MicroRNA-199: A Potential Therapeutic Tool for Hepatocellular Carcinoma in an Experimental Model

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Abstract

Hepatocellular carcinoma is one of the major health problems throughout the world with a very poor prognosis. MicroRNAs are small regulatory non-protein-coding RNA molecules. We aimed at investigating microRNA-199 as a potential therapeutic tool for HCC both in vitro and in an experimental model. A therapeutic strategy based on the effect of microRNAs to target genes responsible for liver cancer was adopted in this work. The ability of these small RNAs to potently influence cellular behavior was also investigated. The role of miR-199a in the development of liver cancer has been identified using a systematic literature search using miRBase. HepG2 cell line was used to test the effect of miRNA199a in vitro. Hepatocellular carcinoma was induced in Male Balb/C mice by diethylnitrosamine (DEN). Mice were treated with miRNA-199a and sacrificed after 16 weeks and blood samples and liver specimens were collected for biochemical and histopathological assessment. Histopathological examination of liver specimens after miRNA 199a treatment showed regression of Hepatocellular carcinoma with restoration of normal architecture. AFP, VEGF and TNFα levels decreased after treatment with miRNA 199a. Caspase 3 and 9; showed decreased expression in animals treated with miRNA 199a than non-treated ones.

Keywords: HCC- MiRNA199a- caspase- VEGF- TNFα

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Materials and Methods

Bioinformatics
We used the Bioinformatics server of miRNA-target prediction (miRBase) (http://www.mirbase.org/index.shtml), which aims to provide integrated interfaces to comprehensive microRNA sequence data, annotation and predicted gene targets. Specific target genes of miR-199a in hepatocellular carcinoma (HCC) were identified by using this server. First, we searched for our target miRNA-199. Then, we selected hsa-mir-199a which referred to Homo sapiens miRNA-199a. Hsa-mir-199a has two mature sequences, hsa-mir-199a-5p and hsa-mir-199a-3p, each mature mir-199a contains a number of target genes and links that were provided to “predicted target” pages. The target genes which are regulated either by hsa-mir-199a-5p or hsa-mir-199a-3p were determined using (miRDB) database (http://www.mirdb.org/index.html). The target scores for each gene and their sequences from (GenBank) were also identified. Generally, there were 562 predicted targets for hsa-miR-199a-5p and 477 predicted targets for hsa-miR-199a-3p in miRDB. Our analysis showed 14 comprehensive potential target genes for hsa-mir-199a-3p and hsa-miR-199a-5p which have been related to HCC.

The Selection of microRNAs
The role of miR-199 in the development of liver cancer especially HCC has been identified using a systematic literature search. The search was conducted in the electronic databases PubMed (https://www.ncbi.nlm.nih.gov/pubmed) and Google Scholar (https://scholar.google.com) until November 2019. The following key words were used: ‘Malignant OR cancer OR tumor OR neoplasm OR carcinoma,’ ‘hepatocellular OR liver OR hepatic OR HCC’ and ‘miR-199 OR miRNA-199 OR microRNA-199 OR mir 199 OR miRNA 199 OR microRNA 199.’

In vitro study
Cell Culture
Human hepatocellular carcinoma HepG2 cell line (provided from Immunology lab, TBRI) was revived and cultured in 75 cm² culture flasks (Greiner bio-one GmbH, Germany) containing Roswell Park Memorial Institute (RPMI) 1640 Medium (Sigma Aldrich, Germany) supplemented with 100 ml/L fetal bovine serum (FBS), 10 g/L glutamine (Bio Whittaker, a Combrex Company, Belgium) and 10 g/L penicillin/streptomycin. HepG2 cells were cultured at 37°C in a 5% CO₂ incubator for 48 hours (hrs) till full confluency, cells were dissociated from the culture flask by 0.025% trypsin (Sigma) and the culture flask was gently shaken to ensure that a single-cell suspension was obtained. Cells were subcultured till reaching the required cell count. The cells were counted using a Neubauer hemocytometer and viability was tested by the trypsan blue dye exclusion assay using trypsan blue (5 g/L; Biochrom KG, Berlin, Germany).

