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Micro-RNA in Hepatocellular Carcinoma - Related Hepatitis C Virus Patients in Correlation to Disease Progression

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Abstract

Hepatocellular carcinoma (HCC) is a multistep heterogeneous disease as it is related to the risk factors such as HBV and HCV infections, including uncontrolled hepatocyte proliferation, invasion of the neighboring tissue and metastasize to distant tissues. There are several factors affecting the course of HCC among the patients such as oncogenes and tumor suppressor genes. Recently, molecular mechanisms have cleared some of the underlying mechanisms of carcinogenesis, especially the microRNAs, the upstream regulators of a large number of critical genes. Mature miRNAs found to be mounted into RISC, which helps in recognizing the complementary binding sites in the 3' untranslated regions of target genes. That binding causes the degradation of/or inhibition of translation of mRNAs. miRNAs have been reported to be deregulated in human cancers demonstrating their double-edged role as a tumor suppressor and as an oncogene. miRNA deregulation is involved in modulating signal pathways of cellular transformation of a normal cell into a cancer cell. miRNAs have been reported to be associated with the processes of carcinogenesis including inflammation, cell-cycle, differentiation, apoptosis, and metastasis. miRNAs have been considered as potential biomarkers in HCC as their development has been attributed to the deregulation of many genes owing to abnormal expression of miRNAs. Herein, the current chapter will focus on studying the regulation of miRNAs in HCC-related HCV patients.

Keywords: miRNA, HCC, HCV, UTR, fibrosis progression

1. Introduction

MicroRNA (miRNA) has been proven as key regulator homeostasis for multiple biological systems, besides modulation of the disease pathology of many cancers. Experimental target

miRNA biogenesis as key regulators using small molecules or other interferences sheds light on its crucial role in regulating posttranscriptional gene expression. Further studies reported the variability of their loci, the genetic organization, and their tissue specificity, besides controlling the translation of target protein and transcriptome in response to physiologic environmental cues, along with their vulnerability to become designated in diseases like cancer and fibrosis, including that related to infection viruses like HCV. Many pathways analysis of targeted genes performed using infection-associated miRNAs showed that the pathways related to signal transduction activation, DNA damage, and cell death were clearly observed in HBV-infected liver, while proteasome, lipid metabolism activation, immune response, and antigen presentation were predominantly in HCV-infected liver. These differences are associated with miRNAs' level in the infected liver and it was confirmed in cell line like Huh7.5 cells in which infectious HBV or HCV clones can be replicated, which proved that miRNAs act as key mediators of HCV and HBV infection and liver disease progression as well; therefore, miRNAs can act as liable therapeutic target molecules in the field of translational medicine.

2. Tissue-specific expression and variation level of miRNAs

MicroRNAs are a class of small, endogenous, conserved, non-coding RNAs with a length of 20–24 ribonucleotide RNA sequence that is biosynthesized through transcription of miRNA genes into primary transcripts (pri-miRNA), which are processed by the drosha, generating a precursor of a length of 70 nucleotides (pre-miRNA) with a hairpin-like structure. A remarkable mechanistic difference in canonical against noncanonical miRNAs is that canonical is drosha-dependent intronic miRNAs and so treated co-transcriptionally in the nucleus with protein-coding transcripts. Pre-miRNA is then processed by dicer in the cytoplasm generating mature miRNA duplexes. Mature miRNAs are then mounted into RISC (an miRNA-induced silencing complex) which helps in recognizing the complementary binding sites in the 3'UTR of target genes. Noncanonical intronic, ones called mirtrons, originate from small introns that are similar to pre-miRNAs and can detour the drosha-processing step [1, 2]. Noncanonical pathway affects common cellular response pathways like proliferation and apoptosis by targeting various mRNA transcripts [1]. miRNA binding causes the degradation of, or inhibition of, translation of mRNAs. miRNAs have been reported to be deregulated in human cancers demonstrating their double-edged role as a tumor suppressor and as an oncogene that offers miR clusters as complex and adaptive regulatory controllers for disease progression. Comparative research assessing the organizational structure for the mammalian genome has noticed enrichment in one of the following: copy number variation, chromosomal deletion or insertion, and single nucleotide polymorphisms (SNPs) that subsidize phenotypic diversity. This diversity is obvious in all aspects of human health and investigated diseases. No wonder there is a mounting gratitude to the variation in miRNAs and their target genes in phenotypic variability. Numerous solid malignancies that included hepatocellular carcinoma (HCC) proved to be correlated with miRNAs located at deleted,

