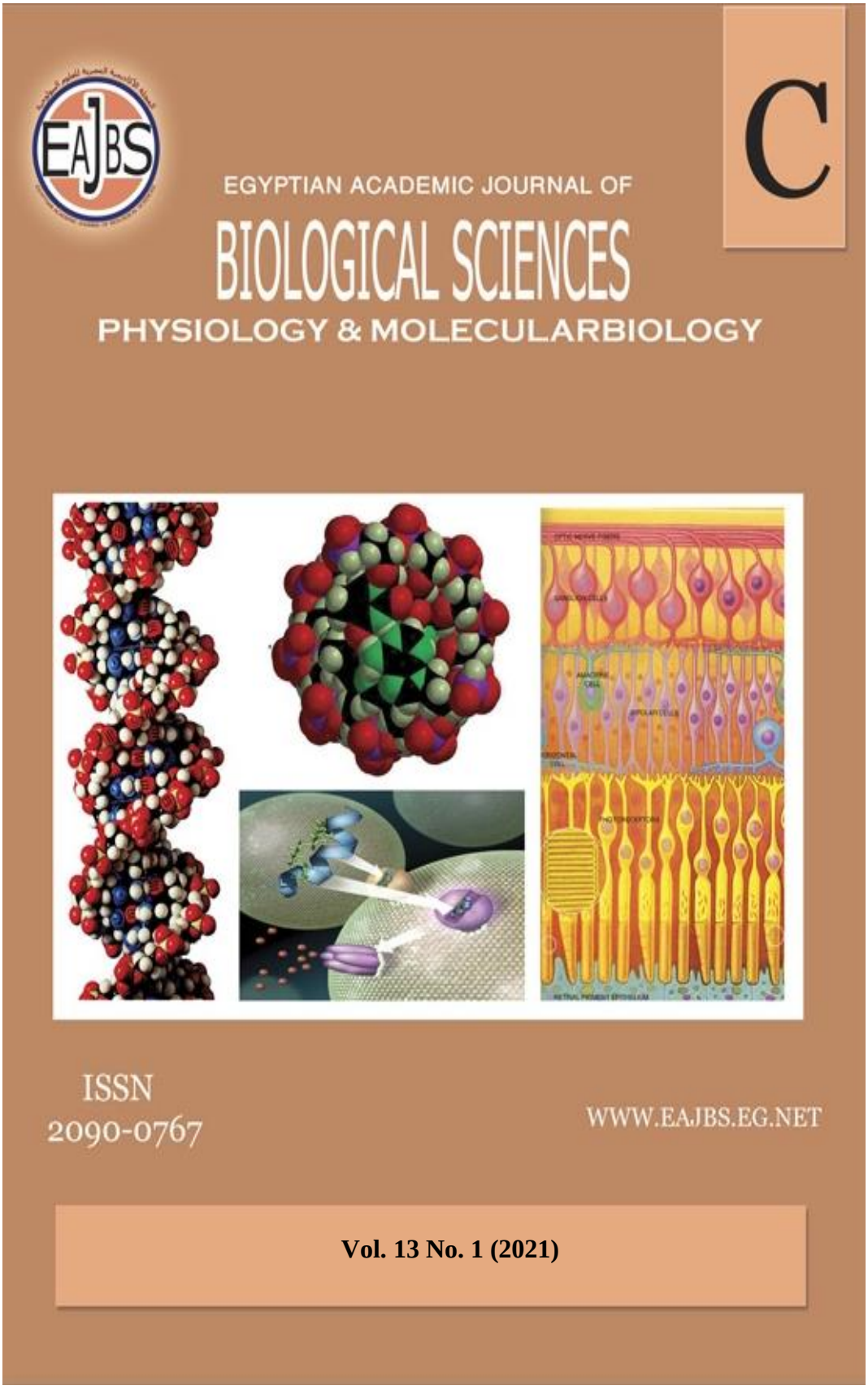

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Molecular Insights into The Biological Basis of Anticancer Efficacy of miRNA Suppression Therapy in HCC Cell Lines

Sania K. Elwia * and Yasmin M. Marei

Department of Medical Biochemistry & Molecular Biology, Benha Faculty of Medicine, Benha University, Qalubyia, Egypt

