Kasr El Eini Street Fom ElKhalig, 11796 Cairo, Egypt. *For Correspondence: abdelhady.abdelwahab@nci.cu.edu.eg*

membrane using the energy of ATP binding and hydrolysis. This leads to reduced intracellular concentration of drugs in cancer cells and deterioration of therapeutic efficacy (Vasiliou and Nebert, 2009; Wilkens, 2015; Kadioglu et al., 2020; Wang et al., 2021). During the past decades, extensive efforts have been made to identify new methods to overcome multidrug resistance (MDR) by targeting the ABC transporters.

MicroRNAs (miRs) are short non-coding RNAs that act as regulators of target mRNAs (Iorio et al., 2005). They have shown promising results in nucleic acid-based drugs, early diagnosis, and disease monitoring (Xu et al., 2018). It has also been shown that miRNAs are involved in drug resistance of tumor cells by targeting -related genes (Iorio et al., 2005; Zheng et al., 2010; Zhuo et al., 2013). MiR-223 has been reported to play a vital role in the chemoresistance mechanism in many human cancers such as breast cancer, gastric cancer, non-small cell lung cancer, and colorectal cancer by regulating the miR-223/FBXW7 pathway (Ding et al., 2018). Another study conducted by Zhou et al, revealed the role of miR-223-3p in modulating doxorubicin-induced autophagy and sensitivity in four miR-223-3p mimic transfected human HCC cell lines (Zhou et al., 2019). Yang et al., (2013) showed that miR-223 played an important role in the regulation of MDR mediated by ABCB1.

The first identified, most studied and cancer-related ABC transporter is P-glycoprotein (P-gp) which is encoded by the *ABCB1* gene. P-gp is also known as Multidrug Resistance Protein-1 (MRP-1), Breast Cancer Resistance Protein (BCRP), and Lung Resistance Protein (LRP). It confers MDR by increasing transmembrane efflux of drugs thus decreasing their concentration in the cytoplasm rendering cancer cells resistant to a wide range of chemotherapeutic drugs (Leonard et al., 2003; Kovalev et al., 2013). Doxorubicin is among 20 or so cytotoxic drugs that are substrates of P-gp (Ozben, 2006). ABCB1 is often upregulated in chemotherapeutic-resistant cancer cell lines and is suggested to contribute to the drug resistance phenomenon (Zhou, 2008). MiRNAs were found to overcome MDR by silencing the activity of ABCB1 efflux pumps or by reducing its expression in various malignancies, including HCC (Duan et al., 2004; Hong et al., 2013). Silencing oncomirs with miRNA inhibitors or restoring tumor suppressor miRNAs with synthetic miRNA mimics has been shown to be a valuable experimental strategy for the treatment of solid and hematological malignancies (Allen and Weiss, 2010; Tagliaferri et al., 2012; Bovell et al., 2013).

Since there is an urgent need to find new strategies to decrease drug resistance, enhance drug response and minimize the cytotoxic side effects, our objective was to identify MDR-related miRNA that can be used as a predictive marker for the response of HCC patients to doxorubicin drug-eluting bead transcatheter chemoembolization (DEB-TACE) treatment in an attempt to ameliorate the efficacy of HCC treatment. To achieve this goal, we evaluated the expression level for MDR-related miRNAs panel in HepG2/doxorubicin resistant cells developed using a single dose mimicking the situation of liver tumor cells surviving TACE treatment. The role of the selected miRNA (miR223-3p) in regulating *ABCB1* gene expression in doxorubicin resistant cells was confirmed. Furthermore, circulating miR-223-3p expression level was found to be correlated with the response of HCC patients to DEB-TACE.

Materials and Methods

Cell culture and resistant cells development

Hepatocellular carcinoma cell line, HepG2, was obtained from VACSERA (the Egyptian Company for Production of Vaccines, Sera, and Drugs). It was cultured in high glucose, L-glutamine containing RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (100 U/ml penicillin, and 100 ug/ml streptomycin) (Sigma Aldrich Chemical Co., St. Louis, MO, USA). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37ºC and were passaged at a 1:4 ratio when confluent, approximately every 3 days.

To mimic the situation of HCC cells surviving TACE treatment, doxorubicin-resistant HepG2 cells (HepG2/Dox) were developed using single-step doxorubicin (Pfizer, New York, USA) treatment as described previously (Buschauer et al., 2018). Briefly, HepG2 cells were exposed to 80% inhibitory concentration dose (IC_{80}=9.9 µg/ml) of doxorubicin for 48 h. Afterward, cells were washed with PBS then cells surviving this treatment (HepG2/Dox) were further cultured in regular, doxorubicin-free medium for 3 weeks. Parental cells (HepG2) were continuously cultured in regular medium without doxorubicin. HepG2 and HepG2/Dox cell lines were passaged when confluent in parallel for the 3 weeks.

Patients/samples collection

A cohort of 60 patients with advanced HCC who were subjected to TACE treatment with drug-eluting bead (DEB) loaded with doxorubicin (50 mg) at the radiodiagnosis clinic of the National Cancer Institute (NCI) between the years 2015 to 2017, was selected. Response to TACE treatment was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST V1.1). Accordingly, patients were divided into responders and non-responders (30/each). Age and gender-matched 30 healthy subjects, with no history of any disease, were enrolled as controls. This study was approved by the Institutional Review Board (IRB approval #201001415034.2) of the NCI, Egypt. Written informed consent was obtained from the patient for publication of this case report and accompanying images.

Venous blood samples (5 ml) were collected from HCC patients prior to TACE treatment as well as the control group under aseptic conditions. Serum was separated immediately by centrifugation and stored immediately at -80°C for quantitation of miRNA expression levels. Different clinical features of the patients were collected from their medical records as summarized in Table 1.

