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MicroRNA-122 restoration modulates alpha-fetoprotein expression in hepatocellular carcinoma treated with doxorubicin

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ABSTRACT

Higher levels of morbidity and mortality are due to malignant tumors and Hepatocellular carcinoma (HCC) is considered of a great percentage. The liver-specific microRNA-122 (mir-122) is frequently down-regulated in HCC and is a promising biomarker for the early HCC diagnosis. Restoration of mir-122 renders HCC cells to be sensitive to doxorubicin. The present study aims to investigate the effect of treatment of HCC cells with doxorubicin and/or mir-122 mimics on AFP expression levels. Using HepG2 cells with different treatments, AFP mRNAs and proteins were isolated and detected by real-time PCR and ELISA, respectively. Our data showed that there was a dynamic change in mir-122 expression as compared to inhibitor negative control. Also, there were no significant differences in AFP expression levels in cells treated with chronic doxorubicin dose then transfected with mir-122 mimics. However, our findings demonstrated statistical significant increase in both molecular and protein expression levels of AFP in cells treated with acute high doxorubicin dose and mir-122 mimics. This could be associated with increasing doxorubicin sensitivity by mir-122 restoration in HepG2 cells. This distinct finding could be attributed to a negative feedback increase in AFP levels to counteract the apoptosis/reactive oxygen species triggered by miR-122 restoration in doxorubicin-treated HepG2 cells.

Key words: microRNA-122, HepG2 cells, AFP, doxorubicin.

INTRODUCTION

Hepatocellular carcinoma (HCC) is considered the main cause for liver cancer. It represents the seventh most common cause of cancer in women, the fifth in men, and the third most frequent mortality cancer cause worldwide [1]. Nearly, 85% of new cases occur in developing countries, with highest incidence was found in certain areas located in Africa, east and Southeast Asia; however, Northern and Western Europe as well as North America were considered areas of lower-incidence [1, 2]. The last decade has witnessed a significant rise in the incidence of HCC with especially high incidence reported in Egypt [3]. Hepatocellular carcinoma evolves through an established background of chronic liver disease like cirrhosis. These chronic diseases include 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[4-7]. Nevertheless, HCC clinical outcome is very poor, that can be explained on the basis of the lack of reliable markers for early diagnosis, resistance to treatment, tumor recurrence, and metastasis [8, 9].

Chemotherapy is an important therapeutic strategy for treatment of cancers. Doxorubicin (Dox) is the most effective cytotoxic agent for HCC treatment. In particular, the drug molecule stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication. Moreover, it has been suggested that doxorubicin activates p53-DNA binding,

leading to induced apoptosis [10]. In another proposed mechanism; semiquinone - the oxidized form of doxorubicin- is an unstable metabolite, that releases free radical species when converted back to doxorubicin . These species can lead to peroxidation of lipid, damage to both ; membrane and DNA damage, oxidative stress, triggers apoptotic pathways and finally leading to cell death [11].

Alpha-fetoprotein (AFP) was first described as a marker for HCC in 1964 by Tatarinov (1964). The first quantitative serum assays for AFP were developed by Ruoslahti and Seppala (1971) [12]. Until now, the most commonly used approach to monitoring patients at high risk for HCC depends on alpha-fetoprotein (AFP) along with ultrasounds every 6-12 months. Numerous studies confirmed that AFP has complicated biological functions, such as promoting liver cancer cell proliferation, inhibition of apoptosis, and immune evasion. Also, the American Association for the Study of Liver Diseases (AASLD) (July 2010) highlighted AFP as both prognostic and diagnostic marker for the surveillance or the diagnosis of HCC in the practice guidelines [13-16]. In this context, the discovery of the central role of microRNAs in human tumorigenesis has opened a new field that may be relevant not only for understanding cancer at the molecular level but also for the development of new diagnostic and therapeutic tools [17].