*E. Mail : sanya.khairy@gmail.com -jessy.marei@gmail.com

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ABSTRACT

***Background*:** MiRNAs are critical in the course and prognosis of the disease and may aid in developing innovative therapies. Due to miRNAs instability and complicated environment, which includes in vivo degradation by nucleases, deregulation of miRNA expression is the hallmark of liver cancer. MiRNAs can function as oncogenes or tumor suppressors; miRNA-222 promotes tumor progression by cancer-related biological processes. MiRNAs are a novel method for cancer gene therapy due to their influence on cell signaling pathways by inhibiting or promoting many related genes. The interaction between genetics and cellular pathways contributes significantly to the onset and progression of HCC

This study aims to illustrate molecular insights into the biological basis of potential anticancer efficacy of anti-microRNA in HCC cell lines

Results: Normalization of dysregulated miRNAs by down-regulation block HCC cell proliferation or increase the sensitivity of liver cancer cells to chemotherapy. Effect of UTMD-mediated with anti-microRNA-222 leads to cell proliferation inhibition and cell apoptosis induction in HepG2 cells line. Inhibition of miR222 expression leads to cell cycle arrest and activation of its target genes.

Conclusion: Ultrasound microbubbles are frequently utilized to investigate gene and miRNA functions. Gene delivery of miR-222 inhibitor by microbubbles/ultrasound is a new therapy and overcome chemotherapy side effects.

INTRODUCTION

Worldwide, hepatocellular carcinoma (HCC) is one of the most frequent and fatal cancers. Unfortunately, due to the limited sensitivity of existing diagnostic approaches, including imaging and detection of alpha-fetoprotein (AFP) levels, HCC is usually diagnosed at a late stage. While recent developments in genomic technology have found several genetic variants in HCC tissues, accessible biomarkers with appropriate sensitivity and specificity for early detection of HCC remain unavailable (Torre LA *et al.*, 2015) (El-Serag *et al.*, 2011).

Hepatocellular carcinoma (HCC) is considered a highly malignant cancer. The incidence is increasing dramatically, with annual new cases estimated at 600,000 worldwide. (Siegel R. *et al.*, 2014) *(Miller K.D et al., 2016).* Only a limited number of cases can be treated with surgery with poor five-year survival. Therefore, miR-222 inhibitors act as chemotherapies to improve HCC patients' survival (Mcglynn K.A. *et al.*, 2015).

In the current study, the aim is to elicit a biological reaction against aberrant cancer cells through new cancer treatments based on changing target gene expression levels. The molecular mechanisms underlying HCC progression are not understood. So, they should be studied to develop effective therapy (Singal A.G., El-Serag H.B. 2015).

MiRNAs are tiny non-coding RNAs that act as post-transcriptional regulators of gene expression (Jeong S.I., *et al.*, 2007) (Nam S.Y. *et al.,* 2014). The miRNAs, based on sequence complementarity level, lead to target mRNAs degradation or mRNA translation suppression, hence inhibiting protein synthesis and achieving gene regulation. The miRNAs, which exhibit tissue and temporal specific expression, are critical negative regulatory RNAs that decrease the expression of other functioning genes. The miRNA dysregulation occurs due to HCC development, so the dysregulated miRNAs are master regulators of the initiation and progression of cancer. MiRNAs directly target protein-coding genes involved in cancer pathogenesis, including cell proliferation, apoptosis, invasion, and metastasis. Depending on their target genes, miRNAs operate as tumor promoters or suppressors (Peng Y. *et al.,* 2016).

MiRNA suppression treatment can reverse miRNA suppression of a target mRNA, resulting in an increase in mRNA expression. Anti-miRNA oligonucleotides (AMOs) bind to the miRNA sense strand, inhibit interactions between miRISC and its target mRNA, prevent mRNA degradation, and therefore permit translation of the mRNA. To increase the efficacy of inhibition, several chemical changes may be made to AMOs, including miRNA inhibitors and antagomirs. Anti-miRNAs are single-stranded RNA molecules that block miRNAs. Anti-miRNAs can precisely attach to and inhibit the action of endogenous miRNA. The miRNA inhibitors were employed in conjunction with the lipofectamine transfection reagent to explore miRNA biological activity via "loss-of-function" (Yong Fu *et al.,* 2019).

MATERIALS AND METHODS

We examined the molecular mechanisms underlying anti-miR-222 by using HepG2 cells. Due to the absence of viral infection, the HepG2 cell line is regarded as a pure cell line of human liver cancer that may be utilised as a model for HCC (Moscato *et al.,* 2015). HepG2 cells are derived from human hepatoblastoma and having a higher degree of malignancy. Its intrinsic activity of drug-metabolizing enzymes is stable and does not decrease with the increasing passages (Yao X.F. *et al.,* 2007). The American Type Culture Collection provided HepG2 cells (ATCC, USA).

