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ORIGINAL ARTICLE

MicroRNA Expression in Focal Nodular Hyperplasia in Comparison with Cirrhosis and Hepatocellular Carcinoma

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Abstract

The liver disease focal nodular hyperplasia (FNH) has several histological features that resemble hepatic cirrhosis. Since cirrhosis may develop further into hepatocellular carcinoma (HCC) contrary to FNH, the aim of the present study was to identify microRNAs (miRNA), which, by their altered expression levels, may be associated with the benign, tumor-like nature of FNH. Altogether 106 surgically removed formalin-fixed paraffin-embedded liver samples were selected, including 22 FNH, 45 cirrhosis, 24 HCC and 15 normal liver tissues. Etiology of the cases of cirrhosis and HCC includes hepatitis C and alcoholism and the HCC cases developed in cirrhotic livers. Relative expression levels of 14 miRNAs were determined using TaqMan MicroRNA Assays. In comparison to normal liver, the levels of miR-34a and miR-224 were elevated not only in FNH but also in cirrhosis and HCC, while the expression of miR-17-5p, miR-18a and miR-210 was decreased in FNH. Further, the levels of miR-21 and miR-222 were increased in cirrhosis and HCC but were decreased in FNH and the expression of miR-17-5p, miR-18a, miR-195 and miR-210 was decreased in FNH as compared with cirrhosis and/or HCC. In conclusion, the elevation of miR-34a and miR-224 may be associated with both benign and malignant proliferative processes, nevertheless the increased expression of oncomiRs miR-21 and miR-222 in cirrhosis and HCC but not in FNH may be related to malignant processes of the liver. The decreased levels of miR-18a, miR-195 and miR-210 may further differentiate FNH from cirrhosis, reflecting the different pathogenesis of these two entities contrary to some histologically similar features.

Keywords Focal nodular hyperplasia · Hepatic cirrhosis · Hepatocellular carcinoma · microRNA · Chronic hepatitis C

Introduction

Histologically, hepatic cirrhosis is characterized by rearrangement of the parenchyma into nodules, excess deposition of extracellular matrix and production of fibrotic tissues [1, 2]. The histologic appearance of focal nodular hyperplasia (FNH) may look very similar to a part of cirrhosis under the microscope [3, 4]; however, by gross examination, FNH appears as a tumor-like hepatic lesion that develops in noncirrhotic liver [5, 6]. Thus, FNH never progresses into hepatocellular carcinoma (HCC), whereas cirrhosis is regarded as a premalignant lesion preceding HCC. The pathogenesis of FNH is unclear, yet FNH is hypothesized to result from congenital or acquired vascular abnormalities leading to either arterial or portal venous thrombosis [3, 7, 8]. In contrast, the most common etiology of cirrhosis includes chronic viral hepatitis (predominantly hepatitis B and hepatitis C viruses), alcoholic liver disease (ALD), metabolic disorders (non-alcoholic steatohepatitis) and autoimmune liver diseases [9, 10].

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MicroRNAs (miRNA) are short regulating noncoding RNA molecules that negatively modulate gene expression at the posttranscriptional level by binding to the 3′ untranslated region of mRNAs, leading to reduced or prevented protein synthesis [1, 11]. miRNAs are essential for fine-tuning the expression of genes involved predominantly in normal cellular processes, such as development, differentiation and proliferation [12, 13]. Over 2500 miRNAs are expressed from the human genome [14], which create a complex regulatory network, since not only miRNAs have multiple target genes but each mRNA may be regulated by several miRNAs. The fact that deregulated miRNA expression compared to the normal state has been observed in many disorders, including inflammatory liver diseases and primary liver cancers [15–19], assigns an important role to miRNAs in liver diseases, which among others may explain the benign or malignant nature of liver diseases. Therefore, the aim of the present study was to detect miRNA expression levels in FNH, cirrhosis, HCC and normal liver for comparison, which could characterize non-tumorous, premalignant and malignant hepatic lesions.

Materials and Methods

Patients

Altogether 106 liver samples were analyzed retrospectively with the permission of the National Medical Ethical Committee (45727–2/2013/EKU). The samples were selected from the archives of 1st Department of Pathology and Experimental Cancer Research as well as 2nd Department of Pathology of Semmelweis University, Budapest, including formalin-fixed paraffin-embedded samples of 22 FNH, 45 hepatic cirrhosis (of which 30 were infection-related cirrhosis and 15 were tumor-surrounding cirrhosis from the studied HCC cases) and 24 HCC cases along with 15 normal liver samples. Patients were between 23 and 82 years of age with an average of 49.5 years, showing a male/female ratio of 40/36. The clinicopathological summary of the patients is shown in Table 1. The cirrhosis samples were obtained from patients upon orthotopic liver transplantation carried out due to vascular and/or parenchymal decompensated cirrhosis. All HCC cases developed in cirrhotic livers and, according to histological grading, they showed a low/high grade tumor ratio of 14/10. Etiology of the cirrhotic and HCC cases included HCV infection, alcohol intake or the combination of these two. Altogether 28 patients with HCV infection had received antiviral treatment; whereas treatment could not be initiated in eight cases of cirrhosis owing to deteriorated state of health, and treatment information was unknown in five cases.

