Published in final edited form as:

Transl Res. 2011 April ; 157(4): 241–252. doi:10.1016/j.trsl.2011.01.008.

MicroRNAs and liver disease

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Abstract

Post-transcriptional regulation of gene expression is now recognized as an important contributor to disease pathogenesis, among whose mechanisms include alterations in the function of stability and translational elements within both coding and non-coding regions of messenger RNA. A major component in this regulatory paradigm is the binding both to RNA stability and also to translational control elements by microRNAs (miRNAs). miRNAs are non-coding endogenously transcribed RNAs that undergo a well characterized series of processing steps that generate short single stranded (~20–22) RNA fragments that bind to complementary regions within a range of targets and in turn lead to mRNA degradation or attenuated translation as a result of trafficking to processing bodies. This article will highlight selected advances in the role of miRNAs in liver disease including non-alcoholic fatty liver disease, viral hepatitis, and hepatocellular carcinoma and will briefly discuss the utility of miRNAs as biomarkers of liver injury and neoplasia.

Introduction

RNA interference (RNAi), discovered by Mello and Fire in the early 1990s [1] provides a common mechanism by which endogenous or exogenously encoded RNAs target mRNA transcripts for degradation or attenuated translation and thereby modulate gene expression. The requisite cell machinery, conserved throughout eukaryotic cells including hepatocytes (illustrated in Figure 1) [2], facilitates posttranscriptional mRNA targeting by endogenous or virally encoded miRNAs. Within the liver, the physiological importance of miRNAs has been demonstrated in metabolism [3], immunity [4], viral hepatitis and oncogenesis. In addition, findings illustrate the importance of RNAi as an experimental tool for gene silencing. This review will briefly describe the biogenesis of miRNAs and the role of microarray technology in detecting of miRNAs. The review will primarily focus on developments in miRNA research as it relates to the pathogenesis of non-alcoholic fatty liver disease (NAFLD), viral hepatitis (C and B), and hepatocellular carcinoma. In addition, the role of miRNAs as biomarkers of liver injury and HCC will be discussed. The data described were found in peer-reviewed literature using Pubmed search (most recent 1-7-11) terms that included microRNA, hepatitis C, hepatitis B, hepatocellular carcinoma, cancer, biomarkers, microarray, bioinformatics, liver injury, and non-alcoholic fatty liver disease.

miRNA Biogenesis

miRNAs are transcribed in mono or polycistronic form as single stranded RNA transcripts from genomic, viral, or plasmid DNA. The resultant transcript, termed pri-miRNA

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(genomically encoded) or shRNA (viral or plasmid encoded) is cleaved in the nucleus by the RNase Drosha to a 60–90 bp hairpin configuration pre-miRNA. The pre-miRNA is exported from the nucleus via a GTP-dependent Ran/Exportin 5 complex. In the cytoplasm, the pre-miRNA undergoes further processing by the Dicer complex to a mature 20–22 base miRNA. The “guide” strand is loaded onto the RNA-induced silencing complex (RISC) [5,6] where it variably directs target transcript cleavage, degradation, or P-body sequestration, based upon the degree of complementarity with its target(s) (Reviewed in [2]) (Figure 1). Each miRNA, by targeting a range of targets (up to hundreds), may broadly modify the cellular transcriptome and maintain balanced cellular physiology. Over or underexpression of a limited number of miRNAs in pathologic conditions (described below) may significantly alter cellular metabolism and other processes resulting in disease.

miRNA Analysis and Bioinformatics

Though the first evidence for miRNA function was observed in 1993 [7], increased understanding for the importance of miRNAs in physiology has occurred in the last 10 years. To date, there have been over 17000 miRNAs described in 142 species, with ~1000 described in humans (miRBase release 16 [8]). As each miRNA can regulate hundreds of target mRNA transcripts, developments in microarray and bioinformatics have been central to understanding miRNA function. A common approach to investigate the role of miRNAs in disease processes is to profile miRNA expression patterns between disease and control tissue (neoplastic vs. non-neoplastic tissue or metastatic vs. non-metastatic cancer). To facilitate non-biased miRNA expression profiling, sensitive microarrays have been developed (reviewed in [9]). There are variations in technique and between commercial microarray vendors, but in general, small amounts of RNA are size fractionated to enrich in miRNA transcripts and reverse transcribed using biotin-conjugated random primers to generate a cDNA library representative of the miRNA population. Covalently modified oligonucleotide probes complementary to guide-strand miRNAs are arrayed and the biotin-labeled cDNA library is hybridized directly on the array slide with a streptavidin-linked fluorophore used for quantitation by laser excitation. Higher signal intensity at a given locus represents higher expression of the miRNA corresponding to the probe at that location. Differential regulation of miRNAs based on microarray data is typically independently confirmed by real-time quantitative PCR.

