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Research Article

Physical, Chemical, Strategies with Antimicrorna and Recombinant Virus to Deliver Genes into Hepatic Tissues. Assement of miRNA Reprogramming and its Gene Networks

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ABSTRACT

BACKGROUND

Hepatocellular carcinoma (HCC) is a deadly malignancy with limited treatment options (1) HCC is a heterogeneous disease ,the occurrence of gene aberrations, cellular context, and environmental influences are good promotor for tumor initiation, progression, and metastasis(2) The molecular mechanisms underlying progression of HCC is not clear. So it is important to investigate the target molecules and molecular mechanisms that sharing in development and progression of HCC for development of effective diagnostic and therapeutic strategies (2).The dysregulation of miRNAs is hall mark of cancer and also play a maior role in the pathogenesis of multiple cancer including HCC,the expression of. miRNAs exert its effect through regulation of oncogenes and tumor suppressor genes that involved in pathway of cell signaling. Therefore MiR-221/222 have been considered either oncogenes or tumor suppressors gene , so it can regulate the cell cycle, apoptosis and metastasis (3).miR-222 is most significantly down-regulated miRNA. the miR-221/222 family having high degree of homology.(4) 5(6).

The oncomiR -222 play important role in development of HCC, the overexpression of miR-222 activate the AKT pathway that promote cellular invasiveness and motility of HCC cells ,miR-222 is considered an oncogenic gene in most epithelial tumors .exert its effect by promoting oncogenic process by targeting PTEN in hepatocellular carcinoma ,Up-regulation of miR–222 induces an proliferation by down-regulating its target P 27Kip1 in ovarian and hepatocellular cancer (7).The tumor-promoting or antitumor activity of miRNAs determined by translation regulatory rate which about >60% of the protein-coding genes . (8)

The modulation of miRNAs occur at different levels, through regulation of miRNA biogenesis, metabolism and function. due to similarity of promoter regions, miRNAs are regulated by transcription.The the dysregulation of miRNA expression in different types of cancer can be explained by dynamic regulatory effect of miRNAs that enables cell adaptation to face changes in the cellular environment,. the binding of Intracellular miRNAs to the mRNAs of target genes with complementary sequences that lead to inhibition of mRNA translation thus reflect role of miRNAs as post-transcriptional regulators of target genes (9)miRNA-therapy based on one of the following : the first is inhibition of proliferation or induction of apoptosis in tumor cells by importing exogenous miRNAs, that act as tumor suppressor miRNAs and also down-regulated in tumor tissues. The second is inhibition of oncogenic miRNAs function, that over-expressed in tumors, derepression of its direct targets genes (10).

Antisense oligonucleotides strategy, including anti-miRNA oligonucleotides, miRNA antagomirs Antagomir is a small synthetic RNA to be complementary to the specific target miRNA moreover it more resistant to degradation (11), the chemically modified antisense oligonucleotides, called antimiRs, that act by sequestion of the mature miRNA in competition with cellular target mRNAs that lead to functional inhibition of the miRNA and also derepression of its direct targets genes and proper optimization of the oligonucleotides for increasing binding affinity, improved nuclease resistance (12).The Small RNAs can be used chemotherapy because it has ability to reprogram and sensitize tumor cells., , the combination of ultrasound with gas-filled microbubbles open new away for therapy ,also provides a safe and noninvasive way for improvement of permeability of tumor vasculature thus it can overcome short half-life in circulation so it promote and control

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release into tumor cells for increasing efficient delivering Small RNAs (13).

Microbubbles are small, gas-filled bubbles, with a diameter range from 0.5 μm and 10 μm, that used as contrast agents in medical imaging and carriers for targeted gene and drug delivery (14) (15) .This study aimed to use of Microbubble as effective method for targeted drug delivery Microbubbles are used for transport of drug or gene to a specific area , and also is used for breakdown of microbubbles ultrasound , that leads to the delivery of the bioactive matter .The Exposure to Ultrasound Microbubble will increase the efficacy of localized drug delivery.and effective roleof miR-222 inhbitor in inhibiton of tumor development and modulating cell proliferation.