MicroRNAs (miRNAs) are a category of endogenous phylogenetically conserved small (22 nucleotides), with singular-strand and unidentified RNA codons. Primary miRNAs, which possess stem-loop structures, are processed into mature miRNAs by Drosha and Dicer RNA polymerase III. These mature miRNAs then associate with the RNA-induced silencing complex, and the resulting complex directly binds to the 3′-untranslated regions of target messenger RNAs to act as suppressors of translation and gene expression. Thus, depending on the target mRNAs, miRNAs are responsible for the control of various biological functions including cell proliferation, apoptosis, differentiation, metabolism, resistance/sensitivity of tumor cells to chemotherapeutic agents, oncogenesis and oncogenic suppression. MicroRNAs have been revealed to be irregularly present in cancers either through up- or down-regulation in neoplastic cells compared with their normal counterparts and deregulated miRNAs have been linked to molecular pathways involved in neoplastic transformation [17–21]. The mechanisms through which miRNA expression is modulated during liver carcinogenesis include chromosomal rearrangements, promoter methylation and control of transcription factors such as c-myc [22]. Recent evidence suggests that AFP can alter mir-29 expression and can induce changes in the methylome of liver cancer cells that are responsible for the more aggressive behavior of HCC [23]. It has been confirmed that deregulation of miRNAs in cancer can be considered as prospective diagnostic biomarkers or as new therapeutic approaches in fighting cancer. The difficulty of miRNA target prediction and biological validation has been a major obstacle to microRNA research [24].

The liver-specific microRNA-122 (miR-122) is frequently down-regulated in primary HCC, which is explained by hypermethylation of mir-122 promoter region. A recent study showed that mir-122 can distinguish HCC from healthy, chronic hepatitis B and cirrhosis groups, moreover it is a favorable biomarker for the early HCC diagnosis [25]. The biological significance of the down-regulation of miR122 expression in HCC has not yet been fully elucidated. It has been shown that the absence of miR-122 expression is closely related to poor prognosis and metastasis of liver cancer [26]. On the other hand, restoration of mir-122 increases the chemotherapeutic sensitivity of HCC cells and minimizes the invasion, migration and growth of metastatic liver cancer cells. Increasing mir-122 levels in HCC with or without antitumor agents may be a promising strategy for HCC treatment [27]. It functions as a potential tumor suppressor in two ways: inhibiting hepatic cell growth by targeting cyclin G1 and promoting apoptosis of hepatic cells by targeting BCL-w and ADAM17 involved in metastasis [28].

To develop targeted cancer therapies, specific aberrantly regulated molecular pathways should be identified to this cancer and useful biomarkers should be discovered that would reflect aberrations in molecular pathways due to the molecular mechanisms of their expression that support their use in a clinical setting [29]. Targeting the liver with microRNAs was reported to be safe and effective at therapeutic doses. Moreover, the liver represents an ideal organ for oligonucleotide-based therapy [17].

In the present study, we explore the effect of acute and chronic doxorubicin doses with/without the restoration of mir-122 on the AFP expression in HepG2 cells.

MATERIALS AND METHODS

1.1. 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 cm^2 flasks in DMEM (Dulbecco's Modified Eagle Medium; Lonza, Belgium); supplemented with 10% Fetal Bovine Serum (Biochrom, Berlin), 1% Penicillin-streptomycin (Lonza, Belgium) and 4 mM L-glutamine (Lonza, Belgium) at 37 ˚C in 5% Co_2 incubator.

1.2. Treatment grouping

In this study, the experimental cell line groups were divided into:

A) Wild HepG2 cells:

a_1) Untreated control (U).

a_2) Transfected with inhibitor negative control (NC).

a_3) Transfected with mir-122 mimics (mir-122).

B) HepG2 cells treated with doxorubicin doses:

❖ First dose-I

b_1) Treated with chronic doxorubicin dose (Dox I).

b_2) Treated with chronic doxorubicin dose then transfected with inhibitor negative control (NC I).

b_3) Treated with chronic doxorubicin dose then transfected with mir-122 mimics (mir-122 I).

❖ Second dose- II

b_4) Treated with acute high doxorubicin dose (Dox II).

b_5) Treated with acute high doxorubicin dose and transfected with mir-122 mimics (D+M II).

1.3. Acute and chronic doxorubicin doses

Wild HepG2 cells were cultured in the presence of 1 µM DOX (Pfizer, USA) for 72 hours *(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.