Once candidate miRNAs for disease processes are identified, the challenge remains to identify and validate physiologic target mRNAs. Advances in bioinformatics and RNA arrays have improved the accuracy of miRNA target identification (reviewed in [10]). Computerized algorithms based on miRNA seed pairing and conservation of miRNA recognition elements (MREs) (targetScan.org, pictar.mdc-berlin.de, microRNA.org and others) allow identification of candidate transcripts but the large number of candidates identified may create challenges in identifying physiologically important targets. *In-vitro* miRNA overexpression or antagonism followed by functional and/or cellular mRNA analysis may identify miRNA-targeted genes. These results can then be narrowed to include only those transcripts with the appropriate MRE. Using these techniques, it may be difficult to distinguish primary (direct targets) vs. secondary (compensatory changes) of miRNA activity. A more direct approach to identifying miRNA targets is to immunoprecipitate RISC-associated proteins and sequence co-precipitated miRNA fragments. Because each miRNA may target up to hundreds of mRNA transcripts, gene ontology and interactome analysis may then allow identification of pathways preferentially targeted by a given miRNA.

Hepatic microRNAs as metabolic modulators and their importance in non-alcoholic fatty liver disease (NAFLD)

miRNAs have been implicated in regulating key hepatic metabolic functions [3] and over the last few years some of the relevant pathways have been selectively interrogated. Initial studies in mice used a loss-of-function approach with either specific antagomirs [11] or by antisense oligonucleotide (ASO) mediated knockdown of miR-122 [12], one of the most abundant miRNAs in adult liver [13]. Either targeting strategy effectively decreased hepatic miR-122 expression in mice, leading to decreased serum cholesterol levels and also decreased expression and/or activity of hepatic HMG-CoA reductase [11,12]. ASO mediated knockdown of miR-122 decreased hepatic lipogenesis and afforded mice protection against high fat diet induced hepatic steatosis and a trend to reduced serum transaminase levels, raising the possibility that therapeutic targeting of miR-122 might be a consideration for patients with metabolic syndrome [12,14]. Antagonism of miR-122 in mouse liver was associated with significant changes (>1.4 fold up- or downregulated) in mRNA expression for a large number of transcripts (>300 in each direction), with enrichment for those mRNAs in which there was at least one copy of the miR-122 seed sequence (CACTCC) within the 3' untranslated region (UTR). These findings were extended in studies using locked nucleic acid (LNA) antagomirs to knock down miR-122 expression, where 199 hepatic mRNA transcripts were observed to be upregulated within 24h of LNA administration [15]. These observations establish a role for miR-122 in hepatic lipid metabolism but illustrate an intrinsic difficulty in assigning pathways and mechanisms resulting from changes even in a single miRNA, because targeting of multiple transcripts may occur through the shared regions of homology to the seed sequence.

The related question of whether miRNA expression profiles are associated with NAFLD and non-alcoholic steatohepatitis (NASH) has also been explored. Sanyal and colleagues reported findings in two groups of subjects, including a group with metabolic syndrome and NASH and a control group matched for body mass index with features of metabolic syndrome but without liver enzyme, ultrasound or histologic evidence of NASH [16]. These investigators found 23 miRs to be upregulated and 23 miRs to be downregulated, with further analysis indicating significant increases in miR-34a and miR-146b and decreased expression of miR-122 in NASH subjects. The functional consequences of miR-122 downregulation in HepG2 cells revealed increased mRNA abundance of sterol regulatory element binding protein 1-c (SREBP-1c), fatty acid synthase (FAS) and HMG-CoA reductase, in keeping with the associations found in subjects with NASH (Table 1). Silencing of miR-122 in HepG2 cells produced a corresponding increase in protein expression for these targets, suggesting that miR-122 silencing mediates effects on key transcriptional regulators of hepatic lipid metabolism, although details of whether these effects are mediated exclusively through augmented mRNA degradation versus translational repression are still to be resolved [16].

The findings in human subjects with NASH showing decreased miR-122 expression might appear somewhat at odds with the findings alluded to above in mice treated by therapeutic targeting of miR-122 in which there was *protection* against high fat induced hepatic steatosis, increased fatty acid oxidation lower plasma cholesterol levels and lower transaminases levels than mice receiving a control ASO [11,12,15]. It is important to bear in mind, however, that a protective effect from preemptive knockdown of miR-122 in high fat fed wild-type mice bears only indirect physiological comparison to steady-state cross sectional observations in obese human subjects with fatty liver disease. The results emphasize the complexity of dissecting cause and effect relationships in hepatic miR-122 expression and metabolic liver disease. Yet another nuance to this complexity has emerged from recent findings in which the role of miR-122 was examined in relation to the known

circadian rhythm of hepatic metabolic functions. miR-122 mRNA was expressed at a relatively constant level throughout the day, consistent with its long half-life (>24h), but ASO mediated knockdown revealed either induction or suppression of hundreds of candidate mRNAs of which circadian transcripts were highly enriched among miR-122 targets [17]. The findings thus strongly imply that miR-122 plays a role in the circadian regulation of hepatic metabolic function although the targets involved are yet to be cataloged [17].