METHODS

HepG2 cell line represents a pure cell line of human liver carcinoma that is usually used as HCC model because there is no viral infection (16) HepG2 cells are derived from human hepatoblastoma and having higher degree of malignancy. And their intrinsic activity of drug-metabolizing enzymes is stable and does not decrease with the increasing passages (17) HepG2 cells were obtained from American Type Culture Collection (ATCC, USA). They were subjected as the following:

Maintenance of HepG2 Cells

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), which is a cell culture medium suitable for growth and maintenance of HepG2 cells, 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).Sequence of anti-miR-222 synthesized and inserted into Bam HI and Hind III sites of the GV249 vector, a vector containing enhanced green fluorescent protein (EGFP). The recombinant plasmid was named EGFP-anti-miR222

Chemically modified antisense oligonucleotides (antagomiR) have been used to inhibit miR expression in vitro. antagomiR-222 used are as follows: 5′-GAAACCCAGCAGACAATGTAGCT-3′ and 5′-GAGACCCAGTAGCCAGATGTAGCT-3′, respectively.Before treatments, cells were divided to study effect of anti-miR222 that mediated by ultrasound microbubbles. as follows

Control group, control plasmid; 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) group, miRNA plasmid + 1 μl Lipofectamine® 2000; vi) Ultrasound + Lipofectamine2000 + plasmid (US + L + P) group, vii) Ultrasound + microbubbles + Lipofectamine 2000 + miRNA plasmid (US + MB + L + P) group,

Transfection

Microbubbles were used at standard condition, according to manufacturer's instructions. The microbubble were mixed with plasmid (1μg/5x106microbubble) in OptiMEM (Gibco, Life technologies Cod. 31985-062) and incubated at room temperature with gentle shaking for 20 minutes. Transfections were achieved by an ultrasound device (Sonitron 2000, Artison corporation®) and compared with standard protocols using lipofectamine 2000 (Cod. 11668027 (18)Thermofisher) ,HepG2 cells were used as a liver cancer model. Microbubbles (were chosen as gene deliver system

The solution of sulfur hexafluoride microbubbles (diameter 1-6 μm)are encapsulated by a phospholipid membrane, that prevents its fast dissolution in blood,the therapeutic applications of microbubbles as gene delivery with combination of microbubbles named sonoporation This process is obtained by exposure of microbubbles to acoustic pressures (MI >0.3-0.6 to produce a mechanic stress driving to the membrane collapse R. K. Jain and T. Stylianopoulos,2010.The destruction of Microbubbles induces a microstream/local microjet surround the bubbles, that leads to increase in permeability among adjacent cell (19)membranes. The pore formation in cell membranes enhance entry into the cell of molecules

Cell transfection and grouping

Transfected cells were plated at low density (2,500 cells per 10-cm plate), grown for 7–10 days, fixed, and stained by crystal. To detect milder effects, cell growth was monitored 96 h after transfection by the MTT [(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium]

MTT assay (in vitro toxicology assay)

The absorbance reading of the blank were subtracted from all samples. Absorbance readings from test samples were then divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation (see formula below). Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation.

The results of MTT assay were found that Ultrasound + microbubbles + Lipofectamine 2000 + miRNA plasmid (US + MB + L + P) group gave the optimal 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).

The Cells were transfected with the recombinant plasmid (EGFP-anti-miR222 and vector plasmid (GV249) through ultrasound microbubble ,Cells were then collected 96 h post-transfection. Cells that were not transfected were set as a blank control.

RNA isolation and determination of target gene expression using quantitative real-time polymerase chain reaction

RNA extraction

Total RNA including miRNA were extracted from the collected cells using RNeasy RNA extraction kit (Qiagen, USA), according to the manufacturer's protocol. The purity & quantity of the iluted RNA were determined using nanodrop 2000 (thermo fisher, USA).

cDNA synthesis and PCR amplification

IScriptTM One-Step RT-PCR Kit with SYBR® Green kit (BioRad, USA) was used for reverse transcription real-time quantitative PCR of the RNA template Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using an RT-PCR kit according to the protocols recommended by the manufacturer. An SYBR green-based RT-PCR assay was performed using the sequence detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). according to the manufacturer's protocol. All primers used were designed . the primers for miR-222 using the U6 as housekeeping gene and p27,P57,PTEN and BMF genes expressions using GAPDH as housekeeping gene

The primers for GAPDH are Forward 5′-GAA GGT GAA GGT CGG AGTC-3′ and 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′) and U6 are 5′-AGC TAC ATC TGG CTA CTG GGT-3′ and 5′-CAA GGA TGA CAC GCA AAT TCG-3′, respectivelyBMF-3′UTR-F (5′-ATACTAGTTGGTGGGGACTT-TTGAGTCT-3′) and BMF-3′ UTR-R (5′-ATAAGCTTGCCCC-TTTCTTCTTCCTCTC-3′) Primers sequences (forward and reverse) were designed as follows: p27, CAGGTCTCCAAGACGACATAGA and CGCCTTTTCGATTCATGTACTGC; p57, AGGTAGCGAGGTGGATCTGTC and GGCCTCTGATTCCCGAGGA The fold change for each target gene was calculated using the comparative Ct (2−ΔΔCT) method.

