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MINIREVIEWS

MicroRNA expression in hepatitis C virus-related malignancies: A brief review

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Abstract

Not only is chronic hepatitis C virus (HCV) infection a major public health problem, but also it can cause hepatocellular carcinoma and, more rarely, non-Hodgkin's lymphoma. These characteristics mean that HCV is the only virus infecting humans that is able to cause two different cancers. The fine pathogenetic and molecular mechanisms by which HCV induces these two malignancies are not completely clear. In the last decade, it has been shown that microRNAs (miRNAs), a class of 21-23-nucleotide molecules modulating post-transcriptional gene expression, make an important contribution to the pathogenesis of several cancers and are also considered highly promising biomarkers. Here, we briefly describe the current knowledge about microRNAs' involvement in HCV-related molecular oncogenesis. We decided to focus our attention on studies fully conducted on *ex vivo* samples with this specific etiology, and on cultured cell lines partially or completely expressing the HCV genome. Some of the results reported in this review are controversial, possibly because of methodological issues, differences in sampling size and features, and ethnicity of patients. What is certain is that miRNAs play a remarkable role in regulating gene expression during oncogenetic processes and in viral infection. A clear understanding of their effects is fundamental to elucidating the mechanisms underlying virus-induced malignancies.

Key words: MicroRNA; Hepatitis C virus; Hepatocellular carcinoma; Lymphoma

Core tip: Not only is chronic hepatitis C virus (HCV) infection a major public health problem, but also it can cause hepatocellular carcinoma (HCC) and, more rarely, non-Hodgkin's lymphoma. The mechanisms by which the virus induces these malignancies are remain unclear, however, it has become evident recently that small molecules regulating gene expression, the microRNAs, could be involved in these processes.

The aim of this review was to establish order in the rich literature concerning microRNAs in HCC and lymphomas, by selecting only the results relating to HCV-induced cancers from the multiple-etiology analyses.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem, with 170 million carriers worldwide and more than 300000 deaths each year attributable to this virus[1]. Hepatocellular carcinoma (HCC) is a major malignancy worldwide, being the second most frequent cause of cancer death in men and the sixth in women, and hepatocarcinogenesis is closely associated with HCV infection[2]. HCV is also involved in the pathogenesis of non-Hodgkin's lymphoma (NHL)[3-5], although less frequently compared with HCC. These characteristics mean that HCV is the only virus infecting humans that is able to cause two different cancers. The exact processes by which HCV induces both HCC and NHL are a challenging topic for scientists, and although some progress has been made recently, many steps in these complex mechanisms remain unknown.

In the last 10 years, the potential involvement of microRNA in the molecular pathogenesis of several diseases, including cancers, has been recognized. MicroRNAs (miRNAs) are endogenous 21-23 nucleotides, non-coding RNA molecules, regulating post-transcriptional gene expression by translational repression or mRNA cleavage[6]. MiRNAs can act both as oncogenes (oncomirs) or as tumor suppressors because their target genes can induce neoplastic transformation[7].

Our understanding of the role of miRNAs in molecular oncogenesis is expanding day by day; therefore, we will review the most interesting miRNAs involved in the pathogenesis of HCV-related malignancies, and highlight their role as biomarkers or their potential as therapeutic targets.

MICRORNAS IN HCV-RELATED HCC

In vitro studies

Several studies on the relationship between HCV biology and host non-coding RNAs have demonstrated that HCV itself takes advantage of some miRNAs for its replication. The role of miR-122 in supporting HCV replication has already led to the development of an experimental anti-HCV drug (Miravirsen) targeting this miRNA[8-12].

At same time, the virus is responsible for the modulation of other cellular miRNAs. In fact, some authors have studied the effect of HCV proteins, mainly the core protein, on cellular miRNA regulation. The HCV core protein seems able to suppress the activity of Dicer, the enzyme that plays a key role in the production of mature miRNAs[13].