In regard to specific metabolic pathways that may be regulated by miRNAs, recent work has implicated miR-122 and miR-422a in the post-transcriptional regulation of Cyp7a1, the rate-limiting enzyme controlling bile acid synthesis in human hepatocytes [18]. Chiang and colleagues demonstrated that both miR-122 and miR-422a decreased reporter activity of a chimeric luciferase construct containing selected 3' UTR cassettes from Cyp7a1 mRNA [18]. It is well established that Cyp7a1 mRNA exhibits rapid turnover in hepatocytes and the 3' UTR is enriched in A+U sequences along with the canonical AUUUAUUA instability motif, suggesting that post-transcriptional regulation of bile acid synthesis may be an attractive model in which to study the role of miRNAs [19] (Table 1).

In keeping with their emerging importance as pleiotropic modulators of key cellular metabolic functions, there was considerable interest in the findings from liver-specific deletion of mature miRNA expression in mice [20,21] particularly in relation to a metabolic phenotype. Liver-specific deletion of mature miRNAs was achieved using conditional *Dicer* deletion (germline deletion is embryonic lethal [22]) in order to disrupt cleavage of pre-microRNAs into their mature processed form. Studies using an Albumin-Cre transgene (*Dicer*-LKO^{alb-Cre}) demonstrated efficient, progressive postnatal *Dicer* deletion with a striking metabolic phenotype at three weeks of age that included hepatic steatosis with increased triglyceride and cholesterol ester accumulation and impaired regulation of blood glucose, with fasting mice becoming rapidly hypoglycemic [20]. This striking phenotype in *Dicer*-LKO^{alb-Cre} mice however contrasts with other findings in which conditional *Dicer* deletion was driven by an Alfa-fetoprotein-Albumin fusion Cre (*Dicer*-LKO^{alfb-Cre}), where *Dicer* expression was decreased at embryonic day 18 and almost completely downregulated at birth [21]. These latter *Dicer*-LKO^{alfb-Cre} livers exhibited no gross metabolic abnormalities and no changes in serum glucose or cholesterol levels. The dramatic differences in the phenotypes presumably reside in the timing for *Dicer* deletion rather than the extent of knockdown of the target since hepatocytes from both lines demonstrated effective downregulation of *Dicer* expression and of mature miRs, including miR-122 [20,21]. Studies in *Dicer*-LKO^{alb-Cre} mice demonstrated that approximately one third of mice older than 6 months developed hepatocellular cancers in which there was variable degrees of hepatic steatosis [20].

The molecular pathways underlying hepatic steatosis phenotype in *Dicer*-LKO^{alb-Cre} liver is yet to be fully explained but there was decreased expression of miR-122 target genes including those involved in cholesterol synthesis [20]. Nevertheless as emphasized in studies summarized above, the dramatic hepatic steatosis associated with *Dicer* deletion presumably reflects changes other than miR-122 dependent pathways since the effects of miR-122 knockdown alone appeared to attenuate hepatic steatosis and future work will be required to clarify the extent to which miR-122 dependent and miR-122 independent pathways modulate hepatic lipid metabolism in-vivo. Other work has suggested that miR-335 may represent a biomarker for hepatic lipid accumulation in mice, since increased accumulation was noted in genetically obese mice (both ob/ob and db/db) in association with hepatic steatosis [23]. This and other recent work have laid the groundwork for future high throughput screens of miRs that might be useful predictors of lipid droplet formation and metabolic liver disease in humans [24] (Table 1).

The importance of combinatorial interactions among miRNAs was further illustrated in recent work in which alterations in hepatic steatosis was produced by administration of an adenovirus encoding a dominant negative c-Jun and the associated changes in miRNA expression functionally examined [25]. These authors found nine miRNAs (including miR-122 and miR-370) to be differentially expressed in the livers of the adenovirus treated mice and demonstrated that increased abundance of miR-370 was associated with increased expression of hepatic lipogenic target mRNAs (including SREBP-1c, Fatty acid synthase (FAS) and DGAT2). The authors went on to demonstrate that transfection of miR-370 itself induced the expression of miR-122 and that knockdown of miR-122 attenuated the effects of miR-370 overexpression [25]. Those findings together suggest that miR-370 modulates hepatic lipogenic genes indirectly through pathways that include miR-122 targets. Feeding wild type mice a high fat diet for periods up to 8 weeks also resulted in increased expression of miR-122 (but not miR-370) in liver, suggesting that dietary modulation of microRNA expression is a relevant consideration [25] (Table 1).

