Chapter 9
MicroRNAs as Novel Targets in Liver Cancer: Facing the Clinical Challenge

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1 Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality accounting for more than 600,000 yearly deaths worldwide [1]. The major etiologic factors underlying chronic liver disease, cirrhosis and, ultimately HCC are well characterized. Among those, chronic viral hepatitis (e.g., hepatitis B (HBV) and C viruses (HCV)) as well as aflatoxin B exposure and ethanol abuse are the most common causes of hepatocarcinogenesis worldwide [2]. Other predisposing factors include nonalcoholic fatty liver disease (NAFLD) and metabolic as well as hereditary disorders. Highest incidences are traditionally seen in third-world regions such as Southeast Asia and sub-Sahara Africa. However, over the last decades generalized vaccination programs for HBV and improved preservation of food led to a reduction in two major etiological factors (i.e. HBV and aflatoxin B) resulting in stabilization of prevalence and declining incidences in these regions. However, due to a steady increase in HCV infections as well as obesity the incidence and, concomitantly, also mortality rates of HCC have almost doubled in the United States and Europe over the past four decades and are predicted to continue rising. Although several confounding factors (e.g. immigration from high incidence countries) contribute to these high numbers in the western world, HCC ranks among the fastest growing causes of cancer related deaths in the USA. Curative therapeutic options include resection or ablation of small HCCs and/or liver transplantation. However, these approaches are frequently limited by the underlying liver disease and at the time of diagnosis less than 20 % of the patients are eligible for curative treatment strategies [3]. These observations clearly indicate that liver cancer has become a major health problem in western countries and underline the importance for a better understanding of the pathophysiology to

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improve the outcome of this deadly disease. Major progress in unraveling the molecular mechanisms in liver diseases associated with increased risk of HCC as well as several cellular alterations that precede HCC have been made over the last 10 years [4, 5]. A major focus of translational research is the inter-relationship of abnormal genomics, epigenomics, proteomics and downstream alterations in molecular signaling pathways. The overall aim of these efforts is to integrate the generated data with clinicopathological features of HCC in order to uncover new diagnostic classes, improve treatment options, and implement effective prevention strategies [6].

Integrity of the epigenome is a key component of organ homeostasis. Growing evidence suggests that disruption of epigenetic regulation is one of the fundamental mechanisms underlying many human diseases including cancer [7, 8]. This epigenetic landscape of alterations adds further complexity to the pathogenesis of solid tumors. Changes in the epigenome are believed to be early events in carcinogenesis preceding allelic imbalances and ultimately lead to cancer progression [9]. Not surprisingly, epigenetic alterations, in particular aberrant expression of microRNAs with subsequent dysregulation of gene expression have been linked to the pathogenesis of chronic liver diseases as well as hepatocarcinogenesis [10]. In this context, the identification of these small regulatory RNAs have greatly advanced our understanding of liver cancer development and aberrant expression of microRNAs is significantly associated with liver cancer initiation, propagation and progression [11]. Emerging evidence further indicates that certain microRNAs directly contribute to cell proliferation, apoptosis, and metastasis of HCC and correlate with several clinicopathological features [12]. Hereby, number of microRNAs have been identified to be involved in the regulation of key protein-coding genes associated with hepatocarcinogenesis such as WNT/β-Catenin, MYC and TGFβ [13, 14]. Therefore, the investigation of abnormal microRNA patterns is a rapidly emerging field of translational science in liver cancer and has great potential to transform current approaches for both diagnosis and therapy, thereby providing the foundation for predictive and preventive personalized medicine (Fig. 9.1).

In this chapter we will review current knowledge of microRNA regulation in the context of liver development, diseases and hepatocarcinogenesis as well their implications for clinical and translational efforts. We will further highlight challenges and limitations for the application of microRNA-based treatment strategies in HCC and underline the implications of next-generation technologies for improving our understanding of the role of this interesting class of molecules in hepatocarcinogenesis [15].

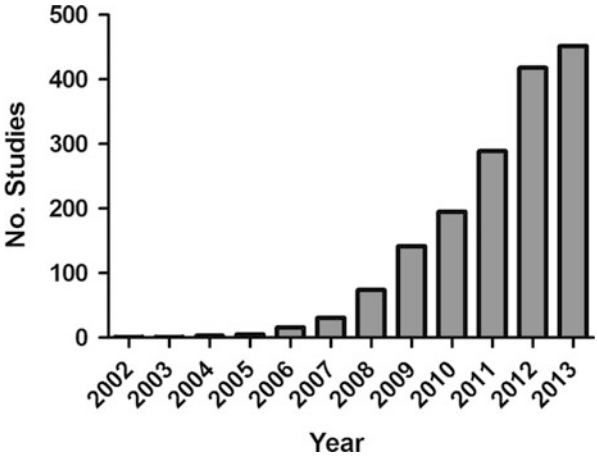

Fig. 9.1 Studies of microRNAs in liver diseases. Timeline for publications of miRNAs in liver diseases was generated using a PubMed query with the keywords "microRNA and liver". From the 1,627 studies included in the query 832 studies (51 %) focused on liver cancer