Some research conducted *in vitro* was designed to better understand the mechanisms by which HCV modulates the expression of miRNAs and promotes evolution towards malignancy.

MiR-198 is downregulated in HCC. Decreased expression of miR-198 is linked to the progression of hepatocarcinogenesis and to the degree of liver injury[14]. An even more recent study conducted *in vitro* showed that miR-198 overexpression in hepatoma cells leads to a marked inhibition of cell growth and migration[15]. These results confirmed the crucial role of miR-198 in hepatocellular carcinogenesis, suggesting that it might act as a potent tumor suppressor by inhibiting cell proliferation and migration (Table 1).

Using miRNA array analysis, Ishida *et al*[16] demonstrated that the expressions of miR-192/miR-215, miR-194, miR-320 and miR-491 were altered by HCV in human hepatoma cell lines. Of these, miR-192/miR-215 and miR-491 were able to enhance the replication of HCV replicon, as well as HCV itself.

A previous microarray analysis identified 108 human miRNAs and 1247 mRNAs whose expression levels changed by more than 2.0-fold in response to HCV infection in a Huh7 cell line. These data indicated that combined miRNA and mRNA profiling might have superior potential as diagnostic and mechanistic features in hepatocarcinogenesis[17].

Salvi *et al*[18] identified differential expression of miRNAs in cirrhotic and non-cirrhotic HCCs. By generating a small RNA library in the human HCC cell line HA22T/VGH, the authors determined the expression levels of the most frequently cloned miRNAs in HCC tissues and in their peritumoral counterparts. This research used an *in vitro* approach to obtain interesting hints for the next part of the study conducted on HCC tissue samples. As better explained in the following section of this review about tissue sample studies, the authors observed miR-24 and miR-27a downregulation in HCCs from cirrhotic liver tissues compared with non-cirrhotic liver samples and upregulation of miR-21.

Braconi *et al*[19], in an *in vitro* study with possible translational impact on clinical practice, hypothesized that HCV viral proteins could modify therapeutic responses to HCC by altering host cell miRNA expression. Using HepG2 cells stably transfected with the full-length HCV genome, these authors demonstrated a five-fold overexpression of miR-193b in these cells. One of the predicted targets of miR-193b is Mcl-1, an antiapoptotic protein able to modulate the response to Sorafenib; therefore, the authors speculated that cells expressing

Table 1 MicroRNAs with an attributed biological significance in hepatitis C virus-related hepatocellular carcinoma development and behavior

Name		Ref.
miR-192/-215	Enhancing HCV replication	[16]
miR-193b	Altered sensitivity to chemotherapy, inhibition of apoptosis	[19]
miR-198	Cell proliferation and migration	[14,15]
miR-320	Cell proliferation	[16]
miR-491	Cell proliferation, inhibition of apoptosis	[16]
miR-24	Liver fibrosis	[17]
miR-122	Modulation of HCV RNA expression	[14]
miR-199a	Poor survival, shorter time to disease recurrence, cell proliferation	[23]

HCV proteins may better respond to this drug because of miRNA-dependent modulation of apoptosis. Therefore, manipulation of miRNA expression (*i.e.*, using miR-193b mimetics) could be a useful strategy to enhance the response to chemotherapy in HCV-related HCC.

HCC tissue sample studies

MiRNAs play a pivotal role in HCV infection and in liver carcinogenesis, but only a few studies have specifically investigated the expression pattern of miRNAs in HCV-associated HCC tissues. While many papers describe deregulation of miRNAs in HCC tissue, the etiology varies in most of them, and only some samples are HCV-positive. In this part of our review we will focus our attention only on studies entirely dedicated to HCV-related HCC.