amplified, or translocated chromosomal regions [3]. Variation in gene expression or regulation affected by expression of the quantitative trait loci is caused due to genetic variants in either cis- or trans-acting SNPs [4]. A remarkable criterion of miRNA binding is their capability to distinguish binding site polymorphism (miRSNPs) in transcribed functional genes, as in the case of miR 214-5p that appears to be dysregulated in HCC [2] and miR-24 in the case of colorectal tumor by the targeting site of polymorphism in the dihydrofolate reductase gene [5]. This binding causes inhibition of translation for its transcripts and can phenocopy the phenotypic character of such a disease with genetic knockouts of the responsible gene [5].

Screening miRNA genetic variation and differential expression level across the human population in healthy and disease patients provides more insights on variable causes of disease progression and susceptibility in addition to physical functionalities [4]. Comparative genomic studies showed that the untranslated regions (UTRs) within the mRNA sequence act as a target sequence even for mRNA-UTR-displaying variants; during miRNA-mRNA adaptive coevolution, the co-expressed miRNA selects its cognate UTR mRNA, which depends on whether the dysregulation of protein output will be harmful, beneficial or inconsequential for the desired effect [6].

Evaluating reports on tissue-specific differential expression of miRNAs showed the cross-regulation feature of miRNAs and its correlation to stability of phenotype differentiation [7], as an example, regulation of neurite outgrowth, dendritic spine size, and neural differentiation that is regulated by overexpression of miR219, miR134, miR128, miR24, miR7, and others [7]. In the same strategy, miR499, miR486, miR208, miR206, miR133, and miR1 proved to control skeletal muscle growth, maintenance and differentiation [8], while miR133 proved to inhibit osteogenic cell-lineage differentiation through controlling Runx2 that is required for bone development, differentiation, and formation. Not only the previously mentioned roles but miRNAs can also exert specialized functions as in case of hypothalamus; fine-tuning expression of oxytocin; and Fos controlled by hyper-expression of both miR24 and miR7 and hence they control lactation and parturition through controlling water in the body [9].

MicroRNA (miRNA) has been proven as key regulator homeostasis for multiple biological systems, besides modulation of the disease pathology of many cancers. Experimental target miRNA biogenesis key regulators using small molecules or other interferences sheds light on its crucial role in regulating posttranscriptional gene expression. Further studies reported the variability of their loci, the genetic organization, and their tissue specificity [10], besides controlling the translation of target protein and transcriptome in response to physiologic environmental cues, along with their vulnerability to become designated in diseases like cancer and fibrosis, including that related to infection viruses like HCV. Many pathways analysis of targeted genes performed using infection-associated miRNAs showed that the pathways related to signal transduction activation, DNA damage, and cell death are clearly observed in HBV-infected liver, while proteasome, lipid metabolism activation, immune response, and antigen presentation were predominantly in HCV-infected liver [2, 3]. These differences are

associated with miRNAs' level in the infected liver and it was confirmed in cell line like Huh7.5 cells in which infectious HBV or HCV clones can be replicated, which proved that miRNAs act as key mediators of HCV and HBV infection and liver disease progression as well; therefore, miRNA can act as liable therapeutic target molecules in the field of translational medicine [11].

Since miRNA discovery as a liable promising class of small non-coding RNAs able to regulate protein translation and stability of mRNA, miRNAs have been implicated as key regulators in many diseases like cancer and autoimmune disease. So there is great effort to leverage knowledge of the miRNA regulatory system to these diseases, especially cancer [12].

3. MicroRNAs and disease susceptibility

Development in the pathobiology of miRNAs sheds light on its crucial character in transcriptome modulation which can reflect cancer state in addition to its application in attenuating possible risks that may be raised during cancer progression [13]. Currently, there is no doubt that down-regulating epithelial markers causes disruption in epithelial mesenchymal that is directly associated with differentiation of epithelial cells in lung cancer, a key developmental pathway in lung cancer progression and metastasis [14]. So expression of miRNA can be used as a progression marker for cancer disease depending on its differential expression during invasion, progression, and metastasis of cancer [3].

