

Are MicroRNAs Reliable Prognostic Biomarkers in Liver Hepatocellular Carcinoma Development?

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Abstract

Hepatocellular carcinoma (HCC) is a complex multi-step process which involves genetic and epigenetic alterations. This disease is often not detected until the later stages of development. Therefore, it is essential to discover and validate new sensitive, non-invasive, diagnostic and prognostic biomarkers which can highlight changes in disease status and liver function. Deregulation of microRNAs activity plays an important role in the pathogenesis of chronic Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) infections, Non-Alcoholic Fatty Liver Disease (NAFLD) and in the progression to HCC. The assessment of serum microRNAs profiles may give insight related to the chronic viral hepatitis molecular mechanisms which lead to HCC. MicroRNAs are involved in the control of the metabolic pathways in infected cells and also the immune-metabolic response to viral infection in liver. Also, microRNAs are crucial regulators of metabolic-related disorders, such as non-alcoholic fatty liver disease. At the moment, in chronic viral hepatitis B, C and NAFLD the reliable diagnosis is liver biopsy with its disadvantages. Therefore, microRNAs detection may improve early identification of individuals with high risk for HCC development. Identification of microRNAs aberrant expression and their oncogenic or tumor suppressor molecular targets can serve as potential biomarkers for the clinical development of new therapies for HCC.

Keywords: MicroRNA; HBV; HCV; HCC; NAFLD; Biomarkers; Viral replication

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid malignant tumours of the liver with an incidence in Romania between 10-20 cases per 100,000 inhabitants per year. HCC is more likely to develop in men than in women [1]. Chronic Hepatitis B virus (HBV) and Hepatitis C Virus (HCV) infected patients have an increased risk for development of cirrhosis and hepatocellular carcinoma. Others major risk factors for HCC include chronic alcohol consumption, Non-Alcoholic Fatty Liver Disease (NAFLD) and dietary aflatoxin exposure [2]. In 2013, the prevalence of HBV in Romania was 7.9% and for HCV was 5.6% [3]. The prevalence of NAFLD was also high, about 20% [4]. The development of tumors, including HCC, is a complex multi-step process which involves the deregulation of multiple intracellular and extracellular signalling pathways [5]. In HCC development and progression have been found genetic and epigenetic alterations of the Retinoblastoma (*RB*), *p53*, rat sarcoma virus oncogene (*RAS*), wingless-type (*WNT*) and transforming growth factor (TGF)- β pathways [5].

In our experience, many patients do not respond to current therapy against hepatitis viruses. Therefore, there is a need for identification and validation of new, specific minimally-invasive biomarkers for early detection of changes in the liver tissue. In this context direct role of microRNAs in controlling cellular pathways is relevant in liver tumorigenesis. The assessment of circulating microRNAs expression in

patient serum could evaluate disease severity and the likelihood of disease progression. Cell-specific microRNAs expressions which are involved in chronic liver pathogenesis and their correlation with chronic HBV and HCV infections are an emerging area of research. Many studies have shown the role of microRNAs alterations in cancer biology, inclusive in HCC, facilitating tumor growth, invasiveness, angiogenesis and avoiding the immune response by controlling targeted messenger RNAs expression which is involved in these processes [6-8]. MicroRNAs act as molecules which trigger a receptor-mediated response. These can be released in extracellular environment in exosomes from biological fluids, acting in a similar way to "hormones" [9]. Discovery of circulating microRNAs offer new tools for non-invasive exploration in different pathologies and microRNAs have the essential characteristics of reliable biomarkers: stability, resistance to RNase digestion, preserved among species [10]. MicroRNAs from the liver may enter in serum passively through apoptosis and necrosis or actively through secretion of exosomes and viral particles [11]. According to recent research, microRNAs have increased the potential for clinical use. Identification of microRNAs aberrant expression and their oncogenic or tumor suppressor molecular targets is necessary for the identification of potential biomarkers and for clinical development of new therapies [12]. The ability of microRNAs to regulate key cellular processes and numerous metabolic pathways by simultaneously directing many targets shows their therapeutic potential [13]. The liver is the main organ responsible for metabolism, detoxification and drug metabolism [14]. Liver injury or inflammation could be reflected by microRNAs expression profiles [15]. The role of miRNAs in chronic HBV and HCV infection has been

reported in many studies adding other dimensions for understanding virus pathogenesis [16-23].

Both viruses (HBV and HCV) cause a systematic metabolic alteration in hepatocyte by affecting different cellular factors and pathways, in order to establish a more permissive microenvironment for viral replication and pathogenic effects [7]. MicroRNAs are involved in controlling the metabolic state of infected cells and also in the immune-metabolic response to liver viral infection [24]. MicroRNAs are also implicated in the regulation of numerous virus-host interactions affecting hepato-carcinogenesis [23]. Replication and propagation of hepatitis viruses could be regulated by host microRNAs which target viral genomes or cellular factors [25]. Some miRNAs facilitate viral replication, while others may even serve as potential anti-viral agents [8].