Neutral Red Cytotoxicity Assay
After trypsinization, cells were seeded in a 96-well microtitre plate (Corning) at a concentration of 5×10⁵ cell/ well. The plates were incubated at 37°C in 5% CO₂ for 24 hrs till confluency. Culture medium containing different concentrations of mir 199 (15, 30, 60, 125, 250, 500, 1000 and 2000 µg/mL), was added in triplicate, cells only were used as untreated control. Plates were incubated for 72 hrs. Culture media were discarded and 50µl of dye medium (1ml Neutral red stock diluted in 10ml RPMI) were added to each well and incubated for 2 hrs. The dye-medium was removed and the plates were washed with PBS, pH = 7.4 three times. 50 µL of acetic acid-ethanol (one ml glacial acetic acid in 100 ml 50% ethanol) were added and the plates were kept on a plate shaker for 10 min at room temperature to extract the dye. The absorbance of the extracted dye was measured by spectrophotometric reading (Spectra max 190 Molecular devices) using 540 nm filter. The mean of three measurements for each concentration was determined.

Calculation: The mean ± SEM of three separate experiments for each concentration was determined.

The inhibition percentage = (A*control - A dose/ A control) ×100

A* is the absorbance at wave length 490-520 nm, Control = Untreated cells, Blank = Media without cells.

Calculations of IC50
Dose-response curves were plotted, and 50% inhibitory concentrations of miRNA (IC₅₀) were calculated through GraphPad Prism 8 program and Microsoft Excel 2010 program. Experiments were carried out in triplicates and the data were presented as mean ± SEM.

In vivo study
Animals
Two weeks old male Balb/C mice were provided from the animal house, Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Animals were maintained less than 12h light/dark cycles, fed on a standard diet and given free access to water. All experimental procedures applied on animals were applied according to guidelines for the care and use of laboratory animals, National Research Council, USA.
Induction of hepatocellular carcinoma

Tumors were induced in male mice by a single intraperitoneal (i.p.) injection of DEN (Sigma, Aldrich) diluted in phosphate buffer saline (PBS) weekly for 16 weeks at a dose of 50 mg/kg body weight using a 29G syringe producing DNA damage that led to cellular changes.

Design of miRNA-199a

MiRNA-199a was manufactured as the following sequence of miR-199a precursor:

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CCCAGUGUUCAAGACUACCUGUUC
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Animal study

Thirty male mice were categorized into three main groups (10 mice / each); Normal control group: were injected i.p. with PBS as a vehicle control; mice in HCC Pathological group received i.p. injection with DEN; mice in miRNA-199a treated group were injected intrahepatically only once with 100 µl of miRNA-199a per animal after one week post-injection of DEN. After 16 weeks post-injection of DEN, animals of each group were euthanized. Retro-orbital sampling was performed in mice under transient anesthesia using isoflurane by penetrating the retro-orbital sinus with a glass capillary. Blood samples were collected in tubes and centrifuged at 2000 xg for 20 min. Serum was transferred into a fresh tube and stored at -80°C for further analysis. After complete death under cervical dislocation, mice were dissected to harvest livers that were washed with PBS, and immediately fixed in 10% buffered formalin for histological evaluation.

Histological examination

Fixed liver tissues were dehydrated in alcohol with growing concentrations: 70% - 85% - 90%, inhibiting tissue damage and water is finally removed by absolute alcohol baths. Then tissues were embedded in hot paraffin. The paraffin blocks were sectioned at 4-5 µm thickness. Then, the sections were deparaffinized and dehydrated through grade ethanol dilutions and finally stained with hematoxylin and eosin staining. The sections were examined under light microscope.

Measurement of tumor markers

Serum concentration of Alfa-Fetoprotein (AFP) was determined by Quantikine® ELISA kits (R&D Systems, Inc., USA).