Cell transfection

Mimic of hsa-miR-223-3p (cat#219600), inhibitor (cat#219300), negative control of mimic (NCm)

(cat#1027280), and negative control of inhibitor (NCi) (cat# 1027271) were purchased from Qiagen (Germany). HepG2/Dox cell line seeded in 6 well plates at 3X10^{5}cells/well were transfected with hsa-miR-223-3p mimic, inhibitor, NCm, or NCi using HiPerfect reagent (Qiagen) according to the manufacturer's protocol, at a final concentration of 15 nM and 50 nM for mimic, NCm and inhibitor, NCi respectively. The transfected cells were harvested for downstream analysis after 24 h.

Cell viability assay

The cytotoxic effect of doxorubicin on the growth of HepG2 and HepG2/Dox cells was assessed using Sulphorhodamine-B (SRB) assay (Sigma-Aldrich Chemical Co., USA) as described previously (Skehan et al., 1990). HepG2 and HepG2/Dox cells (1.0X10^4 cells/well) were seeded in a 96-well plate. The next day, cells were treated with different doses of doxorubicin (0.5, 2, 4, 5, and 8 µg/ml). Cell fixation with 10% trichloroacetic acid for 1 h at 4ºC was done 48h after DOX treatment. Staining with 0.4% (w/v) SRB for 30 min. followed by washing in 1% acetic acid was carried out. SRB-bounded cells were dissolved by 10 mM Tris solution (pH 10.5). The absorbance was measured at 570 nm using a microplate reader (TecanSunriseTM, Germany). The sigmoidal concentration response curve fitting model (Graph Pad, Prism software) was used to calculate the IC_{50} values.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted and purified from transfected and untransfected cells and from patients' sera using miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA concentration was quantified by a NanoDrop-2000 spectrophotometer (ThermoFisher Scientific, USA). Complementary DNA (cDNA) was synthesized from 1 µg RNA using miScript II RNA reverse transcription kit (Qiagen, Germany) following the manufacturer's instructions. Using miRNA databases MiRTarBase (https://www.bio.tools/mirtarbase), microRNA.org (https://www.microrna.org) and Targetscan (http://www.targetscan.org), 14 different miRNAs that target *ABCB1* gene, were identified. Relative expression of ABCB1, as well as that of the miRNAs were estimated by qRT-PCR using miScript SYBR Green reagent kit obtained from Qiagen using cDNA as template. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and RNU6B were used as internal controls for mRNAs and miRNAs, respectively. Primer sequences were obtained from Qiagen (Germany). All the qRT-PCR reactions were performed in triplicate on ViiA™ 7 PCR system (Applied Biosystems, USA). Data were analyzed using ΔΔCt comparative method(Schmittgen and Livak, 2008) and the fold of change =$2^{-\Delta\Delta Ct}$. Gene expression was considered up-regulated if values of fold change were higher than 2.

Western blotting analysis

Cells were harvested and lysed in cold RIPA (150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP-40) lysis buffer containing protease/phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA). Protein concentrations were estimated with Pierce BCA Protein Assay kit (Thermo Scientific, USA). Proteins were resolved by SDS-PAGE and transferred onto a PVDF membrane. The membranes were blocked with 3% BSA then incubated with primary antibodies against P-glycoprotein (ThermoFisher, dilution 1:300 in TBS-T) or β-actin (ThermoFisher, dilution 1:1000 in TBS-T) at 4°C overnight. Following washing, the membranes were incubated with anti-rabbit (for P-glycoprotein) or anti-mouse (for β-actin) secondary antibodies (Sigma Aldrich, dilution 1:2,000). Protein bands were visualized using enhanced chemiluminescence (ECL) reagents and quantified using online Image J 1.52a (Wayne rasband, National institute of health, USA.) software. Western blot analysis was performed 3 times.

Measurement of cellular doxorubicin uptake

Measurement of intracellular concentrations of doxorubicin was assessed using flow cytometry exploiting the fluorescence characteristic of doxorubicin [excitation/emission wavelengths of 470 and 560 nm respectively] as previously described (Kauffman et al., 2016). HepG2, HepG2/Dox, and transfected HepG2/Dox cells were seeded in tissue culture flasks (3.5 x 10^6/flask) until reaching 60% confluency. Doxorubicin (4.47 µg/ml = IC_{50}) was added to each flask and incubated at 37°C for 24h. Cells were washed with cold PBS then harvested and re-suspended in PBS. The acquisition was done on Navois flow cytometer of 10,000 events (Beckman Coulter, Life Sciences) equipped with a 488-nm blue diode laser and a 575/30 filter detector (FL2), analysis was done using Kaluza analysis version 2.1 software.

Apoptosis analysis

Cell apoptosis was determined using an Annexin-V fluorescein isothiocyanate (FITC) apoptosis detection kit (Biospes, Cat# BAD1001), following the manufacturer's instructions. HepG2, HepG2/Dox, and transfected HepG2/Dox cells were treated with doxorubicin (IC_{50}) for 24 h. The cells were harvested in PBS and resuspended in binding buffer with concentration adjustment to be 1×10^6 cells/mL, then 5µL of Annexin-V FITC was added to100µL of cells (1×10^5) for 15 min. at 4°C in the dark then acquisition was done on Navios flow cytometer (Beckman Coulter, Life Sciences)) equipped with 525/40 filter detector (FL1).

Statistical analysis

All data analysis was performed by Graph Pad Prism (San Diego, USA) software. All data were presented as mean±SD or SEM from at least three independent experiments. Student's t-test was used to determine the significance between two different groups. Analysis of variance (ANOVA) test was used when more than two groups were compared. $P<0.05$ was considered statistically significant.

Results

Selection of doxorubicin resistant HepG2 cells

To mimic the development of resistance to doxorubicin

Table 1. Demographic and Clinical Data of Primary HCC Patients Treated with TACE.