Varnholt *et al*[14] also proposed and validated a methodological approach for the study of miRNA expression in retrospective samples. The authors examined 80 miRNAs in a large set of formalin-fixed paraffin-embedded (FFPE) archival primary liver tumors derived from 39 HCV-positive patients, most of whom with cirrhosis. Interestingly, the tissue archive included premalignant dysplastic nodules (DNs) and HCCs, some of which were from the same patient. The authors assessed the availability and quality of RNA in FFPE tissues by comparing snap-frozen mouse liver tissues and FFPE samples, demonstrating that long-term storage did not affect the integrity of the miRNAs. Based on a wide-ranging approach performed on a small group of samples, the authors found 29 dysregulated miRNAs, five of which were consistently altered in all tested samples (miR-122, miR-100, miR10a, miR-198 and miR-145). Further screening of these five miRNAs in a larger set of 52 primary tumors showed a significant upregulation of miR-122 in DNs and in HCC samples, and a highly significant underexpression of miR-198 compared with normal liver parenchyma (Table 2).

Interestingly, the dysregulated miRNAs described in this paper are located on a chromosome region linked to hepatocarcinogenesis[20,21]; however, the specific targets for most of these miRNAs are unknown.

Table 2 Highly dysregulated microRNAs identified in studies regarding hepatitis C virus-related hepatocellular carcinoma

Name		*In vitro*/patient-derived samples	Ref.
miR-24	Down	*In vitro*	[17]
mi-122	Up	Patient derived samples	[14]
miR-149	Up	*In vitro*	[17]
miR-181a	Down	*In vitro*	[17]
miR-192/-215	Up	*In vitro*	[16]
miR-193b	Up	*In vitro*	[19]
miR-194	Up	*In vitro*	[16]
miR-198	Down	*In vitro*	[14,15]
miR-198	Down	Patient derived samples	[14]
miR-199a	Down	Patients derived samples	[23]
miR-210	Down	*In vitro*	[17]
miR-221	Down	*In vitro*	[17]
miR-320	Down	*In vitro*	[16]
miR-373*	Up	*In vitro*	[17]
miR-455-3p	Down	*In vitro*	[17]
miR-491	Down	*In vitro*	[16]
miR-638	Up	*In vitro*	[17]
miR-664	Down	*In vitro*	[17]
miR-887	Up	*In vitro*	[17]
miR-923	Down	*In vitro*	[17]
miR-940	Up	*In vitro*	[17]
miR-1181	Up	*In vitro*	[17]
miR-1234	Up	*In vitro*	[17]
miR-1469	Up	*In vitro*	[17]
Urinary			
miR-516-5p	Down	Patient derived samples	[22]
miR-532	Up	Patient derived samples	[22]
miR-618	Up	Patient derived samples	[22]
miR-625	Up	Patient derived samples	[22]
miR-650	Down	Patient derived samples	[22]

Using miRNA whole-genome expression profiling to identify the urinary miRNA signature specific for HCV-associated HCC, Abdalla *et al*[22] analyzed 32 HCV-associated HCC and 74 HCV-positive patients, comparing them to 12 healthy individuals. The authors focused their analysis on five miRNAs that were dysregulated and whose putative targets could play a role in hepatic carcinogenesis. Interestingly, for miR-618 and miR-650: the sensitivity and specificity of these miRNAs in detecting HCV-associated HCCs were described as having a higher predictive value than the α-feto protein levels.

A wide-ranging analysis was also performed by Diaz *et al*[23] using more than 2000 miRNAs and pre-miRNAs on nine tumor samples and surrounding cirrhotic tissue. The results showed a specific association between 18 miRNAs and HCV-related liver cancer; these miRNAs were involved in the regulation of the p53 tumor suppressor, PTEN phosphatase and retinoic acid signaling, key factors implicated in cell growth that were previously associated with HCC[24,25]. Of note is the issue of miRNA 199a/b-3p, an miRNA highly expressed in normal liver and already found to be downregulated in HCCs of different etiology (HBV chronic infection and alcoholism). The authors confirmed a decrease in the expression levels of this miRNA in HCV-related HCC, suggesting that it has

a role in inducing hepatic malignancy common to different causes.