2 MicroRNAs in Liver Development, Regeneration and Disease

MicroRNAs play a diverse role for liver biology and dysregulation of microRNAs has been implicated in virtually all pathophysiological conditions in the liver [16]. During liver development microRNAs modulate a variety of physiological processes thereby significantly contributing to proper organ homeostasis. Mechanistic analyses of mice with deletion of Dicer1 showed increase hepatocyte turnover at 3 weeks after birth resulting in increased steatosis as well as depletion of glycogen storage underlining the key role of mature microRNAs for liver development. Notably, over time the phenotype was gradually rescued by expansion of Dicer1 competent hepatocytes. Regardless, two thirds of the Dicer1-deficient animals developed hepatocellular carcinomas originating from residual Dicer1 deficient hepatocytes [17]. Interestingly, one of the most striking findings of this study was the almost complete absence of miR-122 in Dicer1-deficient animals. miR-122 is a highly abundant liver specific microRNA accounting for ~70 % of all expressed microRNAs in the liver whilst undetectable in the majority of other organs [18]. Furthermore, miR-122 drives hepatocyte differentiation in a HNF6 dependent manner [19]. The prominent role of miR-122 for hepatocyte metabolism was confirmed in a study by Esau et al. using in vivo antisense knockdown. The inhibition in normal mice resulted in disruption of plasma cholesterol, increased hepatic fatty-acid oxidation, and decreased hepatic fatty-acid and cholesterol synthesis [20]. Conversely, miR-122 inhibition in a diet-induced obesity model resulted in significant attenuation of liver steatosis, as well as a downregulation of several genes involved in lipid homeostasis. A global analyses of 42 microRNAs between human fetal and adult liver tissue identified a number of differentially expressed microRNAs including miR-122, miR-148a, miR-192, miR-194, miR-451, miR-21 and let-7a with a progressive downregulation from fetal to adult livers

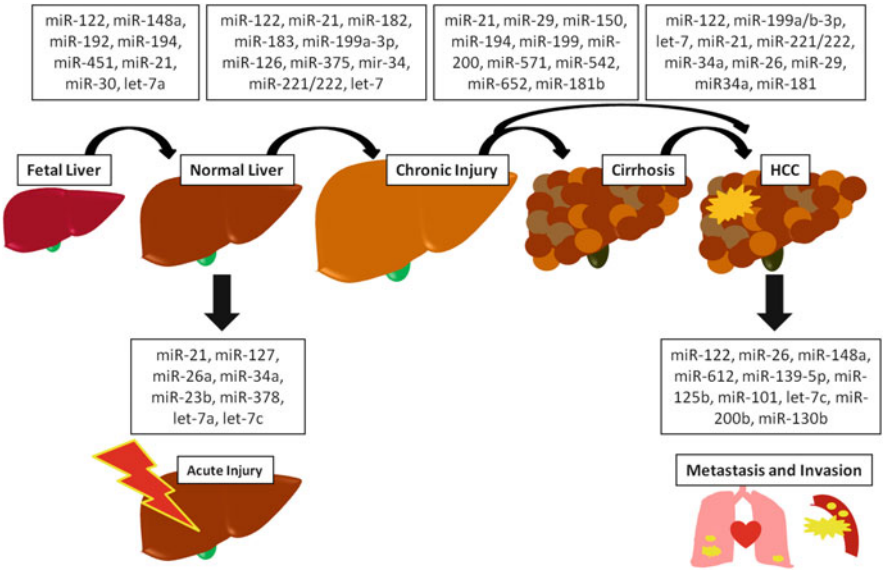

Fig. 9.2 MicroRNAs in liver development and diseases. The figure shows (up to 10) key microRNAs associated with fetal liver development, liver diseases and progression of liver cancer. A striking redundancy of several microRNAs (e.g. miR-122, miR-21, miR-26) is obvious indicating the potential of these microRNAs as targets for therapeutic and/or preventive strategies

(Fig. 9.2) [21]. Little is known about microRNAs that specifically regulate biliary differentiation. A recent study showed that two microRNAs (miR-30a and miR-30c) are critically involved in the development of ductal plate and bile ducts [22]. Consistently, knockdown of miR-30a in zebrafish resulted in defective biliary morphogenesis.