Parameters	Median (IQR) Responders(n=30)	Median (IQR) Non-Responders (n=30)	p-value
Age (year)	62 (47-80)	64 (47-71)	0.9144
Gender (M/F)	23/7 (3.29)	15/15 (1.0)	0.0871
Tumor size (cm)	4.5 (1.5-16.5)	4.7 (2.0-11.5)	0.8506
No of lesions (%)			
One	15 (50)	8 (26)	0.2571
Two	5 (17)	11 (37)	
Multiple	10 (33)	11 (37)	
Child Class (%)			0.4682
A	21 (70)	16 (53)	
B	9 (30)	14 (47)	
AFP (ng/ml)	35.8 (0-1000)	263.5 (1.8-1000)	0.3251
AST (U/L)	53(23-216)	65 (15-144)	0.8976
ALT (U/L)	35 (12-194)	30 (9-57)	0.2278
Alkaline phosphatase (U/L)	138.5 (82-328)	147 (50-287)	0.9704
Total Bilirubin (mg/dL)	1.2 (0.3-4.5)	1.0 (0.4-3.1)	0.4291
Albumin (g/dL)	3.0 (2.2-4)	3.3 (2.7-3.8)	0.2553
Blood urea (mg/dL)	34.5 (17-81)	30.0 (16-89)	0.4496
Serum creatinine (mg/dL)	0.9 (0.6-2.4)	0.8 (.2-1.9)	0.0778
Hemoglobin (g/dL)	11.9 (6.6-14.3)	12.6 (8.8 – 15.5)	0.2501
INR	1.23 (1.0 – 1.61)	1.2 (1.0-1.48)	0.3064

Fisher's exact test for qualitative data between the two groups.; Mann Whitney test for non-parametric quantitative data between the two groups; *: Significant level at P value < 0.05; Abbreviations: IQR, interquartile range; M, Male; F, Female; AFP, alpha fetoprotein; AST, aspartate transferase; ALT, alanine transferase; INR, international normalized ratio.

in HCC after TACE treatment, HepG2 cells were subjected to a single high dose of doxorubicin as described previously (Buschauer et al., 2018). After the determination of the IC_{50} of doxorubicin in the HepG2 cell line, we started the process of selection for the resistant cells by adding a high lethal dose (IC_{80}) of doxorubicin for 48 h. Inverted microscopy examination confirmed that after 48 h incubation with 9.9 µg/ml doxorubicin, 10-20% of HepG2 cells survived (HepG2/Dox) (Figure 1A). These cells, along with parental untreated cells (HepG2), were

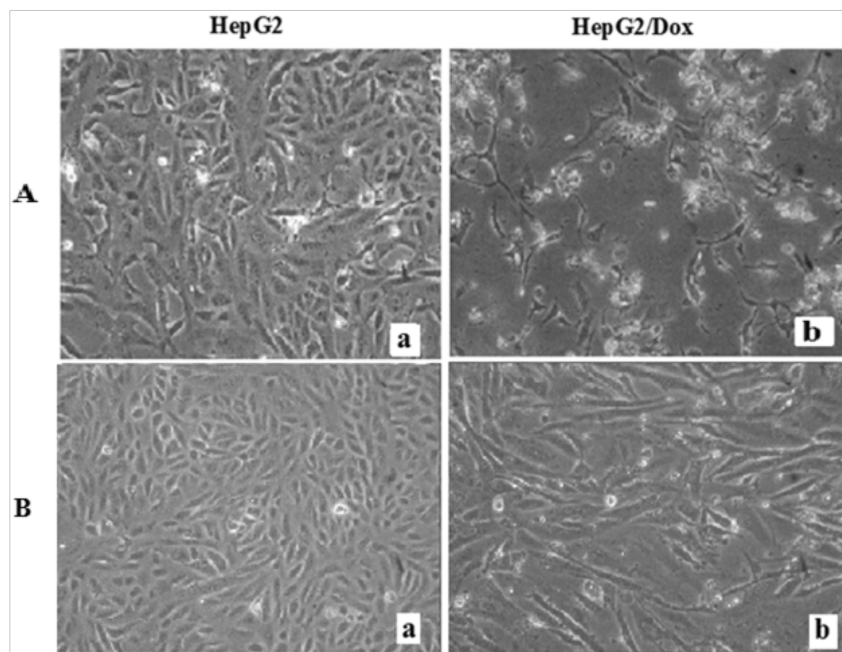

Figure 1. Selection of HCC Cells Surviving Dox Treatment. HepG2 human HCC cell line was treated with the indicated concentration of Dox for 48 h. (A) Inverted microscopy of (a) untreated CTRL cells and (b) cells treated with 9.9 µg/ml Dox after 48h incubation. (B) Inverted microscopy of (a) untreated CTRL cells (HepG2) (b) cells surviving 3 weeks after Dox treatment (HepG2/Dox).

Table 2. Expression Level of MiRNAs in Hepg2/Dox Cells Compared with HepG2 Parental Cells

miRNA	Fold change (±SD)	P-value
hsa-miR-129-5p	1.06±1.08	0.3779
hsa-miR-133	1.36±1.11	0.4355
hsa-miR-145-5p	0.61±0.75	0.2067
hsa-miR-198	13.05±1.07	0.0019**
hsa-miR-223-3p	0.20±0.3	0.0004***
hsa-miR-26a-5p	6.78±8.44	0.3315
hsa-miR-27a-3p	6.19±5.9	0.1124
hsa-miR-298	10.17±11.6	0.0400*
hsa-miR-302b-3p	37.91±0.785	0.0062**
hsa-miR-31-3p	1.97±0.02	0.0541
hsa-miR-31-5p	0.31±0.01	0.4794
hsa-miR-373-3p	2.73±0.23	0.0884
hsa-miR-373-5p	0.29±0.01	0.4725
hsa-miR-451a	1.33±057	0.207

Data are represented as mean values ± SD from three replicates. *, Significantly different (* p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.0004). The statistical significance of the results was analyzed using the two-tailed Student's t test

maintained for 3 subsequent weeks in doxorubicin free media. Monitoring of cell growth and morphology with the inverted microscopy revealed that HepG2/Dox cells developed a spindle-like, outstretched shape (Figure 1B).