1. Stability of HepG2 Cells:

To maintain HepG2 cells stable, they were kept in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). This medium is suitable for the growth and maintenance of HepG2 cells. It was supplemented with 10% fetal bovine serum (FBS) (HyClone; GE Healthcare Life Sciences, USA), 10 ug/ml of insulin (Sigma, USA), and 1% penicillin-streptomycin (10 IU/10 IU per 100 ml).

2. Design of Modified anti-miR Oligonucleotides:

The sequence of anti-miR-222 was synthesized then inserted into Bam HI and Hind III sites of the GV249 vector, which contained enhanced green fluorescent protein (EGFP). The recombinant plasmid was named EGFP-anti-miR222. Anti miR-222 used are as follows: 5′-GAAACCCAGCAGACAATGTAGCT-3′ and 5′-GAGACCCAGTAGCCAGATGTAGCT-3′, respectively.

Before starting treatments, cells were divided into the following groups:

i) Control group.
ii) Microbubbles + plasmid; (MB + P) group.

iii) Ultrasound + miRNA plasmid; (US + P) group.
iv) Ultrasound + microbubbles + plasmid; (US + MB + P).
v) Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) + miRNA plasmid (L + P); miRNA plasmid + 1 µl Lipofectamine® 2000 group
vi) Ultrasound + Lipofectamine2000 + plasmid; (US + L + P) group.
vii) Ultrasound + microbubbles + Lipofectamine 2000 + miRNA plasmid; (US + MB + L + P) group.

3. Transfection:

US and MB therapy is effective in enhancing the delivery of molecules for the treatment of hepatocellular carcinoma. Following the manufacturer's instructions, microbubbles were used in standard condition. Acoustic pressures were applied to the microbubbles (MI > 0.3-0.6), which can result in mechanical stress and membrane collapse (Koczera Petal., 2017*).*

Destruction of microbubbles results in the production of a local microjet surrounding the bubbles, increasing the permeability of neighboring cell membranes. (Shamout F.E. *et al.*,2015). The formation of pores in cell membranes enabled the entry of molecules into the cell, such as plasmid DNA and oligonucleotides (Bouakaz A. *et al.*, 2016) (Hernot S. *et al.*, 2008).

The microbubbles and plasmid (1g/5x106microbubble) were combined in OptiMEM (Gibco, Life technologies Cod. 31985-062) and incubated for 20 minutes at room temperature with moderate shaking. Transfections were carried out using an ultrasonic instrument (Sonitron 2000, Artison corporation®) and compared according to standard protocols using lipofectamine 2000 (Cod. 11668027) (Chunying Shi *et al.*, 2017).

Transfected cells were plated at a low density (2,500 cells per 10 cm plate) and allowed to grow for seven to ten days before being fixed and stained with crystal. To identify milder effects, MTT [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] was used to assess cell growth 96 hours after transfection.

4. MTT Assay (in vitro toxicology assay):

In vitro, the toxicity of unknown substances was assessed by counting live cells after staining with a vital dye. Other approaches include radioisotope incorporation as a proxy for DNA synthesis, automated counter counting, and approaches based on cellular activity and dyes. The MTT method is used to determine the activity of live cells by measuring the activity of mitochondrial dehydrogenases. Absorbance values larger than the control strongly imply cell growth, whereas values less than the control strongly imply cell death or suppression of proliferation.

$$\%\ \text{viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

The results of the MTT assay confirmed that the ultrasound + microbubbles + Lipofectamine 2000 + miRNA plasmid (US + MB + L + P) group gave the optimal result (maximum decrease in cell viability), which means that this group achieved the best transfection efficiency. Therefore, the following experiments were performed in 96-well plates for only two groups (NC and US + MB + L + P groups).

Using an ultrasound microbubble, the cells were transfected with a recombinant plasmid (EGFP-anti-miR222) and a vector plasmid (GV249). The cells were then harvested 96 hours after transfection. Untransfected cells were used as a blank control.

5.* RNA Isolation and Assessment of Target Gene Expression Using a Quantitative Real-Time *PCR:

A. RNA Extraction:

The miRNA was extracted from the collected cells using RNeasy RNA extraction kit (Qiagen, USA) according to the manufacturer's protocol. The quantity of the eluted RNA was determined using nanodrop 2000 (Thermo Fisher, USA).