1.4. Cell transfection with RNA oligonucleotides

HepG2 cells were transfected with100 nM of miscript mir-122 mimics or miScript Inhibitor Negative Control (Qiagen, Valencia, CA) 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. Cell culture media and total RNA were isolated at 72 h after transfection for further analyses.

1.5. Quantitative real-time PCR analysis for miRNA and mRNA expression

For microRNA, miSCript miRNA PCR system (miRneasy mini kit for miRNA extraction, miSCript RT II for miRNA reverse transcription, 10x miR-122a-1 miSCript Primer Assay and miSCript SYBR Green PCR kit for PCR amplification) (Qiagen, Valencia, CA, United States) 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 Triazol (Qiagen, Germany) following the manufacturer's instruction. For quantifying AFP gene by QuantiFast SYBR Green one-step RT-PCR kit (Qiagen, Germany) using MiniOpticon Real-Time PCR System, Bio-Rad, France; the manufacturer steps were followed. Beta-actin mRNA levels were used for normalization with primer sequence of: (F) 5'-CCTTCCTGGGCATGGAGTCCT- 3', (R) 5'-GGAGCAATGATCTTGATCTTC- 3'. AFP primer sequences' (F) 5′-GAAACCCACTGGAGATGAACAGTC-3′, (R) 5′-AAGTGGGATCGATGCAGGA-3′.

The AFP 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 cycler was used for quantitative estimation.

1.6. Determination of AFP using Enzyme-linked immunosorbent assay

Cell-culture supernatants were collected after centrifugation of media at 3000 rpm and 4°C for 10 min. AFP level in the supernatants was examined using an AFP-specific Human ELISA kit supplied by Abcam (UK). According to the kit instructions, standard curve was constructed and used to determine unknown AFP levels.

1.7. Statistical analysis

Statistical calculations were executed using Statistical Package for Social Sciences (SPSS) version 17. The data was represented as the mean ± standard error mean (SEM). A student's *t*- test, in addition to one-way analysis of variance (ANOVA), was used to compare individual data points among each group. A p value of < 0.05 was set as the criterion for statistical significance.

RESULTS

1.1 Level of mir-122 expression in HCC cells after treatment with mir-122 mimic and Inhibitor Negative Control

The dynamic expression of mir-122 after transfection in wild and doxorubicin treated HepG2 cells was demonstrated by isolating 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 as shown in (Fig. 1).

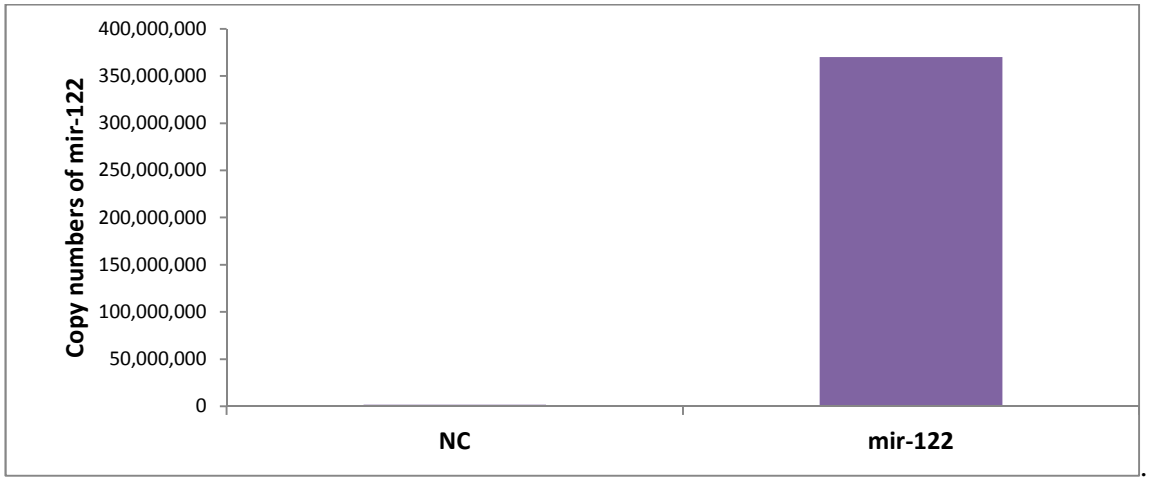

Fig. 1. Copy numbers of mir-122 in cells treated with Inhibitor Negative Control (NC) and mir-122 mimic