Hepatitis C virus and microRNAs

HCV is a hepatotropic virus with positive-sense single-stranded RNA genome, belonging to the flaviviridae family [21,25]. HCV is the only human virus that is able to cause two different cancers: HCC and, rarely, Non-Hodgkin's Lymphoma (NHL) [26]. HCV viral proteins interact with host cellular factors and regulate various signalling pathways to facilitate virus-mediated persistent infection [27]. Current studies showed that virus replication and pathogenesis during HCV infection are modulated by several miRNAs which are key players in virus-host interactions. Also, HCV modulates the expression of miRNAs in order to regulate critical gene networks [27]. The interaction between miRNAs and RNA viruses leads to increased stability of the viral RNA [28]. MicroRNAs activity is one of the main regulatory pathways involved in hepatic lipid and glucose metabolism which is closely linked to hepatitis virus replication [15]. Singaravelu et al. (2015) demonstrated that miR-185 and miR-130b are antiviral hepatocellular factors that regulate immuno-metabolism in infected hepatocytes. HCV-mediated inhibition of these microRNAs expressions has significant effects in liver lipid metabolism. A cellular lipidic environment is crucial to HCV life cycle. HCV-induced lipid accumulation in hepatocytes causes down regulation of miR-185 and miR-130b expression indicating that the virus actively counteracts the host defense [24]. Qisheng Li et al. (2017) have investigated the functions and underlying mechanisms of miR-25, let-7, and miR-130 miRNAs families in modulating chronic HCV infection. The authors proved that these miRNAs could repress multiple essential cellular co-factors of HCV, thus interfering with various concurrent signal pathways that are essential for HCV life cycle. The expression of these HCV-restricting miRNAs was significantly down regulated revealing the roles of these RNA molecules in mediating HCV infection and HCV-related pathogenesis [25].

Let 7 family microRNA suppresses the expression of *RAS* gene. The Ras proteins play a critical role in the development of many types of common human cancers [29]. Studies conducted by Bruni et al. (2011) in cell lines infected with HCV clones shown that miR-128a, miR-196a and miR-142-3p may be differentially regulated by HCV to evade immunity and could be mediators of antiviral response to β -interferon [30]. MicroRNA-122 is one of the first microRNAs to be identified as a "tissue-specific", representing 70% of all hepatic microRNAs. MicroRNA-122 is involved in a complex signalling network in the liver biological processes of development and differentiation, in hepatic lipid metabolism, stress response, and HCC [31,32]. MiR-122 expression is closely correlated with Liver-Enriched Transcription Factors (LEFTs) including Hepatocyte Nuclear Factor (HNF) 6 and 4a

that modulating miR-122 dosage during liver development [33]. Many studies have demonstrated that the most abundant liver-specific miR-122 is associated with lipid and cholesterol metabolism during HCV infection and can increase the abundance of HCV RNA by direct binding to the 5' UTR of the viral genome [20,34,35]. The binding of miR-122 to HCV genome enhances the association of ribosome with viral RNA and has a stimulatory effect on viral translation. The interaction of the complex miR-122-HCV genome with argonaute2 stabilizes and protects viral RNA from degradation by exonucleases [33]. Also, miR-122-HCV RNA complexes might protect the 5' end of the HCV genome from recognition by innate immunity-inducing pattern recognition receptors, such as RIG-I (DDX58) and IFITs (Interferon-induced protein with tetratricopeptide repeats) [36,37].

MicroRNA-122 is up regulated in chronic HCV-infected serum and may serve as a biomarker for early liver inflammation responses in HCV patients [38]. MicroRNA-122 has several target messenger RNAs that encode proteins involved in the development of HCC; such proteins include Prolyl 4-Hydroxylase Subunit α 1 (P4HA1), pyruvate kinase PKM and Mannan-binding lectin Serine Protease 1 (MASP1) [39].