Reduction in dicer expression is often noticed in cancer stem cells like muscle stem cell tumors and rhabdomyosarcoma in periodic cases, shown to be correlated with the down-regulated level of myomiRNAs like miR 133 and miR 1 [15]. OncomiRNAs is a type of miRNA that can decrease tumor suppressor gene expression which leads to phenotype attenuation or promotion for oncogenic characters; miR92, miR21, and miR17 are members of that family that can modulate cell-cycle regulators like P21, PTEN, and E2F that can promote tumor proliferation [3, 12]. On the other hand, tumor-suppressor miRNAs, like let-7, target directly mRNA for silencing [3]; over-expressed Let7 has proved to modulate cell-cycle regulators that lead to tumor invasion and metastasis. So these miRNAs can be used as prognostic tools for cancer incidence or as a parameter for treatment susceptibility. In squamous cell carcinoma and adenocarcinoma, a comparative miRNA expression profiling revealed a significant difference that reached a specific signature to predict overall survival between male smoker patients in addition to the study performed by Liu et al. that clearly declared correlation between overall pathobiology in cancer and tissue-context miRNA expression [15]. Our team recently proved a small panel of four miRNAs that can act as a liable prognostic marker for HCC progression besides its ability to discriminate different stages in hepatocellular carcinoma [2].

3.1. miRNAs and hepatic diseases

Studies for understanding miRNAs in liver diseases showed a significant progression in that field, making liver a promising first organ to achieve precision and targeted therapy. Depending on accumulated studies of miRNA in liver disease, the unique vasculature of the

liver, and the efficiently rapid accumulation of exogenous small RNAs, the liver showed a good target for RNAi targeted therapy. Manipulation of miRNAs in liver diseases proved great evidences in that field; as an example miR122 clearly illustrates a good effective target for ameliorating hepatic steatosis besides many studies that showed miR122 to be a good target in HCV targeted therapy through its role in production of neutralizing antagomirs. MicroRNAs as a key target for viral hepatitis afford another liable possibility for targeting HBV and HCV infection that, by one way or another, causes HCC progression and death upon chronic infection; this targeting prevention will help to reduce HCC risk incidence by regulating many oncogenic miRNAs like miR222, miR221, and miR21 or via tumor suppression ones like miR199 and miR122 that nowadays are used as liable biomarkers in HCC, besides many promising studies that showed its role as prognostic and a marker for therapy response.

3.1.1. miRNA as a metabolic modulator in hepatic diseases

Non-alcoholic fatty liver diseases (NAFLD) are characterized by increased liver fat content and progression to liver inflammation, fibrosis, and ultimately cancer [16, 17]. Obesity, insulin resistance, and diabetes mellitus are risk factors for this disease and it is estimated that NAFLD will be the most common problem in internal medicine by 2020 [18]. Despite the high prevalence of NAFLD, the biology behind the disease progression is not clear and importantly there is no specific treatment for this condition, so osculating necessity for liable biomarkers and discovery of potential drug targets, as researched by Benhamouche-Trouillet et al., showed that miR-21 may be implicated at various steps during the NAFLD disease progression in a cell-specific manner through modulation of PPAR α [19]. Specific conditional dicer1 deletion from embryonic liver leads to disruption in maturation of microRNA from its pre-microRNA form which leads to striking of metabolic phenotypes that include steatosis together with triglyceride and fatty acid accumulation in addition to dysregulation of blood glucose in fasting mice under study [12]. On the other hand, miR-355 has been recently considered as a liable biomarker for hepatic lipid accumulation in rat experiments as its elevated level strongly correlated with obesity in mice in association with liver steatosis [15]; for that reason, a high-throughput screen for miRNAs as a predictor for lipid droplet formation in the liver metabolic disorder in humans is a great demand for disease progression, overall survival rate, and even for prognosis of possible hepatic disorder (as shown in **Figure 1** and **Table 1**).