The role of microRNAs in response to acute liver injury has been addressed in several studies. Hereby, deregulation of several microRNAs (e.g. miR-21, miR-127, miR-26a, miR-34a, miR-23b, let-7a and let-7c) could be associated with proper initiation and termination of proliferative stimuli following a 2/3 partial hepatectomy (PH) in mice [23–26]. Song et al. performed a global analysis of changes in microRNA profiles in response to PH. To specifically address the role of microRNAs for hepatocyte proliferation they utilized a mouse model with hepatocyte-specific inactivation of DiGeorge syndrome critical region gene 8 (DGCR8), an essential component of the microRNA processing pathway [27]. In this model, hepatocytes were microRNA-deficient which caused a significant delay in cell cycle progression particularly in the G(1) to S phase transition. Among the most prominent differentially expressed microRNAs were mir-21 and miR-378. They further showed that miR-21 suppressed DNA synthesis via activation of FoxM1 by directly targeting Btg2, a strong inhibitor of the cell cycle. In continuation of the study, the same group more closely dissected the role of miR-21 in liver injury by using an in vivo antisense oligonucleotide in the PH model [28]. As a result of this

investigations, the authors established a regulatory feedback between mir-21 and cyclin D1 translation that caused the observed delay in G(1) to S phase transition by activating the Akt/mTOR complex (and thus eIF-4 F-mediated translation initiation) in the early phase of liver regeneration.

The majority of HCCs develop on the basis of a chronic inflammation caused by an underlying liver disease and, in more than 80 % of the cases, a pre-existing liver cirrhosis. To gain a complete understanding of the molecular mechanisms of hepatocarcinogenesis the etiology of the liver disease needs to be considered [29]. The major etiologies associated with the evolution of liver cancer are well characterized (e.g., infections with hepatitis B (HBV) and C viruses (HCV) as well as ethanol abuse and aflatoxin B exposure). Other common etiological factors include nonalcoholic fatty liver disease (NAFLD) and metabolic disorders that have become particularly relevant in Western countries due to a sharp increase in prevalence and increasing numbers of HCCs [14].

Already in 2008 a global microRNA analysis showed that HCCs associated with alcoholic liver disease (ALD) display a decrease in miR-126 as well as miR-107 and miR-375 which were specifically associated with HNF1α and β-catenin gene mutations in these patients [30]. Another study performed microRNA expression profiles in two murine models of ALD and NAFLD. In both models the development of steatohepatitis was associated with altered microRNA expression patterns (e.g. miR-705, miR-1224, miR-182, miR-183, miR-199a-3p) [31]. Notably, several of these microRNAs showed a differential regulation between ALD and NAFLD indicating that etiological differences might contribute to activation of different microRNAs. Another common microRNA associated with steatohepatitis in both ALD and NALFD is mir-34. Recently, Min et al. revealed mechanistic insights into the role of miR-34a in NAFLD. They demonstrated that mir-34 down-regulates sirtuin-1 and causes dephosphorylation of AMP kinase and HMGCR, a key regulator of cholesterol synthesis (Fig. 9.1).

Over the last years several studies demonstrated that microRNAs significantly contribute to the regulation of HCV and HBV infections [32]. Several studies addressed the role of microRNAs for HCV infections [10]. HCV infects hepatocytes and replication of the virus is exerted by a unique interaction between host miR-122 and HCV 5′UTR along with proteins of RISC (Ago2, GW182 and HSPs), which results in enhanced replication of HCV virus and mediates stability of the viral RNA [16, 33]. HCV infection leads to hepatocyte damage and subsequent release of pro-inflammatory molecules, which in turn activate immune cells that induce systemic inflammation, fibrogenesis and, ultimately, carcinogenesis. Marquez et al. investigated the role of miR-21 and miR-122 for HCV infection in pre-cirrhotic human patients and in cell culture [34]. Both microRNAs showed deregulated expression in HCV-infected liver tissues when compared to control livers without viral infections. Further, miR-21 induction was positively correlated with fibrotic stage, viral load and serum transaminases levels. Conversely, miR-122 expression inversely correlated with fibrosis and biochemical markers of hepatocyte damage. Besides miR-122 and mir-21 other host microRNAs such as miR-34a, miR-21, miR-146a and miR-125b are increased in patients infected with HCV