Estimation of vascular endothelial growth factor and tumor necrosis factor alfa

Quantitative determination of mouse vascular endothelial growth factor (VEGF) and tumor necrosis factor alfa (TNF α) concentrations in serum sample was done by Quantikine®ELISA kit (R&D Systems, Inc., Minneapolis, USA). The steps were performed according to the manufacturer’s instructions. Optical density was measured at 450 nm.

Estimation of apoptotic markers

Caspase-3 and 9 were estimated by Real time Polymerase chain reaction

RNA extraction and cDNA synthesis

Liver tissue specimens were collected and stored directly at − 80°C for RNA preservation. Total RNA was extracted using Qiagen RNeasy extraction kit (cat no: 74104) according to manufacturer’s kit instructions. RNA concentration was quantified using NanoDrop2000 Spectrophotometer. Reverse transcription was then performed to obtain the cDNA corresponding to the mRNA isolated from the hepatic specimens. cDNA synthesis was conducted using the applied biosystems high capacity cDNA reverse transcription kit. Each reaction consisted of 2 µl reverse transcriptase buffer, 0.8 µl dNTPs, 2 µl random primer, 1 µl reverse transcriptase enzyme, 1 µl RNase Inhibitor, 1 µg of total RNA and completed with RNase-free water to form a total reaction of 20 µl. cDNA was obtained using Biometra T Professional Thermocycler (Germany), under; 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and finally reaction stopped at 4°C.

Quantitative reverse transcription real time PCR (qRT-PCR)

Amplification of cDNA was performed using SYBR Green I (Thermo Scientific) and Real Time (RT)- PCR 7500 (Life Technologies, Applied Biosystems, Foster City, CA, USA). For endogenous control, β-actin was used in each experiment as the housekeeping gene for normalization. Sequence of caspase-9 forward primer was F-‘-GCTGTGTCAGTGTTTGCTACC-3’ and caspase-9 reverse primer sequence was R-‘-CCCAGATGCCATCCAGGTCTC-3’. β-actin forward primer sequence was F-‘-GGGTTGTTGAAGGTCTCAAA-3’ and β-actin reverse primer sequence was R-‘-GGGGTGTTGAAGGTCTCAAA-3’. One tube was used for caspase-9 and another for β-actin. For each qRT-PCR reaction, 2 µl of the cDNA template products, 12.5 µl RT2 SYBR Green ROX qPCR master mix (cat no: 330520, QIAGEN), 1 µl of each of the forward and reverse primers, 8.5 µl of RNAase free water, in a final volume of 25 µl reaction were mixed. Cycling conditions were; initial denaturation step at 95°C for 10 minutes, followed by 45 cycles, each consisting of; denaturation at 95°C for 15sec, annealing step at 60°C for 1 min, and a final extension step at 60°C for 10 min. Melting curve conditions are started right after qRT-PCR completion and are as follows; 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. Relative expression of these markers was quantified by using of comparative threshold cycle (CT) method.

Statistical analysis

One Way ANOVA (parametric) was used to test the effect of experimental periods of the studied parameter. Duncan’s test to homogeneity was used to compare between each two dependent variables. Data were represented as a mean of 10 mice ± SEM. Data were exhibited a significant effect or difference at α=0.001 and α=0.05. The statistical analysis was done by the aid of Statistical Package for the Social Sciences (SPSS) version 24.6.

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Results

Bioinformatics

According to the steps we had discussed previously, we found out that miRNA 199a has more than 400 target genes in the body that reacts differently according to the location of miRNA in the body. Then, we shortlisted our predicted target genes to those with prediction score > 80, as these genes represent the most confident and highly reliable targets. Our shortlist included 162 predicted target genes for hsa-miR-199a-3p and 189 predicted target genes for hsa-miR-199a-5p, from which only 14 genes were found to be related to HCC. We used Cytoscape (https://cytoscape.org) (Figure 1), which is an open source software platform for visualizing complex networks and integrating these with any type of attribute data, to visualize miRNA 199 and its predicted target genes.