Four recent publications have collectively illustrated features of the homeostatic regulation of hepatic cholesterol through the coordinated transcription of SREBP2 and miR-33 [26–29]. The key features include the demonstration that miR-33 is encoded within intronic regions of mouse and human SREBP2 and that both RNAs are coexpressed [26–29]. The functional consequences of miR-33 expression include decreased expression of the cholesterol export pump, ABCA1 [26,27,29] and in addition decreased expression of genes involved in fatty acid oxidation [28]. The net effects and integrated response to cellular cholesterol depletion thus includes a regulated program in which increased SREBP2 transcription upregulates sterol synthesis while miR-33 induction decreases cholesterol export (via decreased ABCA1 expression) and attenuates degradation of intracellular fatty acids [26–29] (Table 1).

Another consideration in regard to the role of miRNAs in NAFLD is highlighted by a recent report examining visceral adipose tissue profiles in a small cohort (12) of subjects that revealed alterations in miRNAs targeting adipokines and cytokines [30].

miRNAs and Hepatitis C virus (HCV)

Experience with RNA interference in plants and invertebrates would argue for a conserved role for miRNA in the innate response to viral infections. However discoveries in human viral infections have revealed unexpected findings that have enlarged an understanding of miRNA function within mammalian cells.

The role of miRNAs in modulating the response to hepatotropic virus infection has been most extensively studied in the setting of HCV infection, the most common etiologic agent underlying chronic hepatitis in the United States. Exposure to HCV leads to chronic infection in the majority of subjects and, as a consequence of infection, typically ranging from two to four decades, individuals are at risk for the development of cirrhosis and hepatocellular cancer [31]. The prevalence of HCV infection in the United States is 1.6% and is the most common indication for liver transplantation. The HCV virus is a positive sense, single-stranded RNA virus of 9.6 kB [32] whose genome includes a 5' noncoding region (NCR) containing four conserved structural domains and an internal ribosomal entry site (IRES) that permits cap-independent translation of viral RNA with minimal requirement for canonical translation factors [33]. The resulting polyprotein consists of four structural and six nonstructural proteins that undergo further proteolysis by viral and host enzymes.

Robust, sustainable cell-culture models of HCV infection first became available in 1999 with the advent of subgenomic replicon systems [34]. A curious feature of these early replicon systems was that efficient replication could be sustained in the Huh7 but not HepG2

cell line, even though both transformed cell lines have their origin in human hepatocellular cancers. The biologic basis for this efficiency was first delineated in 2005 when Jopling, et al. demonstrated that miR-122 was detectable in Huh7 but not HepG2 [35]. Further, the HCV genome contained recognition sites for miR-122's seed sequence within its NCR. In cells stably transfected with a HCV replicon, sequestration of miR-122 with chemically modified ASOs resulted in an 80% decrease in accumulation of replicon RNA. The viral elements that interact with miR-122 have been mapped to two conserved sites in the 5' NCR between stem-loop I and II complementary to the seed sequence of miR-122 [36] (Table 1).

The finding was all the more surprising because it seemed counterintuitive to the traditional notion of RNA interference as an innate antiviral response, such as in plants and invertebrates [37]. Indeed, siRNA targeting of DICER1, Drosha, DGCR8 and the RISC effector complex appears to inhibit HCV replication [38]. While the precise mechanism underlying HCV's interaction with miR-122 is incompletely understood, the position of the miR-122's binding site within the 5' NCR is critical. Translocation of the binding site to the 3'NCR in a luciferase reporter mRNA upregulated reporter activity when miR-122 levels were diminished [36]. miRNA-122 has been postulated to increase both RNA replication and translation, the latter independent of viral replication [39]. Upregulation of translation by miR-122 has been observed in reporter constructs and also in constructs carrying full-length HCV genomes [40] (Table 1).

In a separate experiment, Jangra and colleagues [41] studied mutations in full-length HCV constructs capable of creating infectious virions *in vitro*. Non-overlapping mutations were introduced into either the IRES or miR-122's binding site in separate constructs. Production of infective virus in constructs harboring IRES mutants was downregulated by 28-fold compared to greater than 3000-fold reduction in constructs with disruption of the miR-122 binding site [41].

The importance of these observations is that a complete description of miR-122's role in HCV infection requires looking beyond HCV replication, stability and translation to study other mechanism including post-translational targets or RNA targets relevant to HCV biology. One such target is heme oxygenase-1 (HO-1), the enzyme that catalyzes the degradation of heme to biliverdin. HO-1 is an inducible enzyme upregulated in conditions of oxidative stress. Incubation of biliverdin with cell lines carrying HCV replicons reduced HCV replication by induction of antiviral interferons [42]. HO-1 is transcriptionally repressed by heterodimers comprised of the transcription factor Bach1 and proteins of the Maf family. The 3' NCR of Bach1 contains binding sites for miR-122, whose importance was confirmed by silencing miR-122 which then increased HO-1 mRNA levels 2-fold. Further, silencing of Bach1 by siRNA or chemical means with cobalt protoporphyrin or heme decreased HCV RNA [43] (Table 1).