infection and might contribute to progression of the disease [16]. Additionally, miR-155 and miR-499 in hepatocytes, are associated with the progression from inflammation to cancer via Notch and Wnt signaling [35]. However, due to the prominent role of miR-122, this microRNA is believed to have the greatest potential as a (complementary) target for antiviral therapies and preliminary results from ongoing clinical trials are very promising [36]. miR-122 was also found to be involved in the pathogenesis of HBV infections. A significant down-regulation of mir-122 was observed in chronic HBV infected patients, inversely correlating with viral load. Hereby, miR-122 stimulated HBV replication by up-regulation of cyclin G1 thereby preventing p53-mediated repression of a HBV enhancer element [37]. Several other microRNAs associated with HBV infection promote inflammation induced hepatocarcinogenesis (Fig. 9.2) [32]. Ura et al. further demonstrated that microRNAs associated pathways related to cell death, DNA damage, recombination and signal transduction were activated in HBV-infected livers [38]. However, despite considerable commonalities in the clinical and molecular phenotype (e.g. upregulation of miR-21 and miR-221/222 as well as down-regulation of miR-122, miR-199 and miR-200) there are fundamental differences in the regulation of microRNAs between HBV and HCV during the progression from inflammation over fibrosis to HCC development (e.g. miR-145, miR-9-2, miR-138-1 and miR-2, miR-320, miR-33, miR-10a and 10b, miR-146, miR-220, let-7) [38, 39]. In the context of hepatocarcinogenesis, the let-7 family should be particularly highlighted, since the observed differences might contribute to specific HB-x related oncogenic effects in liver cancer.

Up to now, there are no studies to directly address the effect of aflatoxin B1 on microRNA expression. Due to the interaction with p53, in particular at codon 249, it is reasonable to conceive that aflatoxin could modulate p53 induced microRNAs [32].

Liver fibrosis is the common final path of chronic liver diseases regardless of the etiological differences. It is orchestrated by different resident (e.g. hepatocytes, hepatic stellate cells and Kuppfer cells) and non-resident (e.g. immune cells) cell types and develops on the basis of complex alterations in pro-fibrotic signaling pathways such as TGFβ, SMADs, and MYC (e.g. miR-21, miR-150, miR-194, miR-199, miR-200) that cause a dysbalance between the disposition of extracellular matrix proteins and matrix metalloproteinases [40]. Therefore, microRNAs are involved in regulation of liver fibrosis at multiple levels, i.e. cell type and context dependent [16]. Number of microRNAs have been identified to regulate hepatic stellate cell activation during liver fibrogenesis [41]. Recently, miR-29 was identified to induce fibrogenesis by activating TGF-β as well as NF-κB in hepatic stellate cells [42]. Further, members of the miR-29 family were significantly repressed following CCl4 induction of fibrosis in mice and in livers of patients with advanced fibrosis [43]. Importantly, several microRNAs could also be detected in sera of patients with progressed liver diseases (e.g. miR-571, miR-542, miR-652, miR-181b) suggesting that microRNAs might possess potential as biomarkers during liver fibrogenesis [44, 45].

3 MicroRNAs in Hepatocellular Carcinoma

3.1 MicroRNAs in Hepatocarcinogenesis

Hepatocarcinogenesis is considered a multi-step process that results from sequential alterations of epigenetic and genetic mechanisms leading to a disruption of three core cellular processes i.e. cell fate, cell survival, and genome maintenance, that can promote or "drive" tumorigenesis in the majority of human cancers [46]. During this sequence an activation/inhibition of at least 12 key signaling pathways and downstream molecules such as p53, WNT, β-Catenin, MYC, ErbB family as well as chromatin modifications is observed. MicroRNAs are involved in the regulation of virtually all of these processes and pathways [47]. Given the prominent role of miR-122 for liver homeostasis, several studies demonstrated its relevance also for hepatocarcinogenesis [48, 49]. Mechanistic proof for the tumor suppressive role of miR-122 has been recently revealed by two independent studies. Hsu et al.demonstrated that deletion of mouse miR-122 not only leads to hepatic steatosis and inflammation but also to HCC development. On the molecular level this phenotype was associated with hyperactivity of oncogenic pathways as well as increased infiltration of inflammatory cells that produce pro-tumorigenic cytokines, including IL-6 and TNF [50]. Furthermore, Tsai et al. revealed that loss of miR-122 resulted in phenotypic similarities to common human liver diseases and leads to the activation of several key oncogenic pathways associated with HCC, e.g., TGFβ, MAPK and PTEN [51]. Interestingly, a crucial clinical and functional relevance of miR-122 for human HCC was already established years before these two compelling mechanistic studies [52, 53]. Consistently, miR-122 was preferentially downregulated in a subset of primary tumors that display a particular poor prognosis and showed enrichment of gene sets linked to cancer progression [52]. The authors further identified that miR-122 cooperates with liver-enriched transcription factors such as HNF1A, HNF3A and HNF3B. Functionally, loss of miR-122 resulted in an increase of cell migration and invasion indicating that miR-122 is a marker of hepatocyte-specific differentiation and an important determinant in the control of cell migration and invasion. An epigenetic switch between inflammation and cancer was initiated by a feedback regulation of miR-124, IL6R, STAT3, miR-24, and miR-629 causing sustained oncogenic signaling and downregulation of HNF4α [54]. Furthermore, systemic administration of miR-124, prevented hepatocarcinogenesis by inducing tumor-specific apoptosis without overt liver toxicity indicating the therapeutic potential of this microRNA feedback-inflammatory loop in HCC. Global analyses of microRNAs by next-generation sequencing with differential expression in human normal liver, hepatitis and HCC identified nine microRNAs (miR-122, miR-99a, miR-101, miR-192, miR-199a/b-3p and several let-7 family members) accounting for ∼88.2 % of the "miRNome" in human liver [55]. Further, decreased miR-199a/b-3p expression significantly correlated with survival of HCC patients. Moreover, targeting of miR-199a/b-3p using adeno-associated virus (AAV) 8 inhibited tumor growth via interacting with PAK4/Raf/MEK/ERK pathway. Fornari et al. further