In vitro study

Neutral Red Cytotoxicity Assay

Inhibition effect of the miRNA199a on HepG2 cells was evaluated using the neutral red cytotoxicity assay. The proliferation of HepG2 cells was inhibited on using miRNA199a in a concentration-dependent manner.

Cells were evaluated by morphological changes; wells treated with miRNA199a showed disturbance of the cell monolayer as well as characteristic changes of cell death including granulation, blebbing, shrinkage and nuclear fragmentation. miRNA199a was active in the inhibition of proliferation of HepG2 cells as proved by calculating inhibition activity (%) using graphpad prism 8. IC_{50} was found to be (0.05±0.0025) mg/mL (Figure 2).

Figure 1. Target Genes of miR-199 Using Cytoscape Software

Figure 2. The Inhibition of Proliferation Activity in the HepG2 Cell Line of miRNA 199 Using the Neutral Red Cytotoxic Assay
In vivo study

Histopathologic Examination of Liver Specimens

Microscopic examination of liver sections of mice after DEN injection showed loss of hepatic lobular architecture with severe hydropic degeneration of the hepatocytes. Portal tracts were thickened and extended with chronic inflammatory cells and fibrotic tissue. There was also marked polymorphism, increase in mitotic activity and increase in nuclear size as illustrated in Figure 3B.

After treatment with miRNA-199a, liver specimens showed restoration of normal liver architecture, moderate inflammatory infiltrate, decreased mitotic activity, decreased nuclear size, mild polymorphism compared to the pathological non-treated group (Figure 3C).

Estimation of tumor markers

**AFP**

Our results revealed that AFP level in DEN treated mice serum samples was found to be 14.63±2.32 ng/ml which is higher than normal control (3.38±.64 ng/ml) with very high significance (P <0.001). While there was very high significant decrease in AFP levels in sera of mice treated with mir199 to reach 8.92±.57 ng/ml when compared to mice injected with DEN (P <0.001)
Estimation of vascular endothelial growth factor and tumor necrosis factor alfa

**VEGF**

The results of this study revealed that VEGF level in DEN treated mice serum samples is 371.17±9.64 pg/ml that is higher than normal mice (121.47±3.34 pg/ml) with very high significance (P <0.001). While after treatment with mir199, VEGF level decreased markedly (191.56±6.07 pg/ml) with very high significance compared to mice injected with DEN (P <0.001) (Figure 4B).

**TNFα**

Also there was an elevation in TNFα level (459.27±7.49 pg/ml) with very high significance after injection with DEN when compared to level in normal mice sera (200.37±8.71 pg/ml) (P <0.001). With mir199 treatment, there was a very high significant decrease in level of TNFα (355.41±8.29 pg/ml) in comparison to mice injected with DEN (P <0.001) (Figure 4C).

Estimation of apoptotic markers

**Caspase 3**

Relative quantification value of Caspase 3 was found to be elevated by 4.1 folds after injection with DEN when compared to its value in normal mice sera with very high significance (P <0.001). Our data also showed that there was a very high significant decrease in Relative quantification value of Caspase 3 in miR199 treated mice sera in comparison to mice injected with DEN (P <0.001) (Figure 5A).

**Caspase 9**

Our results showed that Relative quantification value of Caspase 9 in DEN injected mice was higher than its level in normal mice sera by 4.28 folds with very high significance (P <0.001). After treatment with miR199a, there was a very high significant decrease in relative quantification value of caspase 9 in comparison to mice injected only with DEN (P <0.001) (Figure 5B).

Discussion

Hepatocellular carcinoma (HCC) is one of the most common primary malignant tumors in the world and the third leading cause of cancer-related death. It accounts >90% of primary liver malignancies (Bray, 2018; Forner et al., 2018). Treatment lines of HCC include surgery, liver transplantation, ablative therapies, trans-arterial embolization, radiotherapy, and chemotherapy and immunotherapy (Abdalla and Stuart, 2019). New approaches for treatment HCC are being investigated including microRNAs, Golgi-73 Protein (GP73), Glypican-3 (GPC3), Osteopontin (OPN), and more (Tunissioli et al., 2017).