Despite these findings, miR-122 is not required for HCV RNA replication. Later generation replicons, including those cloned from other HCV genotypes, have been shown to successfully replicate in HepG2 cells [44], human cervical cancer derived HeLa cell [45] and mouse liver cells (hepa1-6) [46]. Further, the cell culture findings have yet to be completely correlated with clinical outcomes of infection. For example, there was no correlation between HCV RNA viral load and levels of miR-122 in liver tissue of HCV-infected subjects and non-responders to antiviral therapy tended to have lower pretreatment liver tissue miR-122 levels than responders [47]. In other studies, there was an inverse correlation between hepatic miR-122 expression and severity of hepatic fibrosis [48]. Those reservations noted, there is still compelling evidence that targeting of miR-122 may emerge as a relevant strategy in the treatment of HCV infection. Lanford, et. al. treated chimpanzees chronically infected with HCV with a locked nucleic acid modified oligonucleotide

complementary to miR-122 [49]. HCV RNA levels fell by 2.6 orders of magnitude in the primate receiving the highest dose of the agent and showed histologic improvement in liver biopsy specimens. Further, deep sequencing of the 5'NCR showed no evidence for selection of adaptive mutations to the miR-122 recognition site. The synthesis of small molecule inhibitor and activators of miR-122 raise the possibility for new avenues of treatment of HCV infection [50] (Table 1).

Though miR-122 is the best studied of the miRNAs to interact with HCV, it is not unique. miR-199a also recognizes sequences in the 5' NCR of HCV and downregulates HCV RNA replication [51]. miR-196 also contains within its seed sequence a region complementary to sequences in HCV and both inhibits HCV expression and downregulates Bach1 [52]. miR-196 is also one of eight miRNAs upregulated in response to interferon signaling [53]. It is also worth noting that miR-122 associated suppression of HO-1 was associated with decreased replication of hepatitis B virus [54]. In other words, while targeting miR-122 expression may become a relevant strategy to attenuate HCV replication, the data suggest that such a strategy would increase HBV replication. This would be an important consideration in coinfecting individuals. In other work (expanded below) findings from subjects with HBV and hepatocellular carcinoma (HCC) suggest that miR-152 is frequently downregulated in and inversely correlated with the expression of DNA methyltransferase I [55]. The findings also indicated alterations in global methylation profiles suggesting that the epigenetic changes associated with alterations in miR-152 expression may be useful predictors of HCC in patients with chronic HBV infection (Table 1).

miRNA and Hepatocellular Carcinoma

miRNAs contribute to oncogenesis by mechanisms including decreased expression of tumor suppressor genes (oncomiRNAs) or alternatively as tumor suppressor genes targeting an oncogenic mRNA transcript for destruction (tumor suppressor miRNAs) [56].

miRNA encoding genes are frequently located at sites of DNA deletion or amplification in malignancy [57] and while an association of miRNAs with cancer was earlier demonstrated in the setting of loss of miR15 and miR16 expression in chronic lymphocytic leukemia [58], altered miRNA expression has been associated with numerous cancers including lymphoma [59], breast, prostate, colorectal [60] cancer, and others.

miRNA expression profiling of hepatocellular carcinoma (HCC) was compared in 25 paired human HCC and adjacent non-tumorous (NT) tissue samples by miRNA microarray analysis, revealing increased expression of three miRNAs and decreased expression of four miRNAs in HCC [61]. Increased miR-18 and miR-20 abundance correlated with poor tumor differentiation suggesting that altered miRNA expression may contribute to loss of hepatocyte differentiation. Studies in a rat model of HCC revealed 23 upregulated and 4 downregulated miRNAs with miR-122 the most consistently downregulated miRNA in HCC tissue [62]. These authors also examined human HCC miR-122 expression revealing significantly decreased miR-122 expression in 10 out of 20 HCC tumors and similar downregulation of miR-122 in hepatoma cell lines (HepG2, Hep3B, and H-7 cells) compared to normal liver tissue. These data suggest that HCC tissue may bear characteristic miRNA expression patterns useful in tissue or serum-based diagnostic approaches and implicate miR-122 as among the most characteristically altered species (Table 1).

Identification of dysregulated miRNA expression in HCC has led to miRNA target identification and increased understanding of the molecular basis of HCC. In a microarray-based comparison of miRNA expression between cirrhotic and HCC tissue, thirty-five differentially miRNAs were identified, including several implicated in other human malignancies [63]. These include members of the let-7 family, miR-221, miR-145, and the

normally liver-enriched miR-122a. miR-122a targets in liver predicted *in-silico* using miRanda, TargetScan, and PicTar algorithms implicated cyclin G1, which was then formally evaluated as a miR-122a target. Transfection of miR-122 into HEP3B hepatoma cells decreased Cyclin G1 expression and further analysis revealed an inverse correlation between cyclin G1 protein (western blot) and miR-122a expression (comparing HCC and cirrhotic liver) suggesting that decreased miR-122a may allow overexpression of genes involved in cell-cycle progression and increased risk of malignant transformation (Table 1).