showed an inverse correlation between miR-199a-3p with mTOR and c-Met associated with a shorter time to recurrence after HCC resection [56]. Another global microRNA analysis in 104 HCC, 90 adjacent cirrhotic livers, 21 normal livers as well as in 35 HCC cell lines detected a set of 12 microRNAs (including miR-21, miR-221/222, miR-34a, miR-519a, miR-93, miR-96, and let-7c) associated with malignant progression in liver cancer. Hereby, miR-221/222 were the most upregulated microRNAs in HCC and identified to target the CDK inhibitor p27 as well as DNA damage-inducible transcript 4 (DDIT4), a modulator of mTOR pathway, to enhance cell growth in vitro [57, 58]. Another microRNA with high expression in HCC targeting the PTEN/mTOR pathway is miR-21 [59]. During hepatocarcinogenesis, miR-21 functionally confers to malignant properties such as proliferation, migration, and invasion. Other microRNAs involved in the activation/repression of key signaling pathways and oncogenic molecules in HCC such as c-MYC, c-MET and Hippo are members of the let-7 family, miR-1 as well as miR-375 [60]. Besides the above mentioned studies several other investigations revealed microRNAs with both tumor suppressive (e.g. miR-1, miR-26, miR-29, miR34a, miR-195, miR-223) and oncogenic activity (e.g. Mir-224, Mir-9 and Mir-181) [10, 60]. For some of the mentioned microRNAs (e.g. miR-221, miR-125B, miR-26, miR-122) a prognostic relevance and prediction of drug sensitivity (e.g. interferon) could be demonstrated [11, 61–63].

Besides the malignant transformation and promotion of HCC microRNAs have been implicated to promote or repress the generation of metastatic disease [13]. Not surprisingly, the prominent role of miR-122 was also confirmed in this process. Loss of miR-122 induced the generation of intrahepatic metastasis by promoting angiogenesis via regulation of ADAM17 [53]. A recent study further demonstrated that downregulation of the prognostic microRNA miR-26a also correlated with HCC recurrence and metastasis and was functionally associated with cell proliferation, migration, and invasion [64]. Xu et al. recently showed that miR-148a is repressed by the HBx protein in a p53-dependent manner thereby promoting cancer growth and metastasis through targeting hematopoietic pre-B cell leukemia transcription factor-interacting protein (HPIP) [65]. Inhibition of HPIP expression by miR-148a, reduced the levels of AKT, ERK as well as mTOR through the AKT/ERK/FOXO4/ATF5 pathway. The authors conclude that miR-148a activation or HPIP inhibition may be a useful strategy for cancer treatment. Another recently described tumor suppressive microRNA with anti-metastatic properties is miR-612 [66]. The authors showed that this function was exerted by regulation AKT2 during epithelial-mesenchymal transition (EMT) and metastasis. Consistently, miR-612 levels in HCC patients inversely correlated with tumor size, stage, EMT, and metastasis. Furthermore, miR-612 not only affected local invasion but also intravasation at distant sites indicating that the microRNA is involved in the complete sequence of the metastatic cascade. Two large scale analyses have been conducted to identify metastasis-related microRNAs in HCC [62, 67]. Budhu et al. investigated microRNA profiles in 241 HCC patients and generated a 20-microRNA signature that efficiently predicted the occurrence of venous metastases

and was associated with the patients' outcome [62]. Wong et al. compared microRNA profiles of primary HCC and venous metastasis within 20 matched patients [67]. Interestingly, although non-tumorous livers showed distinct profiles from primary HCCs as well as venous metastases, no apparent differences in the expression pattern of primary HCCs and venous metastases could be detected. However, microRNA expression levels were markedly reduced in venous metastases compared to primary HCCs suggesting that microRNA deregulation occurs early in hepatocarcinogenesis and that the generation of metastasis is aggravated by a stepwise disruption of the deregulated microRNAs.