Caspase-3 activity determination by ELISA

Three wells for each group (NC and USF6 groups) were used. Then the 3 wells of each group were mixed and tested.Caspase-3 activity was measured using Caspase-3 (active) ELISA kit (Invitrogen) which is important in regulation of apoptosis.

Statistical Analysis

The collected data were computed, tabulated & statistically analyzed using SPSS program version (16) software in windows (SPSS Inc. Chicago).The quantitative data of the MTT assay were expressed as median & IQR (Interquartile range). Comparison the quantitative data of the MTT assay were performed using KW (Kruskal Wallis) test. P value ≤ 0.05 is significant.

RESULTS

In the present work, HepG2 cells were transfected with recombinant plasmid containing anti-miR-222. Following 48h of transfection, the effect of anti-miR-221 on HepG2 cells was determined by the following experiments:

Table 1: MTT assay Median and P values of % cell viability in studied groups using MTT assay.

Groups	Group 1	Group 2	Group 3	Group 4	KW	p value
% cell Viability	(NC)	(PC)	(US)	(USF6)		Significant relations
Plate (1)	100	97.88476	93.28957	45.44128	10.53	P= 0.015*
Plate (2)	100	98.78419	96.35258	43.61702		a: G2, G3, G4 versus G1.
Plate (3)	100	96.66908	94.2071	43.73642		b: G3, G4 versus G2
Median	100	97.88 a	94.21 ab	43.74 abc		c: G4 versus G3
IQR	100-100	96.67-98.87	93.29-96.35	43.62-45.44		

Figure1: Median & IQR of % viability in the NC, PC, US & USF6 groups.

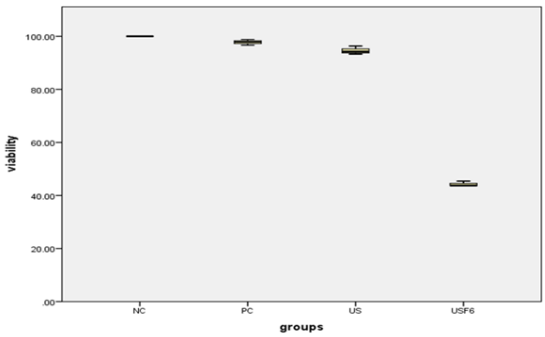


Table (1) and figure (1) show that the % cell viability was significantly decreased in groups 2,3,4 when compared to the NC. Also, the % cell viability was significantly decreased in each of the US & USF6 groups compared to the PC. Moreover, USF6 group showed significant decrease of % cell viability compared to the US group.

The highest significant decrease in cell viability was in the US + MB + L + P group (with % viability 43.74%). This was followed by (US + L + P) group (with % viability 94.21%), then PC group (with % viability(97.88%)

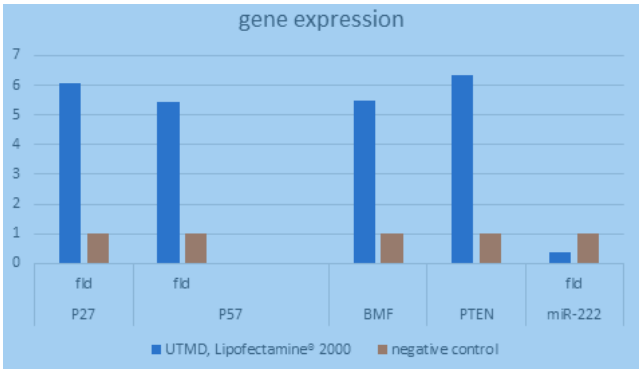
Caspase-3 activity

Table2: Results of caspase-3 enzyme activity assay.

Group tested	Active Caspase-3 Pg/ml
Negative control (NC)	46.8

US + MB + L + P	424.6

Table (2) shows that the activity of caspase-3 enzyme was increased in the US + MB + L + P group when compared to negative control.



DISSUSSION

Hepatocellular carcinoma (HCC) is the fifth most common cancer with high mortality, due to late diagnosis and limited treatment options.(20).