HepG2/Dox cells are approximately 5 times more resistant to Doxorubicin treatment

Resistance of HepG2/Dox cells to Dox treatment was examined using SRB assay. Cells were treated with different concentrations of doxorubicin ranging from 0.5 to 8 µg/ml for 48 h. Figure 2 shows the IC_{50} of parental (HepG2) and HepG2/Dox to be 1.51 and 7.97 µg/ml respectively. This experiment revealed that the HepG2/Dox cells were about 5 times more resistant to killing by doxorubicin than parental cells confirming the development of a resistant cell line.

Identification of miRNA targeting P-glycoprotein expression with possible relation to doxorubicin resistance

To investigate the role of miRNAs in the MDR phenotype, we used miRNA databases MiRTarBase, microRNA.org and Targetscan to select 14 miRNAs that target *ABCB1* gene. These are: hsa-miR-129-5p, hsa-miR-133, hsa-miR-145-5p, hsa-miR-198, hsa-miR-223-3p, hsa-miR-26a-5p, hsa-miR-27a-3p,hsa-miR-298, hsa-miR-302b-3p, hsa-miR-31-3p, hsa-miR-31-5p, hsa-miR-373-3p, hsa-miR-373-5p and hsa-miR-451a. The expression levels of these miRNAs were determined in HepG2/Dox versus parental HepG2. As shown in table 2, the expression levels of miR-198, hsa-miR-223-3p, miR-298, and miR-302b-3p were significantly deregulated in HepG2/Dox compared to parental HepG2. MiR-223-3p was the most highly significantly deregulated miRNA, its expression was 5 times lower in HepG2/Dox compared to parental (HepG2) cells (P-value= 0.0004). Consequently, we decided to study the involvement of miR-223-3p in doxorubicin acquired resistance and response to TACE.

Low levels of miR-223-3p in non-responder patients' sera

Since miR-223-3p showed the most downregulation in HepG2/Dox resistant cells, suggesting a role as an MDR inhibitor, we decided to evaluate the possible correlation between levels of miR-223-3p in HCC patients and response to TACE. To achieve this, we measured the expression level of miR-223-3p in sera of a cohort of patients divided into responders and non-responders (30/each) to DEB-TACE treatment, in addition to 30

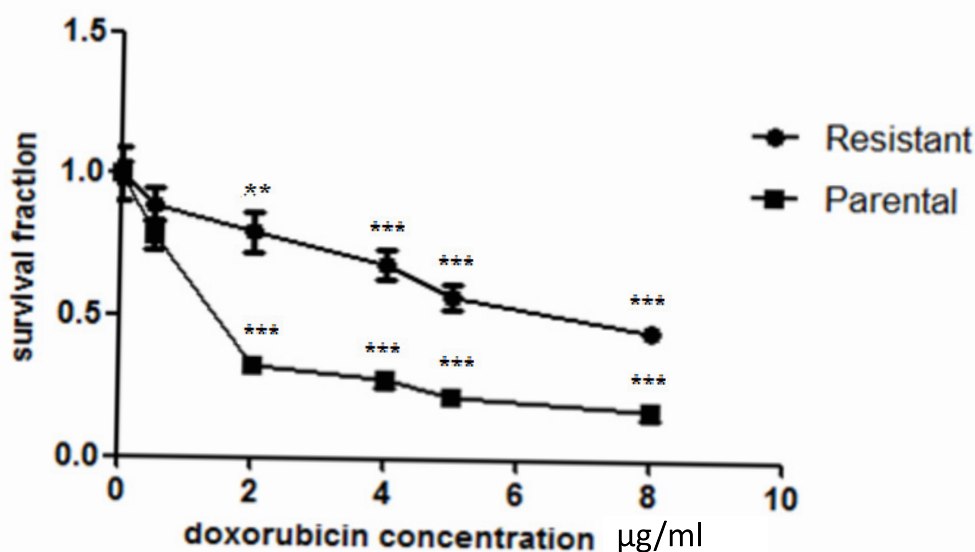

Figure 2. SRB Assay as a Measure of Cell Viability of HepG2 and HepG2/Dox Cells. Dox treatment induced a marked dose-dependent decrease in cell viability in both cell lines. The results are expressed as the mean ± SD of 3 separate experiments performed in triplicates. The results were analyzed using one-way ANOVA followed by Tukey multiple comparison test. **Significant difference from its untreated control (p-value < 0.05), and *** Significant difference from its untreated control (p-value < 0.0001)

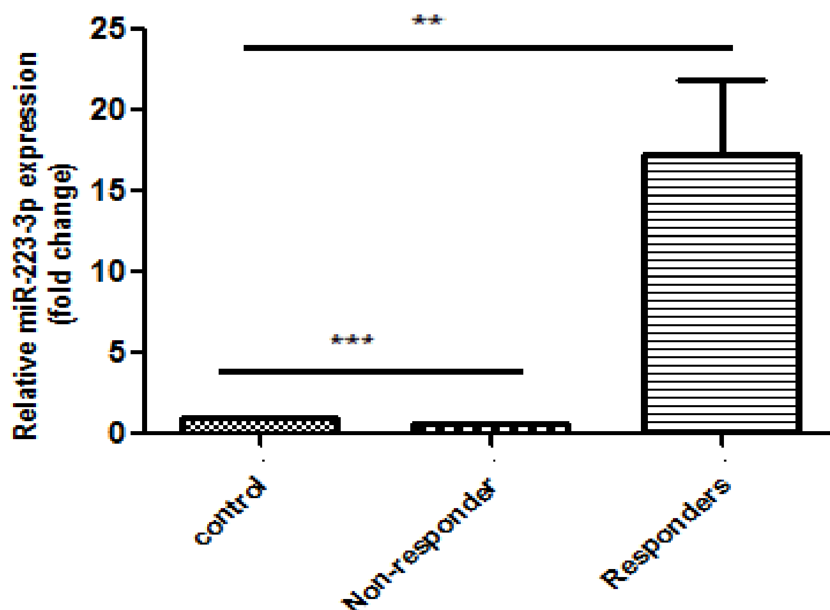

Figure 3. Expression Level of miR-223-3p in Sera of Hepatocellular Carcinoma Patients (Responders/Non-Responders) Using qRT-PCR Assay. The data were normalized by RNU6B as endogenous control and shown as fold change ±SD. All assays were performed at least three times with similar results.