3.2 MicroRNAs for Diagnostic and Prognostic Classification

Prognostic classification of HCC using expression profiles has a long standing history in HCC [14]. In the last years, the power of microRNA profiling for classification of liver cancers has been demonstrated. Profiling of microRNA patterns by microarray revealed subclasses associated with clinico-pathological features as well as mutations in several oncogenic pathways such as β-Catenin and HNF1A [12]. Recently, microRNA profiling of 89 HCC samples using a ligation-mediated amplification method revealed three distinct clusters of HCCs that reflected the clinical behavior of the tumors. The functional role of different identified microRNAs in particular of the miR-517 family was further investigated in cell lines and in an orthotopic mouse model of liver cancer. As a result the authors could associate these microRNAs with increased proliferation, migration, and invasion of HCC cells in vitro and in vivo, indicating the therapeutic potential of microRNA based treatment modalities [68]. Sato et al. examined the microRNA expression profiling in paired tumor and non-tumor liver tissues from 73 HCC patients and constructed prediction models of recurrence-free survival using the Cox proportional hazard model and principal component analysis [69]. As a result, the authors identified 13 and 56 recurrence-related microRNAs in the tumor and non-tumor tissues, respectively. While the number of recurrence-related microRNAs was significantly larger in the non-tumor-derived microRNAs and predicted late recurrence, the tumor-derived microRNAs were superior to predict early recurrence.

MicroRNAs are good biomarkers since they are released through vesicles (microvesicles or exosomes) into the circulation and can be detected in almost all body fluids. The diagnostic power of microRNAs has been addressed in numerous studies [70]. Li et al. performed a microRNA screen in serum of 513 patients (210 controls and 135 HBV-, 48 hepatitis C virus (HCV)-, and 120 HCC-affected individuals) by Solexa sequencing followed by validation with TaqMan probe-based quantitative reverse transcription-PCR [71]. They identified six microRNAs that were significantly upregulated in HCC samples vs control samples. Among those, two microRNAs (miR-375 and miR-92a), were also enriched in patients with HBV infections. Further, a combination of three of these microRNAs (miR-25, miR-375,

and let-7f) accurately classified HCC and control patients. Zhou et al. examined the role of microRNAs for diagnosing HBV-related HCC in plasma of 934 patients [72]. The authors identified microRNAs with high diagnostic accuracy for HCC irrespective of disease status. However, it was particularly useful for the diagnosis of early-stage HBV-related HCCs and could discriminate HCC from healthy, chronic HBV and cirrhosis. Other microRNAs associated with HCC were miR-199a, miR-222, miR-223, miR-21 as well as miR-122 [73–75]. Liu et al. further aimed to investigate if circulating microRNAs could outperform AFP for HCC detection in 96 HCCs [76]. A combined use of miR-15b and miR-130 yielded 98.2 % sensitivity and 91.5 % specificity and detection sensitivity was even higher (96.7 %) in a subgroup of HCCs with low AFP (<20 ng/mL) and identified AFP negative early-stage HCC cases. Altogether, these data provide compelling evidence for the feasibility of circulating miRNAs as biomarkers for HCC diagnosis.

3.3 MicroRNAs as Therapeutic Targets

Due to the striking aberration of several microRNAs in liver diseases as well as liver cancer, microRNAs emerged to attractive molecular targets in liver cancer and great promise rests on these microRNA-based therapeutic approaches to improve the dismal outcome of HCC patients [77, 78]. The first evidence for the efficiency of a microRNA-based treatment strategy stems from Kota et al. [79]. Already in 2009 the authors demonstrate that induction of miR-26a expression in hepatoma cells in vitro induces cell-cycle arrest associated with direct targeting of cyclins D2 and E2. Systemic administration of this microRNA in a mouse model of HCC using AAVs resulted in reduced proliferation and induction of apoptosis in tumor cells without overt cellular toxicity in normal cells, a side-effect that is commonly feared in this context. Two recent studies established the therapeutic potential of targeting miR-221 [80, 81]. Park et al. demonstrated that anti-miR-221 effectively reduced in vivo miR-221 levels and led to subsequent inhibition of tumor cell proliferation as well as increased apoptosis, ultimately leading to improved survival [81]. Callegari et al. utilized a transgenic miR-221 mouse model that exhibits spontaneous nodular liver lesions with further increase in miR-221 expression and a concomitant inhibition of its target protein-coding genes (i.e., cyclin-dependent kinase inhibitor [Cdkn]1b/p27, Cdkn1c/p57, and B-cell lymphoma 2-modifying factor) [80]. Consistently, in vivo delivery of anti-miR-221 oligonucleotides lead to a significant reduction of the number and size of tumor nodules in this model. Ji and colleagues confirmed the therapeutic potential of microRNA based treatment modalities in HCC [63]. In a large series combining different cohorts including 445 HCC patients, they could demonstrate that expression patterns of microRNAs in liver tissue are vastly different between men and women with HCC. Furthermore, the authors identified that miR-26 levels are associated with response to adjuvant therapy with interferon alfa and, more recently, developed a simple and reliable

companion diagnostic (MIR26-DX) to select HCC patients for adjuvant interferon-alpha therapy as a first step to successfully translate information from large scale analyses into the clinics [82].