miR-122 was found to be significantly downregulated in HCC tissue compared to non-tumor adjacent tissue [64]. To identify potential miR-122 target genes, computational models identified 32 transcripts as miR-122 targets revealing targets enriched for genes regulating cell movement, morphology, signaling, and transcription. ADAM17 (a disintegrin and metalloprotease 17), was validated as a negatively regulated target of miR-122 and shown to regulate cell migration and invasiveness of two HCC cell lines (SK-Hep1 and Mahlavu). Rescue of miR-122 expression or RNAi mediated suppression of ADAM17 in Mahlavu cells prior to injection into nude mice significantly decreased tumor growth and angiogenesis. These data suggested that restoration of physiologic miRNA targeting in hepatocytes may decrease the oncogenic properties of hepatoma cells. In contrast to the tumor suppressor qualities of miR-122, miR-221 appears to act as an oncomiR in hepatocytes. miRNA expression profiling in 104 HCC and 90 adjacent cirrhotic liver tissue samples revealed 12 miRNAs linked to progression to HCC [65]. The most upregulated of these, miR-221/222 was transfected into HepG2 cells, leading to increased cell proliferation. Decreased proliferation was observed when cells were treated with an antagomir directed toward miR-221. Injection of miR-221 overexpressing immortalized liver progenitor cells into irradiated nu/nu mice led to decreased tumor latency compared to control immortalized liver progenitor cells alone. mRNA analysis of HCC tissue revealed 15 transcripts containing putative miR-221 binding sites. *In-vitro* miR-221 targeting assays demonstrated potent suppression of DNA-damage inducible transcript 4 (DDIT4) and p27 (Kip-1-CDKN1B), targets involved in limiting cell proliferation. These findings suggest that overexpression of miR-221 appears to have pro-oncogenic consequences (Table 1).

In addition to miRNA-mediated regulation of oncogenes and tumor suppressor genes, mutations or modifications to the templated miRNA sequence may dramatically alter miRNA maturation or targeting efficiency, resulting in oncogenesis. Single nucleotide polymorphisms (SNPs) in miRNA stem-loop structures revealed three SNPs with high allelic frequency (>40%) in the Han Chinese population [66]. One of these, miR-146a was previously reported to be overexpressed in HCC [67]. Genotype distribution analysis comparing HCC with control subjects revealed that the GG genotype was associated with a 2-fold increased risk for HCC compared with the CC genotype. *In-vitro* studies demonstrate that the GG genotype was associated with higher levels of miR-146a production and promotion of cell proliferation in the NIH-3T3 immortalized cell line. These data show that mutations in miRNA encoding loci themselves can increase oncogenic risk (Table 1).

miRNA expression profiles may also predict HCC clinical behavior. In a study examining 482 cancerous and non-cancerous resection specimens from 241 patients, and a cohort of 131 patients, a unique 20-miRNA signature (including miR-122a) was predictive of HCC venous invasion vs. non-metastatic HCC [68]. Prospective validation of this miRNA signature in 110 additional cases demonstrated that miRNA analysis could significantly and independently predict survival and relapse. Though candidate target analysis was not performed in this study, many predicted targets of these 20 miRNAs were previously shown to be included in a 153-mRNA metastasis signature from hepatic tumors [69].

Identification of stem-cell like cells within HCC tissue has allowed investigation into the role of miRNA in this population. Cells with a CD133⁺ surface phenotype display cancer stem cell characteristics including long-term self renewal, tumor initiation, and resistance to chemotherapy [70,71]. Recent data suggests that enrichment with CD133⁺ cells is associated with decreased disease-free and overall patient survival [72]. Quantitative PCR miRNA analysis of CD133⁺ cells derived from HCC tissue and comparison to hepatoma cell lines revealed 8 candidate miRNAs that were differentially regulated [72]. Of these, miR-130b most closely correlated with CD133 expression. miR-130b was preferentially expressed in CD133⁺ cells in resected HCC specimens. Introduction of miR-130b into CD-133⁻ cells resulted in enhanced proliferation, resistance to chemotherapy, and the ability to be passaged from one generation to another. PicTar and Targetscan miR-130b target prediction revealed 289 potential downstream targets. When combined with miRNA microarray analysis of miR-130b transfected cells, three putative miR-130b targets were found, including the tumor suppressor gene *TP53INP1*. *In-vitro* luciferase reporter assays using the *TP53INP1* 3' UTR validated this transcript as a target of miR-130b. These data support the direct role of miR-130b in hepatic neoplasia and suggest a potential role for miR-130b antagonism in HCC therapy.