healthy volunteers. Our results revealed a significant downregulation in miR-223-3p level in non-responder patients (1.6 times decrease, p value=0.0004). Moreover, there was a significant upregulation in its level in the group of responder patients (16.75 times increase, p value=0.002) compared to normal individuals respectively (Figure 3).

miR-223-3p modulates ABCB1 expression

Consistent with a possible role of P-gp in doxorubicin acquired resistance we found that the expression levels of ABCB1 (32 fold of change, p=0.04) (Figure 4A) and

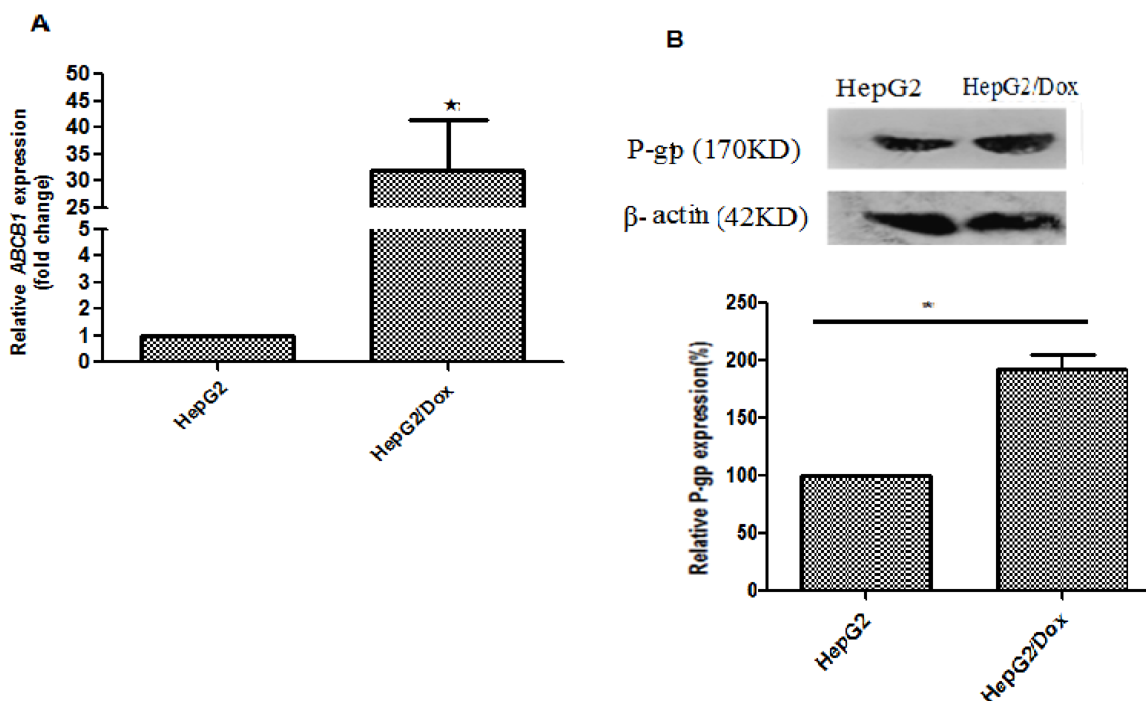

Figure 4. Expression Level of ABCB1 in HepG2/Dox and Its Parental HepG2 Cell Line. (A) ABCB1 mRNA expression was determined by qRT-PCR. The relative amount of ABCB1 was normalized to GAPDH, * p value=0.0443. Data are shown as mean fold change ± SD in HepG2/Dox cell line relative to HepG2 cell line. (B) Western blot analysis revealed the increased protein expression of P-gp in HepG2/Dox compared to HepG2. The figure is a representative of three different experiments with similar results.