3.4 Epigenetic Crosstalk in Liver Cancer

The interaction of different epigenetic layers such as convergence of microRNAs and DNA methylation as well as chromatin modifications is a field of growing scientific interest in translational HCC studies. Takata et al. investigated whether components of microRNA machinery and subsequent functional impairment of microRNAs are involved in hepatocarcinogenesis and identified DDX20 as frequently down-regulated in human hepatocellular carcinomas [83]. Mechanistically, this disruption led to reduced levels of miR-140 resulting in enhanced nuclear factor-κB (NF-κB) activity. Furthermore, the authors identified DNA methyltransferase 1 (Dnmt1) as a bona fide target of miRNA-140. Genetic loss of DDX20 caused increased Dnmt1 expression and hypermethylation of metallothionein gene promoters that enhanced NF-κB activity. In agreement with this finding, miR-140 deficient animals were prone to hepatocarcinogenesis and displayed an overlapping phenotype to that of DDX20 deficiency, suggesting that miRNA-140 plays a central role in DDX20 deficiency-related pathogenesis.

Several studies addressed the cross-talk between microRNA and histone deacetylases [84–86]. Au et al. recently demonstrated that EZH2, a member of the polycomb repressive complex 2 (PRC2) that catalyses histone H3 lysine 27 (H3K27) tri-methylation, epigenetically silenced multiple miRNAs that negatively regulate HCC metastasis [84]. They compared the expression of 90 epigenetic regulators in 38 primary HCC and paired non-tumorous livers and identified that EZH2 was upregulated in more than two thirds of the investigated HCC. The alterations in EZH2 were further associated with HCC progression and multiple HCC metastatic features, including venous and intrahepatic invasion as well as the absence of tumor encapsulation. They further identified a subset of microRNAs that were epigenetically suppressed by EZH2 in human HCC. These included well-characterized tumor-suppressor microRNAs, such as miR-139-5p, miR-125b, miR-101, let-7c, and miR-200b. Another study revealed a regulatory network between HDAC4/Sp1/miR-200a that conferred to aberrant histone acetylation in HCC patients and could potentially be targeted in therapeutic approaches [86]. Buurman et al. further demonstrated that up-regulation of HDAC1-3 in HCC cells reduces the activity of miR-449. They further established that miR-449 binds to the well known oncogene c-MET leading to subsequent down-regulation with downstream activation of apoptosis and reduced proliferation [85]. Another interesting study demonstrated the regulatory cross-talk between MATA1 and microRNAs during hepatocarcinogenesis [87]. The authors identified three novel microRNAs (miR-664, miR-485-3p, and miR-495) that negatively regulate MAT1A expression thereby contributing to a better understanding how

decreased MAT1A levels contribute to liver cancer development. This study has several important mechanistic, technical and clinical implications [88]. The study nicely demonstrates that a tight interaction of different epigenetic layers is an important driver for the development and progression of liver cancer. Consistently, tumors with low microRNA miR-664, miR-485-3p, and miR-495 activity showed higher DNA methylation, increased repressive H3K27me3 levels, lower Let7 expression (via promoter methylation of Lin28B) and vice versa. Additionally, the study confirms that microRNA-based therapy is an effective therapeutic approach for HCC.