Currently, antiviral therapy for chronic HCV infection is based on Direct-Acting Antivirals (DAAs) that target specific viral proteins of the HCV replication cycle [40]. The selection of DAA-resistance mutants and the dissimilar antiviral activities of DAA against different HCV genotypes have shown the need to find a drug with a novel mechanism of action applicable to all HCV genotypes [37]. Involvement of miR-122 in HCV replication has led to the development of an experimental anti-HCV drug (Miravirsen) targeting this microRNA [26]. The HCV antiviral drug miravirsen is a β -D-oxy-locked nucleic acid-modified phosphorothioate antisense oligonucleotide which hybridizes with mature miR-122 and blocking its interaction with HCV RNA [41, 42]. Also, miravirsen binds to the stem-loop structures of pre-miR-122 and pri-miR-122 and suppress miR-122 biogenesis by inhibition of Dicer- and Drosha-mediated processing of miR-122 precursor [41]. The miR-122 binding sites are conserved on all HCV genotypes and subtypes [43]. In vitro studies shown that miravirsen has antiviral activity against all HCV genotypes [42]. This is the first miRNA-targeting antisense agent administered to patients by subcutaneous or intravenous delivery in saline solution [42]. Miravirsen is taken up by hepatocytes and forms highly stable heteroduplexes with miR-122, resulting in depletion of free miR-122 and represses of HCV viral RNA replication [37].

Tumorigenesis and the loss of tumor differentiation in HCC were associated with higher expression levels of miR-92, miR-20, miR-18 and precursor of miR-18 [18].

Hepatitis B virus and microRNAs

HBV is a non-cytopathic enveloped hepadnavirus with a circular, partially double-stranded DNA genome [8]. Chronic hepatitis B infection has many steps: the immune-tolerant phase, the immune-reactive Hepatitis B e Antigen (HBeAg)-positive phase, the inactive HBV carrier state, and HBeAg-negative chronic hepatitis [22]. Host cellular microRNAs are able to promote or repress the HBV lifecycle, either by direct interaction with HBV transcripts or by indirect targeting cellular mediators, involved in the HBV pathogenesis [19]. HBV infection dysregulates cellular miRNAs controlling in this way the host genes expression to promote its replication [21]. HBV encodes HBV-miR-3 which controls the process of self-replication by inhibiting the synthesis of viral proteins and HBV replication [44]. It has been

shown that almost all HBV viral proteins modulate different host miRNAs (let7-family, miR-29a, miR-101, miR-145, miR-21, miR-222) which are involved in the modulation of host hepatocarcinogenesis and immune response [8]. Also, HBV replication is modulated by its own proteins [44]. HBx plays an important role in HBV-related HCC by regulation of apoptosis mediated by *p53*, *FAS* and TGF- β [45]. Single-Nucleotide Polymorphisms (SNPs) in miRNA encoding genes could be associated with the susceptibility to persistent HBV and, consequently, HCC development [8]. It was shown that specific miRNAs are involved in several aspects of HBV biology. Zhang et al. (2015) have identified that miR-216b and miR-188-5p expressions were significantly lower in plasma of patients with HBV related-HCC, compared with the healthy controls, while miR-324-3p, miR-484 and miR-454 have increased expressions. These differentially expressed miRNAs in the plasma of patients with HCC could suggest molecular transformation within the tumorigenesis processes such as abnormal proliferation, invasion and Epithelial-Mesenchymal Transition (EMT) [46]. Price et al. (2017) have identified that in HBeAg-positive patients plasma levels of miR-122-5p, miR-125b-5p, miR-192-5p, miR-193b-3p, and miR-194-5p are higher compared with HBeAg-negative patients. Thus, the association of these microRNAs with HBV DNA and HBsAg levels are correlated with viral replication [22]. It has been reported that liver tissue miR-15a can downregulate Smad7 mRNA and enhance the Transforming Growth Factor β 1 (TGF- β 1) signalling pathway [47]. TGF- β 1 signalling is involved in cell proliferation and tumorigenesis [48]. HBV mRNA acts as an endogenous competitor for down regulating of miR-15a leading to up regulate Smad7 expression. HBV viral strategy to exploit its compact genome for promoting tumorigenesis is involved in the development of HBV-related HCC [47]. HBV has been found to down regulate other miRNAs in order to control various signalling pathways. Increases in circulating miR-122 levels in plasma might be caused by HBV-induced up regulation of miR-122 expression [22]. In the study conducted by Akamatsu et al., (2015) miR-122 was independently related with HBV DNA level, whereas miR-125b was independently associated with levels of HBV DNA, HBsAg and HBeAg. MiR-22 and miR-1275 were independently associated with serum g-glutamyl transpeptidase levels [16]. Wang et al. (2017) found that miR-150, miR-342-3p, miR-663, miR-20b, miR-92a-3p, miR-376c-3p, and miR-92b are specifically altered in HBV-related HCC. These microRNAs regulate various genes that encode for cell cycle and apoptosis regulators [45]. Singh et al. (2018) have analyzed differential expressions of 17 microRNAs in liver biopsy samples from different stages of HBV infection and liver disease: immune-tolerant phase, acute viral hepatitis, no fibrosis, early or late fibrosis, and healthy controls. They showed that in the immune-tolerant group elevated levels of miR-199a-5p, miR-221-3p and let-7a-5p contribute at suppression of innate immune response allowing HBV replication and viral persistence. In the acute viral hepatitis patients, miR-125b-5p and miR-3613-3p were up-regulated, whereas miR-940 was down-regulated, which might affect cell proliferation through the signal transducer and activator of transcription 3 pathway. In early fibrosis, miR-34b-3p, miR-1224-3p, and miR-1227-3p were up-regulated, while miR-499a-5p was down-regulated. They possibly mediate chronic inflammation. In advanced fibrosis, miR-1, miR-10b-5p, miR-96-5p, miR-133b, and miR-671-5p were up-regulated, while miR-20b-5p and miR-455-3p were down-regulated, possibly allowing chronic disease progression [49]. Qiu et al. (2017) demonstrated a positive correlation between let-7a miRNA transcription and HBV replication at patients with HCC, as well as in HCC cell lines. Also, they suggest that let-7a miRNA inhibition could