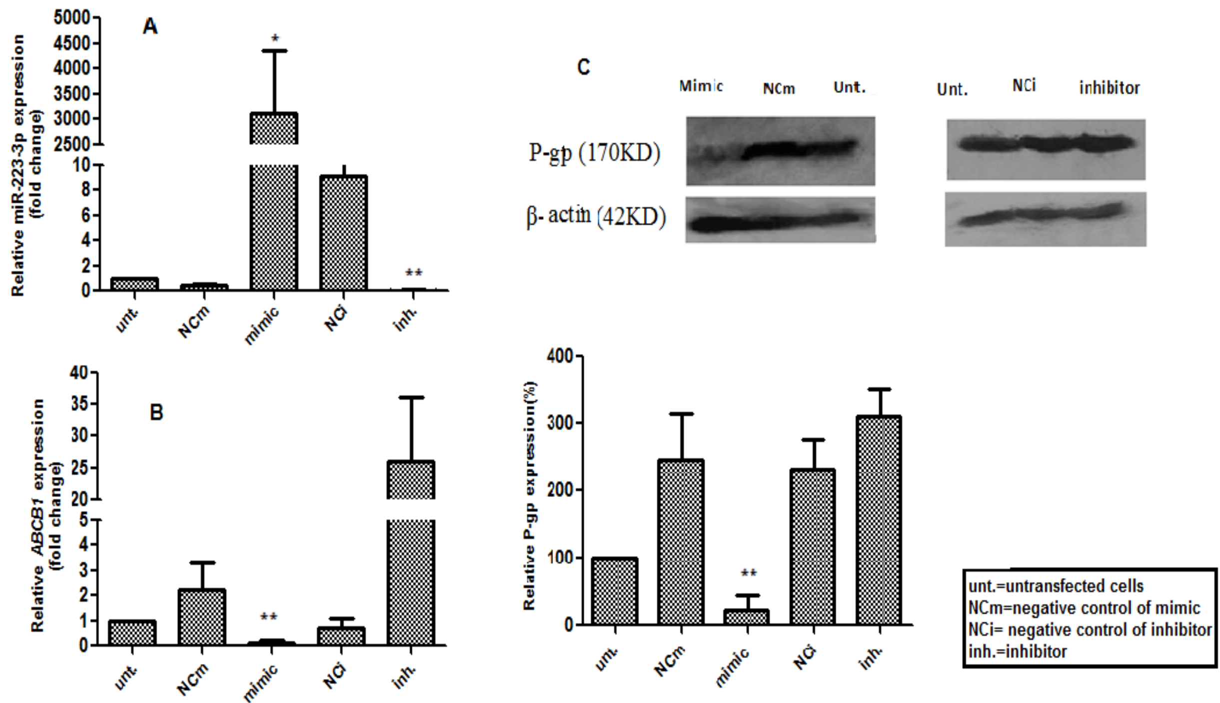

Figure 5. MiR-223-3p Decreases P-gp Expression in HepG2/Dox Cells. (A) qRT-PCR analysis of miR-223-3p expression in HepG2/Dox cells transfected with miR-223-3p mimic/inhibitor or NCs. (B) qRT-PCR analysis of P-gp expression in HepG2/Dox cells transfected with miR-223-3p mimic/inhibitor or NCs. (C) Expression of P-gp protein was examined by western blot analysis after transfection with miR-223-3p mimic, inhibitor or NCs. The assays were performed at least three times with similar results.

P-gp (2 times overexpressed, p=0.0264) (Figure 4B) were significantly higher in the HepG2/Dox cells compared to the parental HepG2 cells.

To confirm whether miR-223-3p is involved in the regulation of the expression of ABCB1 in cells with newly acquired resistance and whether it is associated

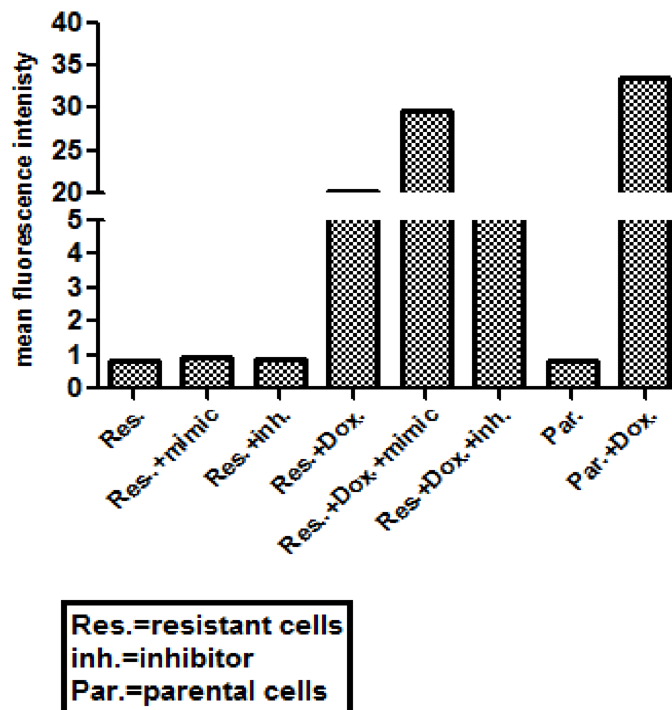

Figure 6. Determination of Intracellular Concentration of Doxorubicin Using Flow Cytometry Assay. Fluorescence intensity was measured at the excitation wavelength of 470 nm and emission wavelength of 549 nm in HepG2, HepG2/Dox and transfected HepG2/Dox using flow Navios cytometer (Beckman Coulter, Life Sciences) and analyzed using Kaluza analysis version 2.1software. The presented bar graph is a representative of several independent flow cytometry experiments.