By merging miRNA expression data, candidate target analysis, and clinical information, increasing data suggests that miRNA expression may predict prognosis and response to chemotherapy. 78 matched cancerous and non-cancerous tissues from HCC patients were evaluated by miRNA profiling [73]. 8 differentially expressed miRNAs were identified and validated by RT-PCR. *In-vitro* analysis demonstrated that overexpression of miR-125b in HepG2 cells impaired cell growth, possibly via modulation of Akt signaling pathways. Kaplan-Meier survival analysis demonstrated that high levels of miR-125b correlated with improved survival in HCC patients. A larger study of 241 patients correlating miRNA expression changes with survival [74], revealed increased miR-26 expression in females than in males and significantly decreased expression in HCC tissue. Transcriptomic analysis of HCC tissue with low miR-26 levels suggested that altered nuclear factor- κ B and IL-6 signaling might play a role in hepatic oncogenesis. Kaplan Meier analysis revealed that increased miR-26 levels were associated with increased patient survival. Conversely, although decreased HCC miR-26 expression predicted poor survival, this pattern of reduced miR-26 expression was associated with a favorable response to interferon therapy. These observations were validated in 214 additional patients with similar findings (Table 1).

Identification of altered miRNAs in HCC may have value in predicting the response to pharmacotherapy. It has been shown that miR-199a-3p is decreased in a variety of malignancies including HCC and through bioinformatic approaches, mammalian target of rapamycin (mTOR), a regulator of cell proliferation, was identified as a potential target of miR-199a-3p. In three HCC cell lines, miR-199-3p expression was inversely related to mTOR expression. *In-vitro* restoration of miR-199a-3p expression in HCC cells resulted in cell cycle arrest, decreased invasion, and increased sensitivity of the cells to doxorubicin challenge. Reduced expression of miR-199a-3p in HCC was associated with a significantly decreased time to recurrence in patients who underwent surgical resection. A similar approach examined miRNA expression profiles in hepatoma cells compared with human hepatocytes. 26 miRNAs including members of the *let-7* family were found to be downregulated in hepatoma cells [75]. This was associated with upregulated expression of *Bcl-xL*, an antiapoptotic protein found increased in HCC tissue. Restoration of *let-7c* and *let-7g* led to decreased *Bcl-xL* expression in Huh7 hepatoma cells, and decreased expression of a *Bcl-xL* 3'-untranslated region-containing reporter mRNA transcript in a target sequence-specific fashion. Restoration of *let-7c* in Huh7 hepatoma cells led to increased sensitivity to staurosporine-induced apoptosis. Conversely, overexpression of *let-7c* in normal hepatocytes had no effect on sensitivity to staurosporine-induced apoptosis.

Transfection of *let-7c* into Huh7 cells similarly dramatically increased sensitivity of the cells to sorafenib. These data strongly suggest that miRNA profiling of HCC tissue may predict response to chemotherapy, and that restoration or rescue of certain downregulated miRNAs may increase pharmacologic efficacy in HCC treatment (Table 1).

In-vivo evidence in murine HCC models further suggests that therapeutic restoration of miRNA deficient in HCC tissue may have anti-tumor effects. Using a liver-specific tetracycline-repressible *MYC* transgene in which mice form HCC-like lesions on withdrawal of doxycycline [76], workers found miR-26 to be dramatically downregulated, consistent with human HCC expression patterns. Expression of miR-26 in HepG2 cells resulted in cell cycle arrest via post-transcriptional repression of Cyclin D2 (*CCND2*) and Cyclin E2 (*CCNE2*). Therapeutic adenoviral delivery of miR-26 in mice with HCC resulted in a dramatic protection from HCC formation by reducing cancer cell proliferation and increasing tumor-specific apoptosis (Table 1). These results strongly suggest that RNAi-based therapeutics may be efficacious in medical treatment of HCC and in neoadjuvant therapy prior to resection or transplantation.

miRNAs as Biomarkers of Liver Injury

Serum levels of alanine aminotransferase (ALT) along with aspartate aminotransferase (AST) are the primary serum biomarker of parenchymal liver injury in a variety of clinical scenarios [77]. However there are significant limitations to the use of aminotransferases as biomarkers of liver injury. First, elevations in serum aminotransferases can reflect non-hepatic injury (particularly skeletal muscle injury), and thus complicate non-invasive assessment of hepatic injury. Second, in situations such as acute acetaminophen toxicity elevations in serum aminotransferases may occur after a critical therapeutic window. Third, serum transaminase concentrations generally do not effectively discriminate between etiologies of liver injury.

In light of these limitations, recent findings highlight the potential for serum transcriptome analysis as biomarkers of both acute and chronic liver injury. Hepatocyte-specific mRNA profiles were analyzed in a rat model of acute chemical (D-galactosamine and acetaminophen)-induced liver [78], demonstrating albumin and $\alpha 1$ -microglobulin/bikunin in peripheral blood after liver injury. Notably, albumin mRNA was detected in serum 2 hours after liver injury, prior to elevations in serum ALT or AST. These transcripts were not elevated after bupivacaine-HCl-induced skeletal muscle damage suggesting that serum mRNA profiles may effectively differentiate hepatocyte injury from alternative sources transaminases and other studies have confirmed this pattern of hepatocyte-specific mRNA release in experimentally-induced liver but not muscle injury [79]. Furthermore, transcriptomic profiling revealed DGAL and APAP-specific patterns of serum mRNA detection suggesting that RNA patterns in serum might provide clues to the etiology of liver injury, and perhaps the offending agent in drug-induced liver injury.