1.2 AFP gene expression levels

To test whether treatment of HepG2 cells with mir-122 mimics and/or acute/chronic doxorubicin doses could affect expression of AFP gene. Thus, AFP gene expression level was detected by real-time PCR. There were variations in gene expression levels among different groups of treatment when compared to negative control. These variations showed a statistical increase in different treatments but it did not reach a significant level except in HepG2 cells treated with acute high dose of doxorubicin then transfected with mir-122 mimics (D+M II) as shown in (Fig.2).

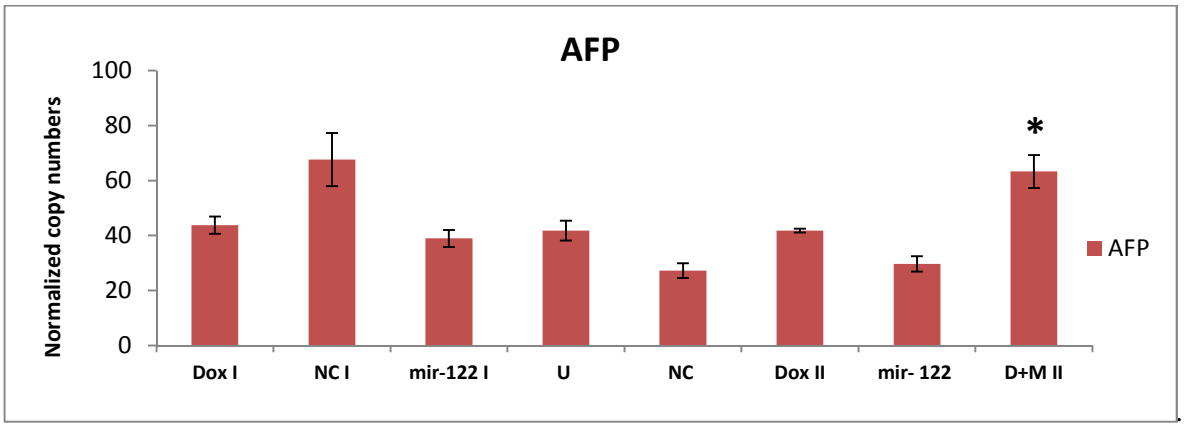

Fig. 2. Statistical significance curve of AFP gene. Dox I; cells treated with chronic doxorubicin dose, NCI; cells treated with chronic doxorubicin dose then transfected with inhibitor negative control, mir-122 I; cells treated with chronic doxorubicin dose then transfected with mir-122 mimics, U; wild HepG2 cells, NC; Wild HepG2 cells transfected with inhibitor negative control, Dox II; HepG2 cells treated with acute high doxorubicin dose, mir-122; 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 means ± SEM. *$P < 0.05$

1.3 AFP protein expression levels by ELISA

AFP gene expression levels were confirmed by evaluating AFP protein levels assayed by *ELISA.* Similar to gene expression, there were differences in the concentration of protein level but without a statistical significant level other than the same group of treatment. Cells treated with acute high doxorubicin dose and transfected with mir-122 mimics (D+M II) showed statistical significant increase, $P< 0.05$, as compared to inhibitor negative control (NC) as represented in (Fig. 3)

DISCUSSION

Hepatocellular carcinoma (HCC) is the most common and one of the leading aggressive of all human cancers. Doxorubicin is the most effective cytotoxic agent for HCC treatment through induction of oxidative stress, and triggering apoptotic pathways [16]. It has been indicated that miRNAs, a class of small noncoding RNAs that regulate gene expression post-transcriptionally, either through directly contribute to HCC by targeting many critical regulatory genes or participate in multiple intracellular signaling pathways that modulate apoptosis, cell cycle checkpoints, and growth-factor-stimulated responses. When defective, these pathways appear to result in malignant transformation and finally HCC development [30].