The conflicting concerns about whether profile expression of miRNA correlated with NASH or NAFLD have also been well investigated. Sanyal [20] research group reported in double subject groups, including NASH and metabolic syndrome group, in addition to the control group; control groups were matched in BMI. His investigators reported 23 up-regulated miRNAs and the same number of down-regulated miRNAs, with detailed interpretation referring to the role of some miRNA expression dysregulation, that is, miR-34a and miR-146b up-regulation besides miR-122 down-regulation in NASH subjects. Odd findings were reported in human subjects with NASH-like decreased expression of miR-122. However, the protective feature arise from silencing of miR-122 specially in high fat-fed mice bears only indirect physiological matter differentiate steady-state cross-sectional investigations in overweight or obese humans subjects with such a fatty liver disease. This result includes the complexity of dissecting effects and causes cross-talk in hepatic expression of miR-122 and metabolic liver disease.

The importance of miRNAs cross-talk analysis was further elucidated in many publications where hepatic steatosis variations was evolved through application of an adenovirus encoding a dominant negative c-Jun and then testing changes in miRNA expression that is associated with it [21]. They found many miRNAs (miR-122 and miR-370 was common among many publications) to be differentially expressed (DE) in liver tissues of mice that were treated by adenovirus and showed that the elevated presence of miR-370 correlated with the osculating expression of hepatic lipogenic target mRNAs (e.g., FAS, SREBP-1c, and DGAT2); these findings suggest that dietary modulation of some miRNA expression is a relevant consideration [21].

Figure 1. miRNA that either up-regulated or down-regulated with its target genes in different stages of HCC disease progression.

Liver diseases	Down-regulated miRNAs	Up-regulated miRNAs	Dysregulated miRNA
Steatohepatitis	miR-21	miR-17	miR-21
	miR-29a	miR-24	miR-33a/b
	miR-130a	miR-27	miR-122
	miR-185	miR-34a	miR-155
	miR-205	miR-103	
	miR-206	miR-107	
	miR-378	miR-122	
	miR-451		

<i>Liver diseases</i>	Down-regulated miRNAs	Up-regulated miRNAs	Dysregulated miRNA
<i>HCV infection</i>	Let-7	miR-21	miR-126
	miR-17	miR-122	miR-192
	miR-27a	miR-141	miR-198
	miR-29a,b,c	miR-146a	miR-345
	miR-130a	miR-192	
	miR-155	miR-215	
	miR-181a	miR-491	
	miR-194		
	miR-196		
	miR-199a		
	miR-221		
	Let-7	miR-18a	miR-233
	miR-1	miR-21	
<i>HCC</i>	miR-15a	miR-92a	
	miR-16	miR-130b	
	miR-26a	miR-141	
	miR-29	miR-155	
	miR-34a	miR-181	
	miR-101	miR-195	
	miR-122	miR-221	
	miR-124	miR-222	
	miR-125b	miR-224	
	miR-126	miR-494	
	miR-138	miR-1269	
	miR-141		
	miR-145		
	miR-146a		
	miR-148		
	miR-195		
	miR-199		
	miR-200		
	miR-223		
	miR-375		

Table 1. Liver diseases in association with the microRNA level in each stage.

3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the enzyme that catalyzes mevalonic acid synthesis rate-limiting step in cholesterol and other isoprenoid production, showed both miR-21 and miR-34a as key player molecules in this step, through controlling dephosphorylation and activation of HMGCR [22]. One requirement for any effort to enhance miRNA levels as therapeutic tools is the well-established pro-oncogenic characters for miRNAs in HCC and its related diseases, which will be discussed later here.

3.1.2. *miRNA and HCV infection susceptibility*

Hepatitis C virus is the sole member of hepacivirus C species that is known to be a blood-borne infectious viral disease causing the significant persistence of liver disease, with around 110–170 million infected patients globally, with nearly two-thirds of this number chronically infected and not less than one-third developing fibrosis and cirrhosis after 20 years of the onset of infection; most of them develop different stages of hepatocellular carcinoma [23]. Possible therapy for chronic hepatitis C (CHC) virus treatment has undergone a great transformation recently; discoveries in viral infections in humans have shown surprising findings that have broadened our understanding of miRNA function within human body.

In the setting of the HCV infection, the role of various miRNAs in modulating the viral infection response has been deeply studied, that clarifies causes of chronic hepatitis C progression in most infected patients and consequences of infection with its manipulation in the risk of developing cirrhosis and HCC [24]. The HCV virus is a positive-sense, single-stranded RNA virus of 9600 base [25]. It contains 5' untranslated region (UTR) that contains four structurally conserved domains besides an internal ribosomal entry site (IRES) which allows viral RNA translation in a cap-independent manner with minimal dependence on canonical translation factors [26]. Translation of viral RNA leads to a polyprotein product that consists of six nonstructural and four structural viral proteins that undergo additional proteolysis by viral and host enzymes [27].