HCC occurrence as multistage of deregulation of the genes that contribute to the cell-cycle control, cell migration and apoptosis. MicroRNAs (miRNAs) are involved in regulation of these events (21)

In this study we aimed to investigate the transfection efficiency of anti-miR-222 on HepG2 cells by using ultrasound microbubbles with lipofectamie 2000 and assessment effect of anti-miR and its gene network on HepG2 cells, were performed The expression of miRNA 222 and CDKN P27,P57,PTEN,BMF were detected by using reverse transcription-polymerase chain reaction. microRNAs reported as therapy for HCC (22)

The delivering of miRNA antagonists to specific tumor sites rather than redundant site is important. for reduction its undesired off-target effects. Oncomirs or tumor suppressor miRNAs exert its effect on organ systems against HCC (23). microRNAs (miRNAs) are short non-coding RNAs ,the interaction of base pairing with their target messenger RNAs thus it regulate gene expression. Dysreguled miRNAs are involved in the pathological initiation and progression of many human diseases. miR-221 and miR-222 (miR-221/222) are two homologous miRNAs, and they are overexpressed in several types of human cancer, The Silencing of miR-221/222 could be anovel a promising therapeutic approach Biomarkers for Diseases (24). miRNA gene delivery systems are used should be effective and non-toxic to humans. since miRNAs have important role in the pathogenesis of HCC, so the generation of regulatory networks that consist of genes and miRNAs in liver tumors should be considered; in this way the biological behaviors of cancer could be recognized (25)ultrasound and microbubbles therapy is important and effective to enhance the delivery molecules to be a therapy for hepatocellular carcinoma (26)ultrasound and microbubble-mediated drug delivery technology can improve targeted drug delivery by reducing drug dose and adverse effects(27) This is carried out through sonoporation that lead to formation of openings in the blood vessels induced by oscillations triggered by ultrasound and the destruction of microbubbles (26)

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

Non-viral gene delivery systems has used for localized delivery,The high molecular weight and negative charge of NA represents a major message to efficient cellular uptake. In many cell types, the size requirement for particle uptake is in the rage up to 200 nm, significantly smaller than the hydro dynamic diameter of DNA of a few thousand base pairs, anchorment of the cell membrane by negatively charged moieties' that lead to electrostatic repulsion of negatively charged of nucleic acid(28).

Non-viral delivery agents are used for improvement of cellular uptake efficiency either through complextion and charge reversal of nucleic acid or through physical method by directly allowing nucleic acid to enter the cell (29).

The cellular uptake of macromolecules and drug carriers is very inefficient without external assistance. Therefore, it is desirable to develop potent delivery systems for achieving effective intracellular delivery of chemic entities. Apart from of the types of delivery strategies, the composition of the cell membrane is critical for delivery efficiency due to the fact that cellular uptake is affected by the interaction between the chemical entity and the plasma membrane(29)

The gene delivery systems include the following ,first plasmid-based gene expression system to control function of a gene within the targeting cell, second a gene for encoding therapeutic protein, third gene delivery system for controlling the delivery of the gene expression plasmid to certain location in the body . The successful gene delivery system requires the foreign genetic molecule to remain stable within the host cells (30 (31).

The gold standard for effcient transfection is non viral vectors that commonly used with Lipofectamine 2000 These non viral vectors have several advantages over viral vectors, such as lower immunogenicity and toxicity, better cell specifcity, better modifiability and enhanced productivity. Moreover They are a better alternative to deliver genes and also responsible for the repair and regeneration of damaged tissues 28 32,33.

Non-viral systems are classified according to preparation into physical or chemical types. The most physical methods are microinjection, electroporation, ultrasound, gene gun, and hydrodynamic applications. physical methods enhance delivery of the gene by application of physical force for increasement the cell membrane permeability. On the other hand chemical methods utilize natural or synthetic carriers to deliver genes into cells through combination with the negatively charged genes. 934)chemical methods that use carrier molecules as cationic lipids, polymers, and nanoparticles for Chemical or biochemical vectors, also known as nonviral vectors are primarily composed of two groups of vectors, namely organic and inorganic vectors. The most used former consists of lipid-based vectors, (28)In this study ,After transfection of miR-222 in HepG2 HCC cell lines, the cell growth was dramatically inhibited .The oncogenic effects

of miR-222 are enhanced by cellular proliferation (35) (miR-222 promotes proliferation through direct targeting of CDKN1B/p27 and CDKN1C/p57 . cells transfected with antagomirs enhance cell growth inhibition

The deregulation of multiple signaling pathways leads to HCC. Initial steps involve the disruption of a set of interdependent pathways controlling cell growth and apoptosis(36)

The vital role of miR-222 inhbitor is inhibiton of tumor development and modulating cell proliferation (37). miR-221/222 can enhance cell growth by targeting the cyclin-dependent kinase inhibitor p27 (38) miR-222 silencing can significant suppress growth of liver cancer cells 39(40). (35.) (41 in agreement with this results miRNA reprogramming and its gene networks in cancer are cosiserded effective target therapeutic strategies with a strong hope for success to fight cancer. (42)

In this study ,apoptosis of HCC cell lines was significantly increase with miR-222 inhibitor miR-222 induce tumor necrosis factor-related apoptosis-inducing ligand resistance and enhance cellular migration by modulating the expression of phosphatase and tensin homolog (PTEN). Additional molecules, including the pro-apoptotic protein B-cell lymphoma 2-modifying factor (BMF) (38).