Table 1 Clinicopathological summary of the patients

	Cirrhosis	HCC	FNH
Number of cases:	45	24	22
Gender			
Male	31	20	1
Female	14	4	21
Average years of age:	53.3	61.5	37.2
(min/max)	(27/81)	(42/82)	(23/66)
Etiology:			
HCV infection	27	8	
HCV infection + alcohol	5	1	
Alcohol intake	9	6	
Unknown	4	9	
Antiviral HCV treatment:			
Received	21	7	
Not received	8		
Unknown	3	2	
End-stage cirrhosis characteristics:			
CHILD A	6		
CHILD B	16		
CHILD C	8		
Ascites	14		
HCC:			
Low grade		14	
High grade		10	

HCC hepatocellular carcinoma, *FNH* focal nodular hyperplasia, *HCV* hepatitis C virus, *CHILD* Child-Turcotte-Pugh Score

Normal liver samples were obtained from deceased patients after organ donation, just prior to ligation of the abdominal aorta and reperfusion.

Histology

Tissue samples were processed according to routine pathology procedures. In brief, the small, 1–3 cm long resected samples were submerged in 10% neutral buffered formalin (in PBS, pH 7.0) and fixed for 24 h at room temperature. Following dehydration in a series of ethanol and xylene, the formalin-fixed samples were embedded in paraffin (FFPE samples). The paraffin embedded samples were cut into 3 to 4 μm thick sections and stained with haematoxylin and eosin.

RNA Isolation

RNA was isolated from several 3 to 4 μm thick FFPE sections using RNeasy FFPE Kit (QIAGEN, Venlo, Netherlands) according to the instructions of the manufacturer with modifications for co-purification of miRNAs [20]. Traces of genomic

DNA were eliminated using Turbo DNase digestion (TURBO DNA-free kit, Ambion, Austin, TX, USA).

Reverse Transcription and Quantitative Polymerase Chain Reaction

Selection of miRNAs to be detected was based on the literature [15, 18, 21–23]. Expression of individual miRNAs was determined using the following TaqMan MicroRNA Assays (Life Technologies of Thermo Fisher Scientific Inc., Foster City, CA, USA): miR-17-5p (ID:000393), miR-18a (ID:002422), miR-21 (ID: 000397), miR-34a (ID:000426), miR-122 (ID:002245), miR-140 (ID:000462), miR-195 (ID:000494), miR-210 (ID:000512), miR-214 (ID:002306), miR-221 (ID:000524), miR-222 (ID:002276), miR-223 (ID:002295), miR-224 (ID:002099) and miR-328 (ID:000543). Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) were performed according to the instructions of the manufacturer. Briefly, RT reaction was carried out using TaqMan MicroRNA Reverse Transcription Kit in a final volume of 7.5 μL containing 10 ng total RNA. The qPCR was performed using TaqMan Universal PCR Master Mix No AmpErase UNG in a final volume of 10 μL containing 0.65 μL RT product. The amplification reaction was run in triplicates on a LightCycler 480 Instrument II (Roche Diagnostics, Indianapolis, IN, USA). Relative expression was calculated by the $2^{-\Delta\Delta Cq}$ formula, applying the average of miR-140 and miR-328 as the most stable reference determined by the NormFinder application [24] and normalized to the median ΔCq value of normal liver samples.

Statistical Analysis

The differences in miRNA expression between normal liver, cirrhosis, HCC and FNH samples were analyzed by means of non-parametric Kruskal-Wallis analysis of variance and median test using STATISTICA software, version 12 (StatSoft Inc., Tulsa, OK, USA). A p value of 0.05 was set as the threshold for statistical significance.