B. cDNA Synthesis and PCR Amplification:

The One-Step RT-PCR Kit with SYBR® Green kit (BioRad, USA) was used according to the recommended protocols. A SYBR green-based RT-PCR assay was performed using a sequence detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

The miR-222 gene expression was done using U6 as a housekeeping gene, and p27, P57, PTEN, and BMF gene expressions were performed using GAPDH as a housekeeping gene. The fold change was estimated for each target gene using the comparative Ct (2−ΔΔCT) approach (Table 1).

Table 1: primer sequence.

Gene	Sequence
GAPDH	Forward 5′-GAA GGT GAA GGT CGG AGTC-3′ Reverse 5′-AAG ATG GTG ATG GGA TTTC-3′.
miR-222 miR-222 inhibitor	5′-ACC CAG UAG CCA GAU GUA GCU-3inhibitor control (5′-CAG UAC UUU UGU GUA GUA CAA-3′)
BMF	3′UTR-F (5′-ATACTAGTTGGTGGGGACTT-TTGAGTCT-3′) and BMF-3′ UTR-R (5′-ATAAGCTTGCCCC-TTTCTTCTTCCTCTC-3′)
p27	CAGGTCTCCAAGACGACATAGA and CGCCTTTTCGATTCATGTACTGC
p57	AGGTAGCGAGGTGGATCTGTC and GGCCTCTGATTCCCGAGGA
PTEN	_fwd CTCCTCTACTCCATTCTTCCC, _rev ACTCCCACCAATGAACAAAC,

Statistical Analysis:

The collected data were computed, tabulated & statistically analyzed using SPSS program version (16) software in windows (SPSS Inc. Chicago).

RESULTS AND DISCUSSION

In the current study, the effect of UTMD-mediated with anti-microRNA was studied 48 h following transfection. The effect of anti-miR-222 on HepG2 cells was determined by the following experiments (Table 2 & Fig. 1).

Table 2: MTT assay.

	Cell % viability
	HepG2
Negative control	100
Positive control	97
(US + MB + L + P)	94
(US + MB + L + P)	45

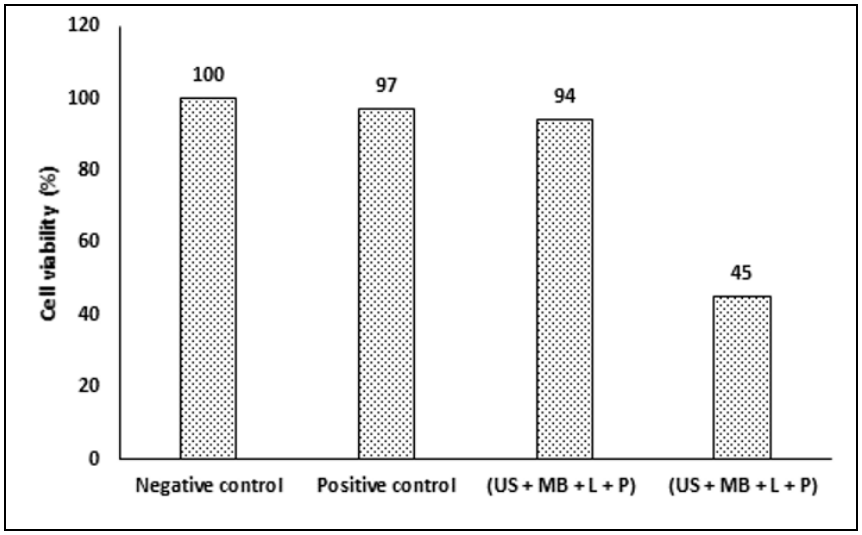

Fig. 1: MTT assay**.**

Ultrasound + microbubbles + Lipofectamine 2000 + miRNA plasmid (US + MB + L + P) group gave the best result (maximum decrease in cell viability), which means that (US + MB + L + P) group achieved the best transfection efficiency. Therefore, the following experiments were performed in 96-well plates for only two groups (NC and US + MB + L + P groups).

As shown in Figure (2) and Table (3), after 48 h of transfection, miR-222 was downregulated with miR-222 inhibitor in the (US + MB + L + P) group, with upregulation in of CDKN1B/p27, P57, PTEN, and BMF gene expression as compared to control.

Table 3: Effect of UTMD-mediated with miR-222 on P27, P57, BMF, PTEN in HCC cell lines by RT-PCR.