This study revealed the inverse correlation between miR-222 and activated caspase-3, as a marker of apoptosis. This explainsed by miR-222 can inhibit apoptosis so miR-222 act as posttranscriptional regulator of both necrosis factor related apoptosis- Gramantieri et al. miR-222 overexpression inhibit apoptosis of HCC- cell lines by targeting bone marrow failure syndromes, and increased apoptotic cell death can be caused by silencing of miR-222 (38). The hypothesis that increased expression of miR-222 downregulate BMF posttranscriptionally. (43),(44) .miR 222 directly regulate apoptosis by targeting CDKN1C/p57.p27; PTEN moreover, inverse relationship, , between CDKN1C/p57.p27 PTEN and miR-221/222 expression in hepatocellular carcinoma 45,46.47.48 in agreement with this results

The positive and negative functions of p27 In regulation of cell proliferation, cell motility and apotosis and enhancement of cellular migration through modulating the expression of phosphatase and tensin homolog (PTEN) , the pro-apoptotic protein B-cell lymphoma 2-modifying factor (BMF) and the DNA damage-inducible transcript 4 (49).

The miR-222 expression level decreased with miR-222 inhibitor, the biggest ΔΔCq was 1.53 (65% knock-down) The target genes of miR-222 involved cell cycle regulation and gene as marker for apoptosis were determined using RT-qPCR. Following transfection. Increase in miR-222 expression and repression of its target genes (such as proapoptotic BMF and cyclin-dependent kinase inhibitor p27/57) have been revealed negatively association of miR-222 with cyclin dependent kinase inhibitors CDKN1B/p27 andi CDKN1B/p27 ,BMF and PTEN n HCC cell line . as shown in Figure(2).Table (3) miRNA over-expression or under expression revealed the correlations between miRNAs and cancer development (50).(51) miRNAs play important role in the occurrence and development of human cancer, The generation of genes and miRNAs in liver tumors that represent regulatory networks should be recognized

The interaction of microRNAs with target gene for HCC. that revealed the biological behaviors of cancer (52).

The biological behaviors of cancer, make miRNAs is a new strategies for cancer gene therapy that explained by role of miRNA expression either inhibition or promotion of many related genes by reduction or increase of mature miRNAs, through its effect on cell-signaling pathways that important in progression and development of cancer, such as cell proliferation, differentiation, mobility and apoptosis ((53)

The Overexpression of miR-221/222 has been reported in a number of malignancies indicating that miR-221/222 may be potential therapeutic targets for epithelial cancer [46].l revealed that cell cycle regulator p27Kip1, as a target of miR-221/miR-222 p27Kip1 and miR-221/222 expression levels inversely correlated The important consequences of miR 222 overexpression on the proliferation rate and the cell cycle , in hepatocellular carcinoma [48., reported that CDKN1C/p57, is also a direct target of miR-222 in the liver, this revealed oncogenic function of miR-222 in hepatocarcinogenesis (54)

PTEN is the second most frequently mutated tumor suppresser gene in cancers after p53. Any Genetic and epigenetic alterations in PTEN gene and its regulatory regions have been involved in many disease (55)

PTEN act a significant and non-redundant tumor suppressor gene Specially in HCC,. Thus dysregulation of PTEN plays an important pathogenic role in the progression of HCC.PTEN has the ability to induce cell death via apoptosis and also mediates interactions with extracellular matrix which result in reduced cell migration (56) .

The expressions of P27kip1, P57kip2 and PTEN are triggered by anti-miR-222. Thus, changes in these factors help to limit the tumor cell-cycle promotion miR-222, which is up-regulated in HCC, thus has an oncogenic role in suppression of CDKN1C/P57 and CDKN1B/P27 gene expressions. So by blocking miR-222 expression in HCC. The use of miRNA silencing technology appears to be a target therapeutic approach for HCC. (57)(48)

In summary, miRNAs are able to regulate gene expression by a variety of mechanisms. This dynamic regulation enables the cell to adapt to changes in the cellular environment, but may also explain the dysregulation of miRNA expression in certain types of cancer.(58) gene therapy is a promising treatment option for a number of diseases such as cancer, , although this technique still risky and under examination to ensure safety and its efficiency . (59)

REFERENCE

1. 1-Zhong Xu, Junjie Hu, Hui Cao, Maria G Pilo, Antonio Cigliano, Zixuan Shao, Meng Xu,Silvia Ribback5, Frank Dombrowski, Diego F Calvisi and Xin Chen Loss of Pten synergizes with c-Met to promote hepatocellular carcinoma development via mTORC2 pathway Experimental & Molecular Medicine (2018)