As previously mentioned in the section regarding *in vitro* studies, Salvi *et al*[18], starting from the screening of a library obtained in a cell line, focused their analysis of HCC tissue samples on selected miRNAs. MiR-24, miR-27a and miR-21 appeared to be the most frequently cloned miRNAs and their expression levels in human HCC tissues were dysregulated. In particular, the authors observed that miR-24 and miR-27a were significantly downregulated in HCCs from cirrhotic liver tissues compared with non-cirrhotic liver samples, while miR-21 was upregulated in HCC tissues compared with the corresponding peritumoral controls. The results described by Salvi *et al*[18] concerning miR-27a are controversial, because other authors have reported upregulation of the same miRNA in hepatocellular carcinoma cells[26].

On this topic, Murakami *et al*[27] analyzed miRNA expression in 24 HCC samples and 22 adjacent non-tumor (NT) tissue samples, most of which were anti-HCV positive. They identified seven mature miRNAs (miR-18, miR-125a, miR-195, miR-199a, miR-199a*, miR-200a, miR-224) and one precursor miRNA (precursor miR-18) that were significantly and differentially expressed in the HCC and corresponding NT specimens. Furthermore, they used this result to build an algorithm that could predict the classification of samples into cancer and non-cancer groups. With the exception of one tumor sample, miRNA profiling allowed for accurate prediction of these groups, with an overall cross-validation accuracy of 97.8%. In addition, they compared miRNA expression in tumors in various differentiation states (well, moderately and poorly differentiated HCC) and found that the degree of tumor differentiation was inversely related to the expression levels of miR-92, miR-20, miR-18, suggesting that these miRNAs contribute to both tumorigenesis and the loss of tumor differentiation[27].

Regarding disease progression to hepatocellular carcinoma, a study by Ura *et al*[28] identified two categories of miRNAs, using a highly sensitive and quantitative RT-PCR method for miRNAs. The first category is associated with HBV or HCV infection; for example, miR-133b was repressed in the HCV group compared with the HBV group and also some hematopoietic-specific miRNAs, such as miR-142-5p, were upregulated in the HCV group. The other miRNAs category comprises those associated with the stages of liver disease and are not virus-related (HBV vs. HCV). The authors found 23 miRNAs that could clearly distinguish chronic hepatitis from HCC and that might be good candidates for molecular targeting to prevent the occurrence of HCC, regulating a common signaling pathway underlying HCC-HBV and HCC-HCV development[28].

A very accurate work of Shirasaki *et al*[29] provided some hints at better clarifying the role of mirR-27a in HCV biology and HCC promotion. With the aim of investigating the role of lipid metabolism in HCV replication and infectivity, the authors established a Huh7.5 cell line transfected with mirR-27a and observed that its target genes are implicated in lipid biosynthesis and transport. Also, overexpression of miR-27a led to a decrease in viral infectivity and to an enhanced *in vitro* response to interferon (IFN). Therefore, the authors suggested that this negative feedback mechanism might contribute to maintenance of a low viral load and to escape from host immune surveillance, resulting in establishment of persistent chronic HCV infection, which is crucial for the development of HCC.

MICRORNAS IN HCV-RELATED LYMPHOMAS

There are few studies available about the involvement of miRNAs in the pathogenesis of HCV-related lymphoma. Conversely, there are several reports regarding deregulation of specific miRNAs in virus-negative lymphomas, including some histotypes typically found to be associated with HCV infection [marginal zone lymphoma (MZL) and diffuse large B cell lymphoma (DLBCL)][30,31].