Subgenomic systems are easier to proceed after discovery of first sustainable cell-culture models for hepatitis viruses, in 1999 [28]. A noticed curious aspect in these early sustainable replicon systems was the successful sustainability of viral replication in Huh7 cell line but not HepG2, albeit both of these transformed cell lines have their origin in hepatocellular cancer in humans. The biologic declaration for this conflicted efficiency was first explained by Jopling et al. in 2005. When he verified that miR-122 has a detectable level in Huh7 but not HepG2 [29], in addition to that, he recently noticed that HCV contains a recognition site for the miR-122's seed sequence in the UTR area of viral genome. The miR-122-interacted viral elements have been mapped to two conserved points within 5' UTR among stem-loop I and/or II, corresponding to the seed sequence of the miR-122 [30]. The finding was more astounding on the grounds that it appeared to be illogical to the customary thought of RNAi as an innate antiviral response like in invertebrates or plants [31]. Inhibition of RISC effector complex molecules like drosha, dicer1, DGCR8, and the RISC using small interfering RNA (siRNA) appears to inhibit HCV replication [32]. Although the mechanism underlying the miR-122 interaction with HCV is not precisely understood, miR-122 binding site position within the 5' UTR proved to be critical, so translocation of this site to the 3' UTR in a luciferase reporter mRNA causes up-regulation in reporter activity upon miR-122 diminished levels [30]. MiR-122 has

been assumed to elevate both replication and translation of RNA, independently from viral replication [33]. Up-regulation of the translation step through miR122 dependent pathway is observed in reporter and full-length HCV genome constructs [34]. Also, Jangra et al. [35] deliberated the mutations of full-length HCV constructs that are capable of generating infectious virions in vitro. He found zero overlapped mutations within the miRNA or IRES binding site in distinct constructs. In those harboring mutations in IRES, infective viral production was down-regulated by more than 28-fold in comparison with constructs with the miR-122 binding site disruption that showed more than a 3000-fold reduction [35]. These observations are important in description of the role of miR-122 in HCV infection that requires searching beyond HCV replication, translation, and stability to investigate more pathways like liable RNA targets in HCV biology or posttranslational targets for it, as an example, heme oxygenase-1 (HO-1) which catalyzes the degradation of heme to biliverdin. Oxidative stress causes HO-1 elevation. Incubation of HCV-infected cell lines with biliverdin causes reduction in HCV amplicons via stimulation of interferon pathways [36]. Heterodimers of BACH1 and a member of the Maf protein family cause transcription repression of HO-1. It was observed that BACH1 3' UTR contains miR-122 binding sites; its function was confirmed to be important by silencing miR-122 that leads to increased HO-1 mRNA levels double fold. Not only that but BACH1 silencing using siRNA or other chemical means like heme or cobalt protoporphyrin also decreased HCV RNA level [37].

However, in these research-based findings, miR-122 proved to be essential in replication of HCV. Later cloned HCV from other genotypes proved to be replicated in some cell lines like HepG2 cells [38], liver cells (hepa1-6) [39], and cervical cancer-derived HeLa cell from humans [40]. In addition, the findings from cell culture are not yet completely correlated with outcomes from clinical infection, like miR-122 level in liver tissue of infected patients and viral load [41]. Moreover, HCV therapy non-responder (NR) patients showed lower pretreatment miR-122 levels in liver tissue biopsies rather than responders [41]. Another study showed inverse correlation between severity of hepatic fibrosis and hepatic miR-122 expression levels [42]. Even bearing in mind those conclusions, there is still convincing evidence that miR-122 targeted therapy may act as a liable strategy in HCV precision medicine. For example, Lanford et al. treated chronically infected chimpanzees with an LNA-modified oligonucleotide against miR-122 [43]. There was a significant drop by 2.6 orders of magnitude of HCV in the chimpanzee that received the optimal dose of this agent besides significant improvement in histologic examination of the liver specimens. Additionally, 5' UTR sequencing indicated no signs for selection of adaptive mutations to the recognition site of miR-122. Similar findings were also reported in human clinical trials, phase II, that test the LNA-modified phosphorothioate antisense DNA oligonucleotide and the anti-miR-122 antagomir (Miravirsen) [44]. These findings were so encouraging to researchers, where the Miravirsen subcutaneous injection reduces serum HCV viral load in a dose-dependent manner, up to a 3-log reduction over 2-5 months [44]. Small molecules synthesis that targets miR-122 raises the possibility for new opportunities in HCV infection treatment [45].