2. 2-Singal AG, El-Serag HB. Hepatocellular carcinoma from epidemiology to prevention: translating knowledge into practice. Clin Gastroenterol Hepatol. 2015;13(12):2140–2151.
3. 3-Pineau P, Volinia S, McJunkin K, Marchio A, Battiston C, Terris B, et al. miR221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci U S A (2010) 107:264–9.
4. 4-Chistiakov DA, Sobenin IA, Orekhov AN, Bobryshev YV. Human miR-221/222 in physiological and atherosclerotic vascular remodeling. Biomed Res Int. 2015;2015:354517.
5. 5-Garofalo M, Quintavalle C, Romano G, Croce CM, Condorelli G. miR221/222 in cancer: their role in tumor progression and response to therapy. Curr Mol Med. 2012;12(1):27–33.
6. 6-Murakami Y, Yasuda T, Saigo K, et al: Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006.25: 2537-2545,
7. 7-Beatriz Aldaz B, Sagardoy A, Nogueira L. et al. Involvement of miRNAs in the differentiation of human glioblastoma multiforme stem-like cells. PLoS One. 2013;8(10):
8. 8-Khare S, Zhang Q and Ibdah JA: Epigenetics of hepatocellular carcinoma: role of microRNA. World J Gastroenterol2013, 19: 5439-5445.
9. 9-Maltby, S.; Plank, M.; Ptaschinski, C.; Mattes, J.; Foster, P.S. MicroRNA function in mast cell biology: Protocols to characterize and modulate microRNA expression. Method Mol. Biol. 2015, 1220, 287–304.
10. 10-Tutar, L.; Tutar, E.; Tutar, Y. MicroRNAs and cancer; an over-view. Curr. Pharm. Biotechnol. 2014, 15, 430–437.
11. 11-Ling, H.; Fabbri, M.; Calin, G.A. MicroRNAs and other noncoding RNAs as targets for anticancer drug development. Nat. Rev. Drug Discov. 2013, 12, 847–865.
12. 12-Majid, S.; Dahiya, R. MicroRNA based therapeutic strategies for cancer: Emphasis on advances in renal cell carcinoma. In MicroRNA Targeted Cancer Therapy; Sarkar, F.H., Ed.; Springer: New York, NY, USA, 2014; pp. 175–188.
13. 13-Tommaso Di Ianni1 , Rajendran J.C. Bose1 , Uday K. Sukumar1 , Sunitha Bachawal1 , Huaijun Wang1 , Arsenii Telichko1 , Carl D. Herickhoff1 , Elise Robinson1 , Sam Baker2 , José G. Vilches-Moure2 , Stephen A. Felt2 , Sanjiv S. Gambhir1,3 , Ramasamy Paulmurugan1,, Jeremy D.. Application of Ultrasound-Targeted Microbubble Destruction-Mediated Exogenous Gene Transfer in Treating Various Renal Diseases,bioRxiv 2019; doi: http://dx.doi.org/10.1101/592931. Hum Gene Ther. 2019 Feb;30(2): 127-138.
14. 14-What are microbubbles? 2018];https://www.news-medical.net/life-sciences/What-are-Microbubbles.aspx 2018
15. 15-Luo MH, Yeh CK, Situ B, Yu JS, Li BC, Chen ZY. Microbubbles: a novel strategy for chemotherapy. Curr Pharm Des. 2017;23:3383–3390.
16. 16-Moscato, Stefania; et al. "Poly(vinyl alcohol)/gelatin Hydrogels Cultured with HepG2 Cells as a 3D Model of Hepatocellular Carcinoma: A Morphological Study". Journal of Functional Biomaterials (2015) (6): 16–32.