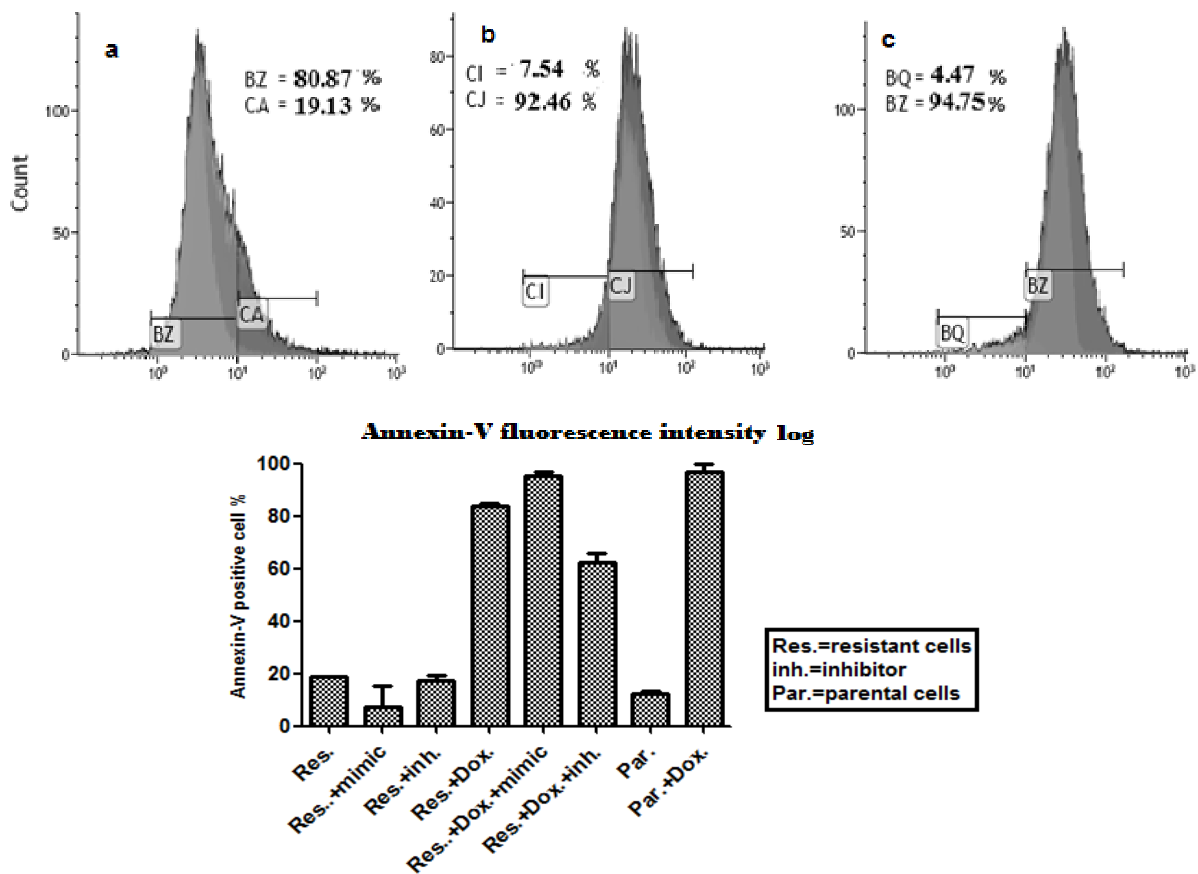

Figure 7. Determination of Apoptosis Using Flow Cytometry Assay. a) Annexin-V positive cell percentage in untreated HepG2/Dox cells. b) Annexin-V positive cell percentage in HepG2/Dox cells treated with IC50 dose of doxorubicin and transfected with miR-223-3p mimic. c) Annexin-V positive cell percentage in HepG2 cells treated with IC_{50} dose of doxorubicin. The presented bar graph is a representative of several independent Annexin-V flow cytometry experiments.

with doxorubicin acquired resistance in liver cancer, we overexpressed or inhibited miR-223-3p in HepG2/Dox cells by transfecting with miR-223-3p mimic, inhibitor negative controls with/without doxorubicin treatment (Figure 5A). Our results indicated that the expression of ABCB1 mRNA was significantly decreased (about 7.7 times, p-value =0.0029) with overexpression of miR-223-3p in HepG2/Dox cells compared to untransfected cells (Figure 5B). Western analysis of P-gp protein confirmed the effect of miR-223-3p on ABCB1 as it showed 6.4 times decrease in the protein expression (p value=0.0022) (Figure 5C).

Consistently, miR-223-3p inhibitor led to an increase in the ABCB1 mRNA and P-gp protein levels. Taken together, these data strongly suggest that upregulation of the miR-223-3p decreased the expression of ABCB1 in HepG2/Dox cells.

miR-223-3p over-expression increase HepG2/Dox sensitivity to Doxorubicin

Flow cytometry analysis showed a remarkable increase in mean fluorescence intensity (MFI) of cellular uptake of doxorubicin (Figure 6) and also an increase in apoptosis in miR-223-3p-mimic transfected HepG2/Dox cells in contrast to miR-223-3p-inhibitor and untransfected cells (Figure 7). This suggests that overexpression of miR-223-3p, thus inhibition of P-gp, in doxorubicin-resistant cells rendered them as sensitive as the parental cells. Conversely, anti-miR-223-3p-transfected HepG2/Dox cells showed more resistance to doxorubicin than the corresponding untransfected controls.

Discussion

The prevalence and mortality of hepatocellular carcinoma (HCC) have been globally rapidly increasing in the last years. Treatment options are varied and continuously evolving. The choice of treatment is determined based on different factors such as tumor size, number, location, and the status of the patient's liver. Although TACE is the recommended first-line therapy for patients with unresectable intermediate stage HCC (stage B), it has a poor long-term outcome with the reported five-year survival rates ranging from 1-8% (Bruix and Llovet, 2002; Kong et al., 2018; Salgia and Mendiratta, 2021). Given the extreme variability of patients' responses to TACE, it is important to find markers that allow for the identification of responders from non-responder patients to design better treatment strategies.

Rapid drug resistance development and toxicities

lead to failure of cancer regression and remission (Fodale et al., 2011; Jindal et al., 2019). Statistical data indicate that over 90% of the mortality of HCC patients is related to multidrug resistance (MDR) (Si et al., 2019). Various studies have shown that MDR of tumor cell lines can be reversed by inhibiting P-glycoprotein expression and function, thus restoring their sensitivity to chemotherapeutic drugs (Zhu et al., 2012). Some miRNAs were found to modulate MDR by targeting P-glycoprotein-encoding ABCB1 expression in many types of cancers (e.g.: colorectal, breast, and leukemia) (Kovalchuk et al., 2008; Zhao et al., 2010; Feng et al., 2011; Kopp et al., 2012). MiRNA-223 (Yang et al., 2013) and miRNA-133a (Ma et al., 2015) were shown to target ABCB1 in SMMC7721 and HepG2 cells and ABCC1 in HepG2 cells, increasing the sensitivity of these HCC cells to Doxorubicin. Furthermore, other studies reported that miR-222 modulates P-gp expression in human colorectal carcinoma cells and serves as a chemosensitizing agent through suppression of ADAM-17-mediated MDR (Xu et al., 2012). In Yang et al., (2013) study, the group compared how the levels of P-glycoprotein in different cell lines affected response to Doxorubicin. In addition, some studies have demonstrated that several miRNAs (miR-1271, miR-4492, miR-214, miR-125b, miR-26a, miR-106b, miR-107, miR-133b, miR-590-5p, miR-21, miR-29a-3p, miR-122, miR-1268a, miR-199a/b-3p, miR-196a2 rs11614913, miR-499a rs3746444, miR-335, miR-10a, miR-23a, miR-24, miR-27a, miR-30c, miR-30e, miR-31, miR-200 a/b, miR-1285-3p, miR-4741, and miR-210) were associated with TACE treatment response (Bozzato et al., 2022).