In a similar mouse model of acetaminophen-induced liver injury, the utility of miRNAs were assessed [80]. Acetaminophen-induced liver injury resulted in a significant increase in microarray-determined serum concentration of hepatocyte-specific miRNAs including mir-122 and mir-192. Increased abundance of these miRNAs in serum was dose-dependent and occurred within one-hour after acetaminophen exposure, prior to increases in serum transaminase concentration. A similar study investigating the utility of miRNAs as biomarkers of either liver, muscle, or brain injury using a rat model revealed that miR-122 and miR-133a were specific serum markers of liver and muscle injury respectively, whereas AST and ALT were elevated in experimentally-induced injury to either tissue [81] (Table 1). Though there are a limited number of studies to date, the data described above highlights the

strong potential for serum RNA analysis, particularly miRNA, in detection and investigation of liver injury.

miRNAs as Biomarkers for Hepatocellular Carcinoma

Cirrhosis, regardless of the cause, is a significant risk factor for HCC formation and the early detection of tumors is an important challenge. Current recommendations include ultrasound imaging every 6–12 months, which carries significant cost, has imperfect sensitivity and specificity, and are not available to all patients. Recent data suggests that serum miRNA analysis may be effective for detection of HCC. Using a murine *MYC*-induced HCC model, serum miRNA analysis revealed altered patterns of miRNA in mice with HCC compared to control mice [82] (Table 1). Regression of HCC in these mice was accompanied by normalization of miRNA expression patterns. A recent study in humans reported serum miRNA profiles in patients with HCC compared to healthy controls and patients with chronic hepatitis B hepatitis [83]. The findings revealed elevations in miR-21, miR-122, and miR-233 in patients with HCC and hepatitis B compared to healthy control patients. Serum miR-21 and miR-122 were also significantly higher in patients with chronic hepatitis B compared with subjects with HCC. Elevations in these miRNAs were interpreted to represent a consequence of liver injury rather than tumor itself but no comparison of miRNA profiles was undertaken in serum from patients with HCC and those with cirrhosis. These data suggest that serum-based miRNA analysis may complement and extend current HCC screening strategies, and may increase availability of HCC screening in high-risk populations.

Acknowledgments

We apologize to colleagues whose work we were unable to cite due to space limitations. Work cited in this review was supported by grants HL-38180, DK-52574 and DK-52560 (to NOD) and by a Fellow to Faculty transition award from the Foundation for Digestive Health and Nutrition (to TAK). The authors have no potential conflicts of interest to declare.

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Figure 1. miRNA Biogenesis

miRNAs are transcribed in mono or polycistronic form as single stranded, hairpin configuration pri-miRNAs. These are cleaved by Drosha in the nucleus to form 60–90 base pair pre-miRNAs. Pre-miRNAs are exported via Ran/Exportin 5 to the cytoplasm. Exportin 5 shuttles back to the nucleus while the pre-miRNA is further processed by Dicer to 20–22 base pair fragments. The guide strand is incorporated into the RNA-induced silencing complex (RISC) to direct Argonaute (the catalytically active RNase), GW182 family members, and other RISC components to target mRNAs in a sequence-specific fashion. The remaining “passenger” strand is degraded. The target mRNA fate is determined, in part, by the degree of complementarity to the guide miRNA resulting in target cleavage, P-body sequestration, non-RNAi mediated degradation, or altered translation.

Table 1

Disease	Upregulated in Disease	Down regulated in Disease	Cell Culture/Animal Modifier of Disease Parameters	Associated with Outcome or Therapy Response	Ref.
NAFLD	miR-24a, miR-146b	miR-122	miR-122, miR-335, miR-370, miR-33	miR-122	3, 12, 16, 23, 25, 26, 28, 29
Hepatitis C	miR-122		miR-122, miR-199a, miR-196	miR-122	35, 36, 41, 48, 52
Hepatitis B		miR-152			55
Hepatocellular Carcinoma	miR-18, miR-20, miR-221, miR-146a	miR-122, <i>let-7</i> family, miR-145, miR-26	miR-122, miR-125b, miR-130b	miR-221/222, miR-125b, miR-26, miR-199a-3p, <i>let-7c</i> , and others	61, 65, 67, 69, 72, 73, 74
Bile Acid Metabolism			miR-122, miR-422a		18
Serum Biomarker of Hepatocyte Injury	miR-122, miR-192, miR-133a				80, 81
Serum Biomarker of HCC	<i>let-7c</i> , <i>let-7g</i> , miR-132, miR-149, miR-152, miR-122, miR-21, miR-233				74, 82, 83