		Fold Change				
		P27	**P57**	**BMF**	**PTEN**	**miR-222**
US + MB + L + P	6.07725	5.45913	5.36702	6.33902	6.6431	0.4678538
NC	1	1	1	1	1	

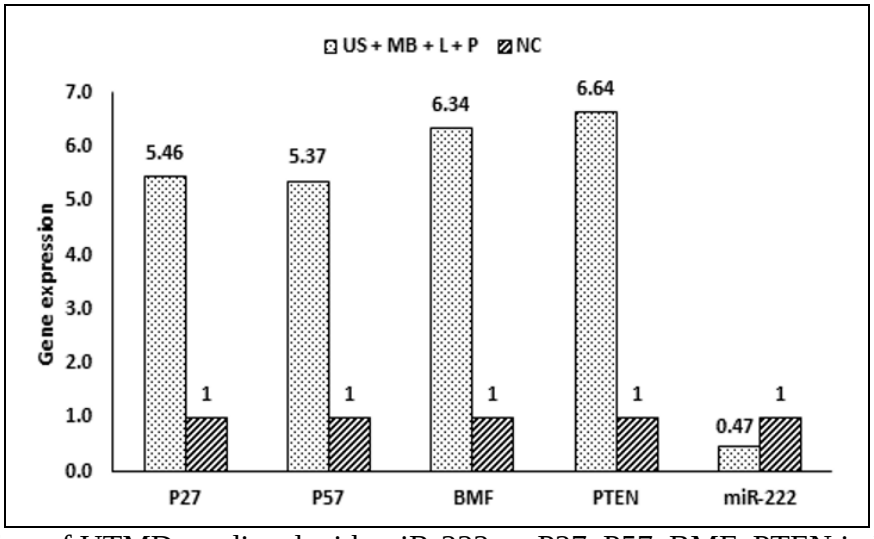

Fig. 2: Effect of UTMD-mediated with miR-222 on P27, P57, BMF, PTEN in HCC cell lines by RT-PCR.

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and is associated with a high death rate due to late detection and limited therapeutic choices. (Yu Jin *et al.,* 2019). Apoptosis is a vital process in carcinogenesis. The development and progression of liver tumors are related to the deficiency of apoptotic activator (P53 has a crucial role in this process) (Brito A.F. *et al.*, 2015). The most molecular changes in HCC are mutations in the TP53 gene and the P53 protein (Gomes A.R. *et al.* 2015) (Villanueva A.. *et al.,* 2011) (Delire B. *et al.,* 2015).

Under normal conditions, there is low concertation of P53 in the cells. However, transcriptional activation of this protein may occur in response to DNA damages, hypoxia, or deregulated cell cycle progression. P53 is required for the maintenance of the cell cycle, the balance of cell proliferation and apoptosis, DNA repair, the response to cellular stress, and angiogenesis suppression (Fabregat I., 2009).

After p53, PTEN is the most commonly mutated tumor suppressor gene in malignancies. Numerous disorders have been linked to genetic and epigenetic abnormalities in the PTEN gene and its regulatory areas (Ayesha *et al.,* 2017). PTEN acts as a tumor suppressor gene in HCC. PTEN dysregulation contributes to HCC progression. PTEN plays a vital role in inducing apoptosis interactions with the extracellular matrix, leading to reduced cell migration.

In the current research, we assessed the anti-miR-222 effect on HepG2 cells using ultrasound microbubbles with lipofectamine 2000 as a novel treatment for hepatocellular carcinoma (HCC). HepG2 cells treated with anti-miR-222 can maintain tumor suppressor genes biological function, keeping the balance between cell proliferation and apoptosis

In this study, the application of UTMD-mediated gene therapy is a common non-viral vector. UTMD, in combination with Lipofectamine® 2000, can transfect miRNA 222 inhibitor.

Non-viral delivery agents enhance cellular uptake through the complexation and reverse charge of nucleic acid or through a physical method by allowing the nucleic acid to enter the cell directly. The higher molecular weight and negative charge of NA are considered a strong message for cellular uptake (Pan Wu *et al.*,2018) (Renshuai *et al.*,2019).

The non-viral vectors have several advantages compared to viral vectors, such as lower immunogenicity, no toxicity, better cell specificity, and better modification. Moreover, they have a role in the repair and regeneration of damaged tissues (Pan Wu *et al.,* 2018) (Wang W. *et al.,* 2016) (Cordeiro R.A. *et al.,* 2017).