MiRNAs are conservative noncoding RNA, of around 19–25 nucleotides in length (Berindan-Neagoe et al., 2014; Gebert and MacRae, 2019). It is responsible for mRNA degradation or inhibiting its transcription (Rupaimoole and Slack, 2017; Ha and Kim, 2019). This may play a role in the mechanism of tumor formation (Giordano and Columbano, 2013; Berindan-Neagoe et al., 2014; Frampton et al., 2015) or tumor suppression through blocking cell cycle, increasing apoptosis, and reducing tumor angiogenesis and metastasis by inhibiting migration and invasion (Murakami et al., 2006; Hou et al., 2011).

In our study, we aimed to investigate microRNA-199 as a potential therapeutic tool for HCC both in vitro and in an experimental model. The role of miR-199a in the development of liver cancer has been identified using a systematic literature search. Specific target genes of miR-199a in hepatocellular carcinoma were identified using various online bioinformatics tools. We reveal the functionality of this micro-RNA through target prediction and functional annotations. In the current study, in silico analysis using novel servers (miRbase, miRDB) has demonstrated the individual target genes of miR-199a and further investigated its function in HCC progression.

Due to the high cost of isolation and culture, limited availability, short life span, metabolic and genetic differences, loss of functions and hepatic phenotype in two-dimensional culture systems; human hepatocytes are not used for in vitro cytotoxicity studies (Štampar et al., 2019).

HepG2 cells are immortalized cell line consisting of human well-differentiated hepatocellular carcinoma cells of a 15-year-old Caucasian male. It is an ideal in vitro...
MicroRNA-199 for Hepatocellular Carcinoma Treatment

In conclusion, miRNA 199a treatment with a single intrahepatic injection could improve both histopathologic structure and decrease AFP, VEGF and TNFα levels in HCC animal model. MiRNA 199a decreased apoptosis as proved by lowering expression of caspase 3 and 9.

Author Contribution Statement

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References

Abdalla E, Stuart KE (2019). Overview of Treatment approaches for Hepatocellular Carcinoma. Exp Toxicol Pathol, 66, 429-36.

Alexander MS, Kawahara G, Motohashi N, et al (2013). MicroRNA-199a is induced in dystrophic muscle and affects WNT signaling, cell proliferation, and myogenic differentiation. Cell Death Differ, 20, 1194–208.

An M, Kwon K, Park I, et al (2017). Extracellular matrix-derived extracellular vesicles promote cardiomyocyte growth and electrical activity in engineered cardiac atria. Biomaterials, 146, 49–59.

Bartel DP (2018). Metazoan MicroRNAs. Cell, 173, 20–51.

Berindan-Neagoe I, Monroig P, Pasculli B, Calin G (2014). MicroRNAome genome: A treasure for cancer diagnosis and therapy. CA Cancer J Clin, 64, 311-36.

Bray F (2018). Global cancer statistics: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin, 68, 394-424.

Chu R, Mo G, Duan Z, et al (2014). miRNAs affect development of hepatocellular carcinoma via dysregulation of their biogenesis and expression. Cell Commun Signal, 12, 45.

Collins SD, Yuen G, Tu T, et al (2019). In Vitro Models of the Liver: Disease Modeling, Drug Discovery and Clinical Applications. In: Timitz-Parker JEE, editor. Hepatocellular Carcinoma [Internet]. Brisbane (AU): Codon Publications; 2019 Oct 24. Chapter 3. Available from: https://www.ncbi.nlm.nih.gov/books/NBK549191/ doi: 10.15586/hepatocellularcarcinoma.2019.ch3.

Connor F, Rayner TF, Aitken SJ, et al (2018). Mutational landscape of a chemically induced mouse model of liver cancer. J Hepatol, 69, 840-50.