In our study, we investigated whether changes in the expression levels of a miRNA that modulate MDR can be used to predict response to TACE treatment. To identify such miRNAs, we determined the differential expression of 14 miRNAs known to affect P-gp expression, in a cell line system (HepG2/Dox) developed to imitate the drug resistance that happens in HCC patients after TACE treatment. These cells were significantly less susceptible to killing by doxorubicin compared to parental HepG2 cells, confirming their increased resistance to doxorubicin. A concomitant increase in the expression level of P-glycoprotein, both at the mRNA and the protein levels in (HepG2/Dox) suggested that P-gp might be involved in the transition to the more resistant phenotype since P-gp is known to play a major role in the increased efflux of chemotherapeutic drugs associated with resistance of cancer cells to doxorubicin.

Our second aim was to confirm that the selected miRNA (miR-223-3p) is associated with doxorubicin resistance in our developed HepG2/Dox cell line and that it can be used to revert to the parental doxorubicin-sensitive HepG2 cells phenotype, thus suggesting possible use as a candidate for miRNA-based combinatorial HCC therapy with conventional chemotherapy.

Of fourteen miRNAs that are known to target P-gp expression, we found that hsa-miR-198, hsa-miR-223-3p, hsa-miR-298, and hsa-miR-302b-3p levels were deregulated in dox-resistant cells compared to parental cells. We identified miR-223-3p as the most significantly downregulated miRNA.

To investigate whether increasing the level of miR-223-3p could restore sensitivity to doxorubicin, we transfected the resistant HepG2/Dox cells with hsa-miR-223-3p mimic, which caused down-regulation in the expression of P-gp both at the mRNA level (ABCB1) and the protein level (P-gp) compared to untransfected HepG2/Dox cells. In addition, transfection of HepG2/Dox cells with miR-223-3p inhibitor resulted in the expected up-regulation of P-gp expression confirming that miR-223-3p still regulates P-gp expression in our resistant HepG2/Dox cells. This is consistent with the previous study done by Yang and colleagues who showed that miR-223 directly suppresses ABCB1 expression in different parental HCC cell lines in response to doxorubicin IC50 addition (Yang et al., 2013).

Interestingly, treatment of the resistant HepG2/Dox cells, over-expressing miR-223-3p with doxorubicin, restored their sensitivity to the drug, inducing apoptosis levels similar to that caused in parental HepG2 cells (92.4% of cells were Annexin V positive). The increase in doxorubicin fluorescence detected by flow cytometry analysis suggested that this is due to increased doxorubicin cellular accumulation resulting from the inhibited P-gp pump, which reduced intracellular drug efflux and increased intracellular drug concentration, enhancing the sensitivity of resistant cells to the chemotherapeutic drug. This is consistent with previous results showing that, as a tumor suppressor, miR-223-3p promotes HCC cell apoptosis by targeting the rapamycin pathway (Dong et al., 2017). Taken together, these findings suggest that correcting the expression of this miRNA in doxorubicin-resistant cells can overcome drug resistance and revert resistant cells to parental phenotype.

To assess the possible use of miR-223-3p as a biomarker in predicting HCC patients' response to TACE, we measured the level of this miRNA in sera of a group of 60 HCC patients, in addition to 30 healthy volunteers. The patients were divided into 2 groups, responders and non-responders, according to their response to TACE treatment. Our results revealed a significant downregulation in miR-223-3p levels in the non-responder patients. On the other hand, there was a significant upregulation in the miR-223-3p levels in the group of responder patients compared to the control group. This result further emphathises the important role miR-223-3p plays as an MDR-specific miRNA in HCC patients. The prognostic value of miR-223-3p has previously been suggested in other cancers. In rectal cancer, it was shown that miR-223 has the ability to predict response to chemoradiotherapy (Li et al., 2011) and in bladder carcinoma specimens low miR-223-3p levels were associated with poor prognosis (Hotchi, 2012).

It would be interesting to confirm the effect of miR223-3p on other resistant HCC cell lines and in relation to other chemotherapeutic drugs. In addition, it is important to perform this study on a larger number of patients.

In conclusion, the present study suggests a potential role for miR-223-3p in predicting HCC patients' response to TACE, which is becoming increasingly essential to help in designing new treatment strategies to decrease the

effects of MDR. Since miR223-3p was found to modulate doxorubicin resistance in hepatocellular carcinoma cells by regulating P-gp expression level, it is conceivable that miR-223-3p, in addition to serving as a novel biomarker tool, could be involved in the development of new therapeutic strategies for advanced HCC management.

Author Contribution Statement

Conceptualization: A.A.A.; Investigation: E.F, R.E.; Formal analysis: E.F.; Funding acquisition: A.A.A.; Methodology: A.A.A, E.N.; Project administration: A.A.A; Resources: A.A.A; Supervision: A.A.A, E.N.; Visualization: E. H., E. N., A.A.A.; Writing - Original Draft: E.F., E.N.; Writing - Review & Editing: E. N., A.A.A.; Writing – Review: S.S., E. M.

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Ethics approval

This study was approved by the Institutional Review Board (IRB approval #201001415034.2) of the NCI, Egypt. Written informed consent was obtained from the patient for publication of this case report and accompanying images.

Conflict of interest

The authors declare that they have no conflict of interest.

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