However, miR-122 is the finest studied HCV-related miRNAs; it is not sole. There are others like MiR-199a that recognize HCV 5' UTR and so suppress viral load [46]. Also, MiR196 down-regulates BACH1 [47], and MiR-196 is up-regulated in response to interferon

signaling [48]. Besides, immune-regulatory miRNA-155 is induced by antiviral TLR3/4 signals [49]. Elevated MiR-155 levels in HCV-infected patients appeared to be inversely proportional to serum viral loads, signifying its relevant antiviral effect through suppression of Tim-3 (HAVCR2) that acts as an immune signaling modulator that is elevated in NK cells of HCV-infected patients where its up-regulation leads to inhibition of Tim-3 that causes an escalating production of interferon- γ (IFN γ) [50]. Not only that but MiR-155 also shows antiviral effects against other viruses like HIV infection through TLR3/TLR4 signaling pathway that prevents macrophage infection by HIV. Last but not least, miR-21, like many miRNAs, is induced by HCV infection but its induction aids HCV and escapes the immune response through directly suppressing interleukin-1 receptor-associated kinase 1 (IRAK1) and myeloid differentiation factor 88 (MyD88) [51] that is mandatory for mediating induced interferon response (type I) upon HCV infection. So, augmentation of miRNA expression levels and activity may prove a valuable aspect in HCV treatment and probably other viral infections.

4. MicroRNA inhibitors as a promising therapeutic approach

From the therapeutic point of view, silencing some miRNAs that encode potential vital or good protein-coding genes that is mandatory to preserve our health status. Inhibitors for these miRNAs have been considered for prospective therapeutic agents. Several approaches include direct delivery of miRNA-ASO or expressing it via mini-circle or viral vectors, which have been recognized for effective miRNA knockdown in vitro and in vivo too. Lately, miRNA-ASO-based therapy has been applied in humans and promising ongoing clinical trials considering liver sicknesses [52].

5. Conclusions

In conclusion, miRNA is a vital feature of assurance and monitoring throughout the tissue growth and disease states. In the near term, there will be much to be learned about adaptive or maladaptive states by an investigative way of differential expression of many miRNAs that is affected by the miRNA genetic architecture, mirtrons, and clusters in addition to SNP in miRNA or polymorphism in their target mRNA. There are diverse approaches of miRNA regulatory mechanism of action, for example negative, positive feedback and cross-regulatory through which various biological processes can be monitored, modulated or even resolved its signaling pathways, include fibrosis, viral infection and cancer. Possibly the micromanagement and homeostasis of these systems of regulatory miRNAs, when disturbed, can attain novel wannabe steady state of interacted interfaces that show an undesired effect in disease progression and severity especially in viral-related cancer cases like HCC. So, an enhanced sympathetic of these miRNA regulatory networks, in addition to improved therapeutic tools for controlling miRNA expression or their targets toward healthy regulatory states, will gain

more interest over the coming years. Indeed, modern advanced miRNA precision-based medicine will undergo advanced phases of clinical trials that will afford more understandings into the biosafety and bioavailability in addition to the efficacy of miRNA as therapy and diagnostic tools.

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Conflict of interest

The author declares that there is no conflict of interest for this chapter.

Acronyms and abbreviations

CHC	chronic hepatitis C
E2F	elongation factor 2
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HO-1	heme oxygenase-1
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
IRES	internal ribosomal entry site
LNA	locked nucleic acid
miR	micro-ribonucleic acid
NAFLD	non-alcoholic fatty liver diseases
NASH	non-alcoholic steatohepatitis
PTEN	phosphatase and tensin homolog
RISC	RNA-induced silencing complex
UTR	untranslated region

Notes/Thanks/Other declarations

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