Donato M, Tolosa L, Gómez-Lechón M (2014). Culture and functional characterization of human hepatoma HepG2 cells. Protocols In Vitro Hepatocyte Res, 1250, 77-93.

Fang L, Wang X, Sun B, et al (2017). Expression, regulation and mechanism of action of the miR-17-92 cluster in tumor cells. Int J Mol Med, 40, 1624–30.

Fornari F, Milazzo M, Chieco P, et al (2010). MiR-199a-3p induces the apoptosis of human hepatoma HepG2 cells. Cell Death Dis, 8, e2706.

Giordano S, Columbano A (2013). MicroRNAs: new tools for diagnosis, prognosis, and therapy in hepatocellular carcinoma?. Hepatology, 57, 840-7.

Guo X, Guo S, Pan L, et al (2017). Anti-microRNA-21/221 and microRNA-199a transfected by ultrasound microbubbles induces the apoptosis of human hepatoma HepG2 cells. Oncol Lett, 13, 3669-75.

Ha M, Kim V (2019). Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol, 20, 5-20.

Heindryckx F, Collie I, Van Vlierberge H (2009). Experimental mouse models for hepatocellular carcinoma research. Int J Exp Pathol, 90, 367–86.

Hou J, Lin L, Zhou W, et al (2011). Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as a therapeutic target for hepatocellular carcinoma. Cancer Cell, 19, 232-43.

Hou J, Lin L, Zhou W, et al (2011). Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as a therapeutic target for hepatocellular carcinoma. Cancer Cell, 19, 232-43.

Hsu CY, Hsieh TH, Tsai CF, et al (2014). Mirna-199a-5p regulates VEGFA in endometrial mesenchymal stem cells and contributes to the pathogenesis of endometriosis. J Pathol, 232, 330–43.

Jiang XP, Ai W-B, Wan L-Y, Zhang Y-Q, Wu J-F (2017). The roles of microRNA families in hepatic fibrosis. Cell Biosci, 7, 34.

Jilkova ZM, Kuyucu AZ, Kurma K, et al (2018). Combination of AKT inhibitor ARQ 092 and sorafenib potentiates inhibition of tumor progression in cirrhotic rat model of hepatocellular carcinoma. Oncotarget, 9, 1145–58.

Jovanovic M, Hengartner MO (2006). miRNAs and apoptosis: RNAs to die for. Oncogene, 25, 6176-87.

Kamel R, Amr K, Alify M, et al (2016). Relation between microRNAs and Apoptosis in Hepatocellular Carcinoma. Open Access Maced J Med Sci, 4, 31-7.

Kohn-Gaone J, Dwyer BJ, Grzelak CA, et al (2016). Divergent inflammatory, fibrogenic, and liver progenitor cell dynamics in two common mouse models of chronic liver injury. Am J Pathol, 186, 1762–74.

Li D, Liu X, Lin L, et al (2011). MicroRNA-99a inhibits hepatocellular carcinoma growth and correlates with prognosis of patients with hepatocellular carcinoma. J Biol Chem, 286, 36677-85.

Li Z, Liu L, Hou N, et al (2016). mir-199-sponge transgenic mice develop physiological cardiac hypertrophy. Cardiovasc Res, 110, 258-67.

Lino Cardenas CL, Henauoi IS, Courcet O, et al (2013). miR-199a-5p is upregulated during fibrogenic response to tissue injury and mediates TGF beta-induced lung fibroblast activation by targeting caveolin-1. PLoS Genet, 9, e1003291.

Murakami Y, Yasuda T, Saigo K, et al (2006). Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene, 25, 2537–45.

Nikolic M, Sustersic T, Filipovic N (2018). In vitro models and on-chip systems: Biomaterial Interaction Studies with Tissues Generated Using Lung Epithelial and Liver Metabolic Cell Lines. Front Bioengineering Biotechnol, 6.