In this study, the cell growth was dramatically inhibited after transfection of miR-222 in HepG2 HCC cell lines. As shown in Figure (1), Table (2) This finding is consistent with (Li X. *et al.*,2014), who concluded that miR-222 inhibitor effectively inhibits tumor development and modulates cell proliferation. The miR-222 oncogenic effects are augmented by cellular proliferation (Pineau P. *et al.*,2010) *and* CDKN1B/p27 and CDKN1C/p57 direct targeting *(Gramantieri L et al., 2009).*

The (miR-221/222) are two homologous miRNAs grouped on the chromosome X short arm (Gui B. *et al.*,2017) (Bandopadhyay M. *et al.,* 2014) (Felicetti F. *et al.* 2008). They exert an oncogenic effect by targeting PTEN in hepatocellular carcinoma. In ovarian and hepatocellular carcinoma, up-regulation of miR–222 promotes proliferation by down-regulating its target P27Kip 1 (Beatriz *et al.,* 2013) (Kawaguchi T. *et al.* 2013).

In line with the current study results, the implication of anti-miR-222 cause arrest cell growth of cancer cell according to (He XX *et al.,* 2014), (Yuan Q. *et al.,* 2013), (Pineau P. *et al.,* 2010), and (Xinmin Guo *et al.,* 2017).

In the current study, the increase in HCC cell lines apoptosis with miR-222 inhibitor could be explained by inducing

tumor necrosis factor-related apoptosis by miR-222 that promotes cellular migration through modulating phosphatase and tensin homolog (PTEN) expression. Pro-apoptotic protein B-cell lymphoma 2-modifying factor (BMF) has the same effect (Gramantieri L. *et al.,* 2009).

The level of miR-222 expression declined with the miR-222 inhibitor; the biggest ΔΔCq was 1.53 (65% knock-down). The target genes of cell cycle regulation and marker for apoptosis were determined using RT-qPCR. In Figure (2), Table (3). primer sequences in Table (1)

When miR-222 is transfected into HCC, the cell lines show inhibition of miR222 expression, which leads to cell cycle arrest and activation of CDKN1B/p27- that regress tumor progression, and induction of apoptosis in HepG2 cells, which may be due to oxidative stress. Therefore, the miR-222 inhibitor is considered a new chemotherapeutic for the treatment of HCC.

The target gene of miR-221 and miR-222 is CDKN1B/p27. It affects HCC prognosis. In the current study, we concluded that the cyclin-dependent kinase inhibitor (CDKI) CDKN1C/p57 is also a direct target of miR-221. The miR-222, when transfected into the HCC cell lines, leads to CDKN1B/p27 and CDKN1C/p57 upregulation.

The inverse association between miR-222 and PTEN as an apoptotic marker was revealed in this study. This finding could be explained by the fact that miR-222 can block apoptosis, so miR-222 acts as a post-transcriptional regulator of both necrosis factor-related apoptosis. (Ramjaun A.R. *et al.,* 2007) (Breuhahn K. *et al.,* 2006).

By targeting bone marrow failure syndromes, miR-222 overexpression suppresses apoptosis in HCC cell lines. The silencing of miR-222 can result in an increase in apoptotic cell death (Gramantieri L. *et al.,* 2009). The miR-222 regulates apoptosis directly by targeting CDKN1C/p57, p27, and PTEN.

The interaction of microRNAs with the target gene in HCC reflects the biological behaviors of cancer (Gong J. *et al.,* 2015). The critical consequences of miR-222 overexpression on the cell proliferation and the cell cycle in hepatocellular carcinoma reported that CDKN1C/p57 is the direct target gene of miR-222 in the liver, and this revealed miR-222 oncogenic role in hepatocellular carcinoma pathogenesis.

CONCLUSION

Anti-miR-222 triggers the changes in expressions of P27kip1, P57kip2, and PTEN through the combination of ultrasound with gas-filled Microbubbles. This limits cell-cycle progression and keeps the balance between cell proliferation and apoptosis.

List of Abbreviations:

HCC: Hepatocellular carcinoma
HBV: Hepatitis B virus or hepatitis C virus
HCV: Hepatitis C virus
AFP: Alpha-fetoprotein
DMEM: Dulbecco's modified Eagle's medium
FBS: Fetal bovine serum
EGFP: Enhanced green fluorescent protein
PTEN: phosphatase and tennis homolog
lncRNA: interfering long non-coding RNA
PDCD: programmed cell death 4
ER: endoplasmic reticulum
EGFP: Epidermal growth factor
CDKN1B/p27: Cyclin-dependent kinase
CDKN1B/p57: Cyclin-dependent kinase inhibitors
UTMD: Ultrasound-targeted microbubble destruction
BMF: B-cell lymphoma-2 modifying factor

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