The first study introducing this issue was a large-scale miRNA expression profiling analysis by Peveling-Oberhag *et al*[32], who compared the expression of 381 miRNAs in microdissected splenic marginal zone lymphoma (SMZL) (HCV-positive and -negative) and in normal splenic tissues. From this analysis, 12 miRNAs were identified as dysregulated in SMZLs compared with normal splenic controls. The majority of these miRNAs were already indicated as molecular players in cancers and lymphomas; however, one of these 12 altered miRNAs, *i.e*., miR-26b, was significantly downregulated in HCV-positive lymphomas compared with HCV-negative SMZLs. MiR-26b is has tumor-suppressive activity and its expression was found to be decreased only in HCV-related SMZLs; therefore, the authors speculated that a specific virus-related mechanism was involved in this particular miRNA.

These data were supported by a study performed in different categories of HCV subjects by Fognani *et al*[33] and Gragnani *et al*[34]. MiR-26b downregulation was reported in peripheral blood mononuclear cells (PBMCs) from HCV-related lymphoma patients and in PBMCs isolated from HCV-positive subjects with mixed cryoglobulinemia, a clinically benign condition that predisposes to the development of a frank malignancy[3].

The same authors also described a significant increase in the expressions of miR-21, miR-16 and miR-155 in PBMCs from only HCV-related NHL patients[33]. These three miRNAs have been previously termed "oncomiRNAs" because their overexpression has been associated with several cancers[32,35-37].

Taken together, these results indicated that miR-26b

is a key factor in HCV-related lymphomagenesis, and suggested that it might be useful as a peripheral biomarker. Recently, a study by Bruni *et al*[38] failed to report miR-26b dysregulation in paraffin-embedded samples derived from five HCV-positive NHL patients (as well as five HBV-positive and five HCV/HBV-negative patients). The very limited number of cases could be the reason for these conflicting results. In their sampling, the authors observed a decreased level of miR-92a exclusively in HBV/HCV-negative NHL patients, while miR-30b was significantly increased only in HCV-positive samples. The analysis of a small subset of nodal marginal zone lymphomas highlighted three miRNAs that also seemed to be associated with HCV infection (miR-223, miR-29a and miR-29b). The authors themselves declared that caution is needed about the interpretation of such data, because of the limited number of analyzed samples.

Another recently published study reported that, six and 14 out of 542 mature miRNAs, were over-regulated and under-regulated, respectively, in HCV-associated diffuse large B-cell lymphoma (DLBCL) compared with non-infected ones[39]. From the analysis of HCV-positive DLBCL, compared with non-infected DLBCL samples, a significant correlation was found between high miR-576-5p and miR-129-3p levels, low miR-522 and miR-512-3p levels, and germinal center B cell-like DLBCL immunophenotype. Moreover, miR-25-5p was significantly correlated with poor prognosis and low miR-542-3p, miR-651-5p, miR-549a and high miR-369-5p correlated with advanced stage.

In summary, all the reported results suggest that differences in miRNA expression exist between HCV-related and HCV-negative NHLs, and further studies will be useful to ascertain if miRNAs are involved in the pathogenesis of HCV-related lymphomas and whether they can be used as biomarkers.

CONCLUSION

This brief review suggested that although miRNA profiling in HCV-related malignancies is an issue of high impact, there is little available research that is completely dedicated to this specific viral etiology. As is often common in miRNA expression studies, the different experimental approaches, limited number of tumor samples, the RNA used for normalization, the ethnicity of patients and, no less important, the kind of tissue used for comparison (normal liver or adjacent non tumor parenchyma) can lead to different and, occasionally, controversial results.

From a technical point of view, different biases could affect miRNA quantification in biological samples, ranging from extraction problems[40,41] to incomplete hybridization during quantitative real time polymerase chain reaction or sequencing for miRNA quantitative profiling[42,43]. Although some high efficiency methods have been proposed recently[44,45], the application of circulating miRNAs in clinical practice still requires highly reproducible techniques for isolation and quantification of biological samples. For this reason, the interesting data reported in this review could represent a starting point for new studies aimed at the thorough investigation of targets and mechanisms of the suggested miRNAs, as well as for the identification of new epigenetic regulators.

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