17. 17-YaoX.F. and ZhongL.F Application of human hepatoma cell line HepG2 and its progress in the detection of genotoxicantsShijie Huaren Xiaohua Zazhi (2007)15145-150
18. 18-Chunying Shi,Yu Zhang,Haichao Yang,Tianxiu Dong,Yaodong Chen,Yutong Xu,Xiuhua Yang,PengfeiLiuUltrasoundtargetedmicrobubbledestruction mediated Foxp3 knockdown may suppress the tumor growth of HCC mice by relieving immunosuppressive Tregs function ,Experimental and molecular medicine 2017
19. 19-Tzu-Yin W., Wilson K., Machtaler S., Willmann J) Ultrasound and microbubble guided drug delivery: mechanistic understanding and clinical implications. Curr Pharmaceut Biotechnol . (2013)14: 743–752.
20. 20-Yu Jin Ye Shen Wong Brian K Goh Caroline G L Lee Circulating microRNAs as Potential Diagnostic and Prognostic Biomarkers in Hepatocellular Carcinoma
21. Scientific Reports 2019 ,9(1)019-46872-8
22. 21-aRavalli RN. Development of microRNA therapeutics for hepatocellular carcinoma. Diagnostics 2013; 3: 170-191
23. 22-Chunying Shi Yu ZhangHaichao Yang,Tianxiu Dong,Yaodong ChenYutong XuXiuhua Yang,Pengfei Liu,Guo X, Guo S, Pan L, et al.): Anti-microRNA-21/221 and microRNA-199a transfected by ultrasound microbubbles induces the apoptosis of human hepatoma HepG2 cells. Oncol Lett(2017); 13: 3669-75.
24. 23-Gaurav Roy1, Papai Roy2MicroRNAs in hepatocellular carcinoma – therapeutics and beyond: A systematic Year : 2017 | Volume : 2 | Issue : 2 | Page : 10-16
25. 24-Jialin Song, Yuanming Ouyang, Weien Yuan Potential Value of miR-221/222 as Diagnostic, Prognostic, and Therapeutic Biomarkers for Diseases
26. Front. Immunol. 2017 doi: 10.3389/fimmu.2017.00056
27. 25-Weidan Ji , Bin Sun † and Changqing Su Targeting MicroRNAs in Cancer Gene Therapy genes,2017 8(1): 21.
28. 26-Sayan Mullick Chowdhury, Taehwa Lee, and Jürgen K. Willmann Ultrasound guided drug delivery in cancer,2019 Mar 12. doi: 10.1186/s40824-019-0156-z
29. Send to
30. 27-Ferrara K1, Pollard R, Borden M.Ultrasound microbubble contrast agents: fundamentals and application to gene and drug deliveryAnnu Rev Biomed Eng. 2007;9:415-47.
31. 28-.Pan Wu, Haojiao Chen, Ronghua Jin, Tingting Weng, Jon Kee Ho, Chuangang You,Liping Zhang, Xingang Wang, and Chunmao Han Non-viral gene delivery systems for tissue repair and regeneration Transl Med. 2018; 16: 29
32. 29-Renshuai Zhanga , Xiaofei Qina , Fandong Kongb , Pengwei Chenb and Guojun Panc DRUG DELIVERY 2019, VOL. 26, NO. 1, 328–342 https://doi.org/10.1080/10717544.2019.1582730
33. 30-Park, D W, Hoffman, A S, Pu, S, Stayton, P S, Design and development of polymers for gene delivery Nature Rev Drug Discovery 2005; 4:581–593.
34. 31- Mali S. Delivery systems for gene therapy. Indian J Human Genet. 2013;19:3–8.
35. 32-Wang W, Balk M, Deng Z, Wischke C, Gossen M, Behl M, et al. Engineering biodegradable micelles of polyethylenimine-based amphiphilic block copolymers for efcient DNA and siRNA delivery. J Control Release. 2016;242:71–9.