3.5 MicroRNAs and Hepatic Cancer Stem Cells

The two dominant models of carcinogenesis postulate stochastic (clonal evolution) or hierarchic organization of tumors (cancer stem cell model) [89]. The latter places a cancer stem cell (CSC) with functional properties similar to untransformed adult stem cells at the germinal center of tumor evolution. Over the past few years, compelling evidence has emerged in support of this hierarchic cancer model for many solid tumors including hepatocellular cancers [61]. The CSCs are held responsible not only for tumor initiation but also for the generation of distant metastasis and relapse after therapy [90]. These characteristics are particularly relevant for a multi-resistant tumor entity like human hepatocellular carcinoma and may herald a paradigm shift in the management of this deadly disease. Accordingly, intense research focused on the identification of microRNAs with functional consequences for hepatic CSCs to identify novel therapeutic targets [91]. Ji et al. performed a global microarray-based microRNA profiling in EpCAM-positive putative liver CSCs and demonstrated that the highly conserved miR-181 family members were activated in the isolated liver CSCs [92]. They further demonstrated that miR-181 was highly expressed in fetal livers as well as in isolated hepatic stem cells. Importantly, inhibition of miR-181 led to a reduction in frequency of CSCs. Mechanistically, miR-181 could directly target hepatic transcriptional regulators of differentiation such as caudal type homeobox transcription factor 2 (CDX2) and GATA binding protein 6 (GATA6) as well as the inhibitor of Wnt/β-catenin signaling nemo-like kinase (NLK). Wu et al. further suggested that hepatic CSCs may originate from hepatic progenitor cells that are continuously exposed to TGF-β stimulation in cirrhotic liver [93]. They then demonstrated that inhibition of microRNA-216a/PTEN/Akt signaling could be a novel strategy for HCC prevention and therapy by targeting of hepatic CSCs. In extension of their previous investigations on CD133 as a marker of liver CSCs [94], Ma et al. identified differential microRNA expression profiles in CD133+ and $CD133^-$ cells from human HCC clinical specimens and cell lines [95]. As a result of this investigation they demonstrated that miR-130b is significantly activated in CSCs. Functional studies on miR-130b lentiviral-transduced $CD133^-$ cells further demonstrated superior chemoresistance, enhanced tumorigenicity in vivo, and a greater

potential for self renewal. Conversely, inhibition of miR-130b in CD133+ CSCs yielded an opposing effect. The authors further established TP53INP1, a known target of miR-130b, as a crucial regulator of self renewal and tumorigenicity. Furthermore, a recent study conducted on 65 hepatoblastomas (HBs) showed that undifferentiated aggressive HBs overexpressed the miR-371-3 cluster with concomitant down-regulation of the miR-100/let-7a-2/miR-125b-1 cluster, which caused an activation of gene sets enriched in embryonic stem cells [96]. ChIP and Myc inhibition assays in hepatoma cells demonstrated that both microRNA clusters are regulated by Myc. A four-miR signature representative of these clusters efficiently stratified HB as well as HCC according to their prognosis. These data suggests that Myc-driven reprogramming of microRNA expression patterns contributes to the aggressive phenotype of liver tumors originated from hepatic progenitor cells.

4 Outlook and Conclusions

Over the last years several microRNAs with differential expression during the development of liver diseases and hepatocarcinogenesis have been identified. Compelling evidence from integrative and mechanistic microRNA profiling studies supports a role for microRNAs for almost all essential processes in liver cancer progression by directly targeting large number of key pathways and molecules. Consequently, microRNAs emerged to promising diagnostic and therapeutic targets. The advent of novel technological platforms further enabled a feasible and safe use of miRNA mimics or "antagomirs" as therapeutics and has already contributed to the development of miRNA-based therapies for HCC. However, despite this growing translational interest in the field microRNAs and microRNA-based therapies, there remain several conceptual and technical issues and challenges [60]. Computational target prediction software are unsensitive and unspecific which underlines a need for thorough experimental validation in authentic tumors [97]. Many of the known microRNAs are believed to regulate multiple target genes. Similarly, microRNA-based gene regulation is supposed to be overlapping with multiple microRNAs contributing to gene expression of one target gene. Therefore, inhibition of a single microRNA might only lead to slight changes in the gene expression of its targets and, therefore, might not be very efficient [8]. Additionally, the regulatory effect of microRNA, with very few exceptions (e.g. miR-122), is unspecific and affects number of cellular downstream targets. Therefore, therapeutic strategies might cause off-target effects and cellular toxicity due to the lack of cell type specificity. In case of the liver where a variety of resident and non-resident cells with opposing effects on hepatocarcinogenesis orchestrate organ homeostasis, this could cause substantial additional problems. Another unanswered but critical question relates to the systemic delivery of microRNA-based therapies for authentic liver tumors. Although results from recent studies indicate that systemic administration of "antagomirs" and miRNA mimics can be safely

performed, considerably more efforts are needed before a broad clinical translation is plausible [10]. Furthermore, the majority of studies are performed on progressed HCC rather than the early phase of malignant transformation. Due to the clinical difficulty of diagnosing and obtaining these samples, better animal models of hepatocarcinogenesis, in which distinct lesions at different stages of progression can be characterized, are urgently needed. Most importantly, up to now, none of the experimental findings have been translated into clinical practice [10]. To serve this unmet need and fully unravel the clinical potential of regulatory microRNAs, several critical technical burdens, such as the best detections method and the ideal sample type as well as the isolation method, have to be overcome.

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