Okeke E, Davwar PM, Roberts L, et al (2019). Epidemiology of Liver Cancer in Africa: Current and Future Trends. Semin Liver Dis, doi: 10.1055/s-0039-3399566.

Oliveira P, Colaço A, Chaves R, et al (2007). Chemical carcinogenesis. Ann Brazil Acad Sci, 79, 593-616.

Pascale R, Simile M, Peitta G, et al (2007). Experimental models to define the genetic predisposition to liver cancer. Calvisi Cancers, 11, 1450-76.

Rashed WM, Kandeil MA, Mahmoud MO, Ezzat S (2020). Hepatocellular Carcinoma (HCC) in Egypt: A comprehensive overview. J Egypt Nat Cancer Inst, 32, 5.

Rodt GS, Macek Jilkova Z, Zeybek Kuyucu A, et al (2017). Efficacy of AKT inhibitor ARQ 092 compared with Sorafenib in a Cirrhotic Rat Model with Hepatocellular Carcinoma. Mol Cancer Ther, 16, 2157-65.
Rupaimoole R, Slack F (2017). MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discovery*, **16**, 203-22.
Sanchez-Perez Y, Carrasco-Legleu C, Garcia-Cuellar C, et al (2005). Oxidative stress in carcinogenesis. Correlation between lipid peroxidation and induction of preneoplastic lesions in rat hepatocarcinogenesis. *Cancer Lett*, **217**, 25–32.
Santos NP, Colaço AA, Oliveira PA (2017). Animal models as a tool in hepatocellular carcinoma research: A Review. *Tumor Biol.*, **39**, 1010428317695923.
Schiffer E, Housset C, Cacheux W, et al (2005). Gefitinib: an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. *Hepatology*, **41**, 307-14.
Song XW, Li Q, Lin L, et al (2010). MicroRNAs are dynamically regulated in hypertrophic hearts, and miR-199a is essential for the maintenance of cell size in cardiomyocytes. *J Cell Physiol*, **225**, 437–43.
Štampar M, Tomc J, Filipič M, Žegura B (2019). Development of in vitro 3D cell model from hepatocellular carcinoma (HepG2) cell line and its application for genotoxicity testing. *Arch Toxicol*, **93**, 3321-33.
Teoh NC, Dan YY, Swisshelm K, et al (2008). Defective DNA strand break repair causes chromosomal instability and accelerates liver carcinogenesis in mice. *Hepatology*, **47**, 2078–88.
Tolba R, Kraus T, Liedtke C, Schwarz M, Weiskirchen R (2015). Diethylamino (DEN)-induced carcinogenic liver injury in mice. *Lab Anim*, **49**, 59-69.
Tunissioli NM, Castanhole-Nunes MM, Biselli-Chicote PM, Pavarino EC, Da Silva RF (2017). Hepatocellular carcinoma: A Comprehensive Review of Biomarkers, Clinical Aspects, and Therapy. *Asian Pac J Cancer Prev*, **18**, 863-72.
Uehara T, Pogribny IP, Rusyn I (2014). The DEN and CCl4-induced mouse model of fibrosis and inflammation-associated hepatocellular carcinoma. *Curr Protoc Pharmacol*, **66**, doi: 10.1002/0471141755.ph1430s66.
Yan M, Yang S, Meng F, et al (2018). MicroRNA 199a-5p induces apoptosis by targeting JunB. *Sci Rep*, **8**, 6699.
Yang JD, Hainaut P, Gores GJ, et al (2019). A global view of hepatocellular carcinoma: trends, risk, prevention and management. *Gastroenterol Hepatol*, **16**, 589-604.
Zhang J, Jin H, Liu H, et al (2014). miRNA-99a directly regulates AGO2 through translational repression in hepatocellular carcinoma. *Oncogenesis*, **3**, e97.
Zhu F, Nair RR, Fisher EM (2019). Cunningham TJ. Humanising the mouse genome piece by piece. *Nat Commun*, **10**, 1845.