36. 33-Cordeiro RA, Santo D, Farinha D, Serra A, Faneca H, Coelho JFJ. High transfection efciency promoted by tailor-made cationic tri-block copolymer-based nanoparticles. Acta Biomater. 2017;47:113–23.
37. 34https://www.researchgate.net/publication/257924981_Gene_Delivery_Systems_Recent_Progress_in_Viral_and_Non-Viral_Therapy [accessed Oct 20 2019]
38. 35-Pineau P, Volinia S, McJunkin K, Marchio A, Battiston C, Terris B, et al. miR221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci U S A (2010) 107:264–9. doi:10.1073/pnas.0907904107
39. 36-Laura Gramantieri, Francesca Fornari, Manuela Ferracin,Angelo Veronese,Silvia Sabbioni, George Adrian Calin, Gian Luca Grazi, Carlo Maria Croce,Luigi Bolondi, and Massimo Negrini, MicroRNA-221 Targets Bmf in Hepatocellular Carcinoma and Correlates with Tumor Multifocality,Clin Cancer Res. 2009 Aug 15; 15(16): 5073–5081.
40. 37-Li X, Stevens PD, Liu J, Yang H, Wang W, Wang C, Zeng Z, Schmidt MD, Yang M, Lee EY, et al. PHLPP is a negative regulator of RAF1, which reduces colorectal cancer cell motility and prevents tumor progression in mice. Gastroenterology. 2014;146(5):1301–12. e1301-1310.
41. 38-Gramantieri L, Fornari F, Ferracin M, et al: MicroRNA-221 targets Bmf in hepatocellular carcinoma and correlates with tumor multifocality. Clin Cancer Res. 15:5073–5081. 2009
42. 39-He XX, Guo AY, Xu CR, Chang Y, Xiang GY, Gong J, et al. Bioinformatics analysis identifies miR-221 as a core regulator in hepatocellular carcinoma and its silencing suppresses tumor properties. Oncol Rep (2014) 32:1200–10. doi:10.3892/or.2014.3306
43. 40-Yuan Q, Loya K, Rani B, Mobus S, Balakrishnan A, Lamle J, Cathomen T, Vogel A, Manns MP, Ott M, et al: MicroRNA-221 overexpression accelerates hepatocyte proliferation during liver regeneration. Hepatology 2013, 57:299–310.
44. 41-Xinmin Guo,1 Shunhua Guo,1 Liwen Pan,2 Li Ruan,3 Yingshi Gu,1 and Jichuang Lai1Anti-microRNA-21/221 and microRNA-199a transfected by ultrasound microbubbles induces the apoptosis of human hepatoma HepG2 cellsOncol Lett. 2017 May; 13(5): 3669–3675.
45. 42-Weidan Ji , Bin Sun † and Changqing Su .Ultrasound-guided drug delivery in cancer Targeting MicroRNAs in Cancer Gene Therapy genes.
46. 43-Ramjaun AR, Tomlinson S, Eddaoudi A, Downward J. Upregulation of two BH3-only proteins, Bmf and Bim, during TGF beta-induced apoptosis. Oncogene (2007) 26:970–81.
47. 44-Breuhahn K, Longerich T, Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. Oncogene (2006) 25:3787–800.
48. 45-Zhang C, Zhang J, Zhang A, et al CDKN1C/p57 a novel target of miR-221/222 in human epithelial cancers. Int J Oncol. 2010; 37(6):1621–1626. 46-Galardi S, Mercatelli N, Farace MG, Ciafrè SA. NF-kB and c-Jun induce the expression of the oncogenic miR-221 and miR-222 in prostate carcinoma and glioblastoma cells. Nucleic Acids Research. 2011;39(9):3892–3902
49. 47-Garofalo M, Quintavalle C, Di Leva G, et al. MicroRNA signatures of TRAIL resistance in human non-small cell lung cancer. Oncogene. 2008; 27(27):3845–3855.
50. 48-Fornari F, Gramantieri L, Ferracin M, et al. MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. Oncogene. 2008; 27(43): 5651–5661.
51. 49-Molina JR, Agarwal NK, Morales FC, Hayashi Y, Aldape KD, Cote G, Georgescu MM. PTEN, NHERF1 and PHLPP form a tumor suppressor network that is disabled in glioblastoma. Oncogene. 2012;31(10):1264–74.
52. 50-Iorio, M.V.; Croce, C.M. MicroRNA dysregulation in cancer: Diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol. Med. 2012, 4, 143–159.]
53. 51-Kasinski, A.L.; Slack, F.J. Epigenetics and genetics. MicroRNAs en route to the clinic: Progress in validating and targeting microRNAs for cancer therapy. Nat. Rev. Cancer 2011, 11, 849–864. [CrossRef] [PubMed
54. 52-Gong J, He XX, Tian A. Emerging role of microRNA in hepatocellular carcinoma (Review).Oncol Lett. 2015 Mar;9(3): 1027-1033.
55. 53-Huang Q, Zhang XW, Ma YS, et al. Up-regulated microRNA-299 corrected with poor prognosis of glioblastoma multiforme patients by targeting ELL2. Jpn J Clin Oncol. 2017;47(7):590-596
56. 54-Siomi H Siomi MC) On the road to reading the RNA-interference code. Nature (2009),457:396–404.l
57. 55 Ayesha Khalid, Tabinda Hussain, Sobia Manzoor, Muhammad Saalim and Saba Khaliq PTEN: A potential prognostic marker in virus-induced hepatocellular carcinoma journals.sagepub.com/home/tub,2017: 1 –10
58. 56-Tamura M, Gu J, Matsumoto K, et al. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 1998; 280(5369): 1614–16177
59. 57-yooN So, chuN Sm, haN eh, choi J, JaNG SJ, Koh Sa, hwaNG S, yu e. Deregulated expression of microRNA-221 with the potential for prognostic biomarkers in surgically resected hepatocellular carcinoma. Hum Pathol 2011; 42: 1391-1400
60. 58-Krol J, Loedige I and Filipowicz W: The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet. 2010,11:597–610.
61. 59-YK Sung and SW Kim Recent advances in the development of gene delivery systems,Biomater Res. 2019; 23: 8.