be used as an antiviral therapy to inhibit HBV replication and to prevent the development of HCC [50].

NAFLD and microRNAs

NAFLD is a multifactorial disease that involves a variety of liver injury which may end up as HCC. This disease is defined as evidence of hepatic steatosis and is closely associated with metabolic syndrome, and cardiovascular diseases [14,51,52]. Progression of NAFLD to fibrosis is a result of multiple factors interaction, such as insulin resistance, lipotoxicity, dietary, fatty acids, lipopolysaccharide generated by gut microbiota, inflammation, oxidative stress, genetic and epigenetic [51]. Liver biopsy still represents the only reliable method to establish non-alcoholic fatty liver disease severity and HCC [53]. Ideal biomarkers for follow-up the evolution of NAFLD to HCC should be minimally invasive, accurate, specific and sensitive. They need to be clinically validated. The discovery of the important role of miRNAs in glucose and lipid metabolisms regulation reveals a connection between miRNAs and metabolic liver disorders. NAFLD is strongly associated with metabolic syndrome [54]. Several microRNAs have been extensively investigated and accepted to be potent regulators in the pathogenesis of NAFLD. Zhu et al. (2017) showed that the expression levels of miR-33, miR-33-5p and miR-33-3p were significantly decreased in high-fat-diet-induced NAFLD. These microRNAs interact with apelin (*apln*) and versican (*vcn*) genes which have been reported to be associated with NAFLD [51]. Also in this study, the expression levels of miR-200b-3p, miR-200b-5p and miR-200c-3p were higher in the liver of NAFLD rats. These microRNAs and their target genes involved in cholesterol biosynthetic processes might be crucial regulators in the pathogenesis of NAFLD [51]. MicroRNA-34a has a crucial role in the regulation of liver inflammation and it is associated with significantly increased severity in NAFLD. Up-regulation of miR-34a in liver steatosis is an important part of a protective negative regulatory feedback mechanism intended to limit disease progression by preventing excessive lipid accumulation in the liver [55]. MicroRNA-34a could be used as a biomarker for disease severity in patients with NAFLD [52]. Serum miR-122 and miR-192 have a dual behavior in NAFLD: increases in mild fibrosis and decreases in the most severe stages [53]. Feng et al. (2014) showed inflammatory factors are crucial mediators of the aberrant expression of hepatic miRNAs associated with NAFLD. Thus, they found that miR-200a, miR-200b, miR-200c, miR-146a, miR-146b and miR-152 were up-regulated both in fatty liver tissues and in cultured cells with FFAs and proinflammatory factors [56]. Nie et al. (2018) showed that the miR-182, miR-29b-3p and miR-741-3p are up-regulated in rats with NAFLD suggesting that these differentially expressed microRNAs may be involved in the pathogenesis of NAFLD [14].

Conclusion

Promising research centered on miRNAs expressions in chronic liver diseases progress emphasizes the possibility of miRNAs becoming valuable biomarkers and useful as predictors for liver injury prognosis and transition to HCC. Correlation of microRNAs pattern expression with clinical and pathological parameters indicates that microRNAs could become a useful molecular biomarker for hepatocarcinogenesis with different etiologies. The assessment of aberrantly expressed microRNA in chronic liver diseases could reveal new mechanisms of tumorigenesis. Virus encoded miRNA (HBV) may provide new potential biomarkers for chronic HBV infection suggesting a new insight of HBV replication mechanisms and regulation. Serum

microRNA levels can reflect differences in the etiology and stage of chronic viral hepatitis. The complex miRNA-mRNA regulatory systems will advance our understanding of viral (HBV, HCV) -host interactions and disease mechanisms for developing innovative, pre-emptive diagnosis approaches.

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