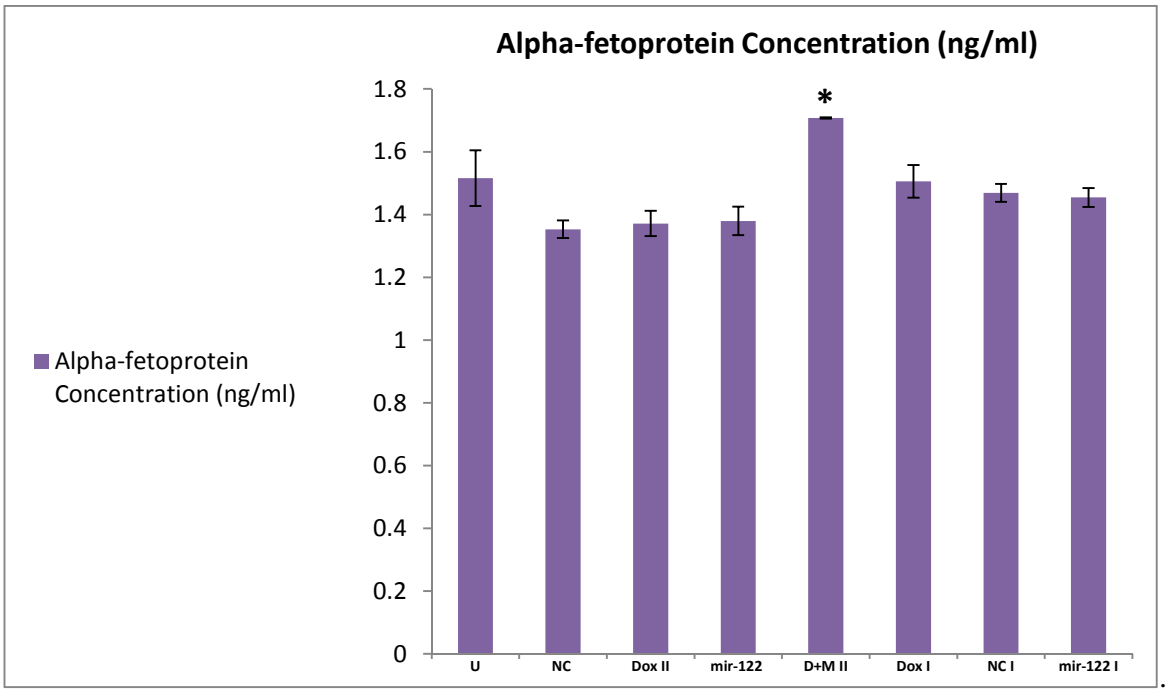

Fig. 3. Alpha-fetoprotein expression levels. The mean value of the triplicate readings for each treatment was calculated. Unknown sample concentration was determined from the standard curve. U; wild HepG2 cells, NC; Wild HepG2 cells transfected with inhibitor negative control, Dox II; HepG2 cells treated with acute high doxorubicin dose, mir-122; HepG2 cells transfected with mir-122 mimics, D+M II; HepG2 cells treated with acute high doxorubicin dose and transfected with mir-122 mimics, Dox I; cells treated with chronic doxorubicin dose, NCI; cells treated with chronic doxorubicin dose then transfected with inhibitor negative control and mir-122 I; cells treated with chronic doxorubicin dose then transfected with mir-122 mimics. Data was presented as means ± SEM. *$P < 0.05$

MiR-122 is a liver-specific miRNA representing about 70% of the liver miRNA population, and it has been characterized for its multiple roles in liver physiology, metabolism and modulation of HCV replication. Notably, its loss or down-regulation has been associated with human HCC development and progression [26].

Alpha-fetoprotein (AFP) has been considered as a useful diagnostic and prognostic tool for chemotherapy treatment [31]. Early reports including studies on choriocarcinomas, revealed that serum or tissue AFP level does not correlate with tumour mass [32]. On the contrary, positive correlations concerning the same parameters have also been demonstrated by patient studies or model systems using mice transfected with PLC/PRF/5 human hepatocellular carcinoma cell line [33].

In the present study, the effect of acute/chronic doxorubicin dose on AFP expression levels in HepG2 cells has no significant changes as compared to control cells. Our data was confirmed with another study that used human well-differentiated hepatocellular carcinoma HuH-7 cell line treated with different concentrations of doxorubicin, in which there was no change in AFP secreted as treated with chemotherapy concentrations of therapeutical use [34].

Our investigation indicated that transfection of HepG2 cells with mir-122 mimics doesn't affect the AFP expression levels. Some reports were in line with our results, verifying no statistically significant correlation between mir-122 levels of expression and AFP in HCC patients [14, 17].

On the other hand, there was a study confirmed that mir-122-CUX1 pathway is responsible for regulating AFP expression in multiple HCC cell lines, where the functional silencing or down-regulation of mir-122 leads to higher

levels of CUX1 protein expression, as well ZBTB20 is expressed due to an increase in the expression of mir-214. Repression of ZBTB 20 leads to an increase in AFP expression [29].

Both mir-122 and mir-214 were found to be decreased in about 70% of HCCs [24, 35-37]. So that further explanations are required to discuss the unusual behavior of mir-122/CUX1/mir-214/ZBTB 20/AFP pathway.

In our study, we noticed a statistical significant increase in AFP expression levels in cells treated with high acute dose of doxorubicin and mir-122 mimics. This result could be explained according some previous studies that indicated the role of mir-122 restoration in increasing doxorubicin sensitivity of HCC-derived cell lines [17, 38]. In addition, as previously mentioned, doxorubicin has a role in oxidative stress through releasing of reactive oxygen species that can lead to peroxidation of lipids, damage to both membrane and DNA damage. These force the cell to trigger apoptotic pathways and finally cell death [11]. Moreover, AFP; purified from human HepG2 cell line media; is bifunctional protein acting in cellular defense against oxidative stress both as a copper transporting and as a superoxide radical scavenger [39].

However, another study stated that AFP knockdown in HepG2 cells aids in inhibition of hepatoma cell growth through promoting apoptosis. Besides, this study added that AFP down-regulation efficiently down regulates intracellular survivin gene expression [40]. Also, an Egyptian study and other old studies demonstrated that AFP exhibited stimulatory effects at low concentrations in both normal liver and HCC; where intact AFP significantly decreased expression of cyclin-dependant kinase inhibitor p27, and increased expression of proliferating cell nuclear antigen (PCNA). Stimulation of cell proliferation is accompanied by inhibition of apoptosis [41-44].

While, other authors studied the proliferation inhibitory effect of AFP in *vitro* and in *vivo* of MCF-7 human breast cancer and prostate cancer cell lines. They concluded that AFP had antitumor activity through inhibition of MCF-7 growth [45-48]. However, in case of metastatic human breast cancer cell line (MDA-MB-231) other researchers didn't observe antitumor activity of human AFP [49-51].

Collectively, the present study revealed that AFP levels were significantly increased in HepG2 cells treated with acute high dose of doxorubicin with restoration of mir-122. This could be attributed to the increased sensitivity to doxorubicin which consequently, activated apoptosis through releasing of reactive oxygen species. While, treatment of our cells with chronic or high acute doses of doxorubicin without mir-122 restoration didn't exhibit any significant changes in AFP expression levels. This could be explained on the basis that, the produced level of reactive oxygen species by doxorubicin alone is not sufficient to increase AFP expression. According to microRNA databases; AFP isn't predicted/validated as direct target gene for mir-122 thus, it is accepted in our study to record no significant changes in AFP levels in cells transfected with mir-122 mimics (mir-122 and mir-122 I). Consequently, it is satisfied to find no significant changes in AFP levels in cells treated with chronic doxorubicin dose then transfected with mir-122 mimics. Accordingly, our results indicate the impact role of mir-122 restoration in facilitating of doxorubicin entry that evokes AFP expression.

In conclusion, this study shows that restoration of mir-122 in doxorubicin treated HepG2 cells increases their expression of AFP, both at mRNA and protein levels. This distinct finding could be attributed to a negative feedback increase in AFP levels to counteract the apoptosis/reactive oxygen species triggered by miR-122 restoration in Dox-treated HepG2 cells.

Further researches are recommended to define the conflicting dual regulatory cascades of AFP in pathways of apoptosis and antioxidant process.

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