Results

Histology

The FNHs in the present study showed tumor-like appearances by gross examination, with typical central scarring and nodular architecture (Fig. 1a) in contrast to the diffuse nodularization observed in cirrhosis (Fig. 1b). Histologically, the FNH cases were of the classic type based on the Bordeaux classification [25]: nodules of varying sizes, fibrous septa and ductal reaction could be demonstrated (Fig. 1c), which were apparently similar to structural alterations seen in cirrhosis (Fig. 1d).

miRNA Expression in FNH, Cirrhosis and HCC as Compared with Normal Liver

The relative miRNA expression levels determined in FNH, cirrhosis, HCC and normal liver samples are shown on Fig. 2. When compared to normal liver, the levels of miR-34a and miR-224 were increased in each diseased samples, FNH, cirrhosis and HCC ($p < 0.001$); while 5 miRNAs showed decreased expression. Namely, miR-17-5p, miR-18a and miR-210 were decreased in FNH ($p < 0.03$), miR-17-5p and miR-221 in cirrhosis ($p < 0.01$) and miR-223 in HCC ($p < 0.0001$).

miRNA Expression in FNH as Compared with Cirrhosis and HCC

In FNH, intriguingly, the levels of miRNAs were reduced in comparison to cirrhosis and/or HCC (Fig. 2). Expression of miR-18a, miR-21 and miR-222 was decreased in FNH as compared with both cirrhosis and HCC ($p < 0.01$); while miR-195, miR-210 were decreased as compared with cirrhosis ($p < 0.04$) and miR-17-5p, miR-221 were decreased as compared with HCC ($p < 0.04$). The level of miR-195 was increased and the expression of miR-221 was decreased in cirrhosis when compared with HCC ($p < 0.02$). In addition, the levels of miR-18a, miR-21 and miR-222 were also decreased in FNH when compared only to HCV infection-related cirrhosis samples ($p < 0.04$, data not shown).

Discussion

Hepatic cirrhosis and FNH share certain similar histological features, however FNH is a tumor-like focal lesion in a noncirrhotic liver, which, in contrast to cirrhosis, never progresses to HCC. The nodular structure within FNH is the result of malformed vessels and a pathologic blood supply featuring fibrous septa and ductular proliferation [26], where the nodular hyperplastic parenchyma is completely or incompletely surrounded by fibrous septa and the hepatocytes retain their normal phenotype. In contrast, the morphology of cirrhosis results from a complex process involving wound-healing reaction, oxidative stress-related molecular mechanisms, tissue hypoxia, an anaerobic proinflammatory environment and epigenetic modification with the contribution of hepatic stellate cells and other extracellular matrix producing cells [27].

Since altered miRNA expression has been reported in hepatic fibrosis [11, 18, 19, 28, 29] and miRNAs have been suggested to play important role in liver carcinogenesis [15, 18, 21–23], the miRNA expression pattern may provide clues

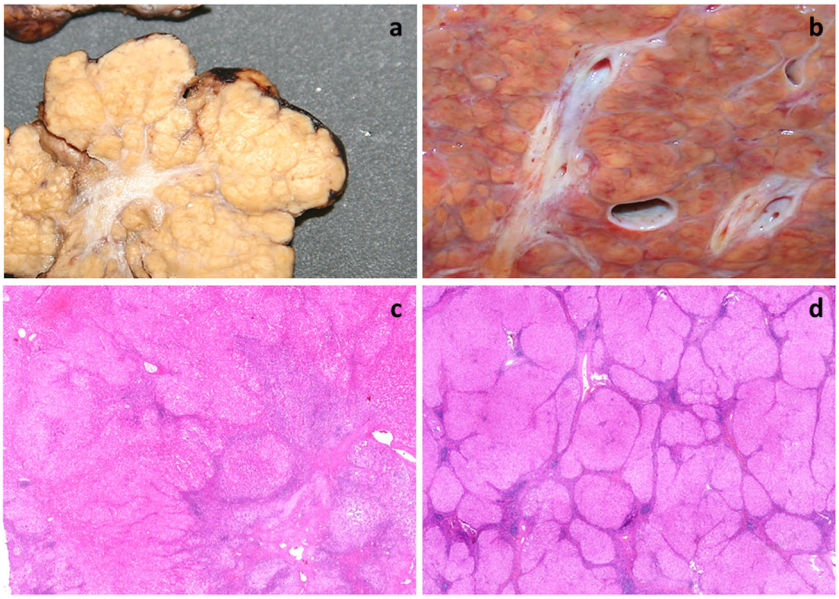

Fig. 1 Characteristics of focal nodular hyperplasia (FNH) in comparison to cirrhosis observed by gross examination (**a**–**b**) and by histology (**c**–**d**). **a** FNH with central scarring and nodular architecture; **b** cirrhosis showing diffuse nodularization; **c** FNH with nodules of varying sizes, fibrous septa and ductular reaction, apparently resembling cirrhosis **d** by histology (H&E ×150)

to biological processes involved in the diseases. In the present study, we aimed to detect alterations in miRNA expression that may indicate the benign proliferation characteristic of FNH contrary to cirrhosis and HCC.

The detected miRNAs showed a predominance of decreased levels. However, miR-34a and miR-224 were increased not only in FNH but also in cirrhosis and HCC in comparison to normal liver. This is in accordance with the

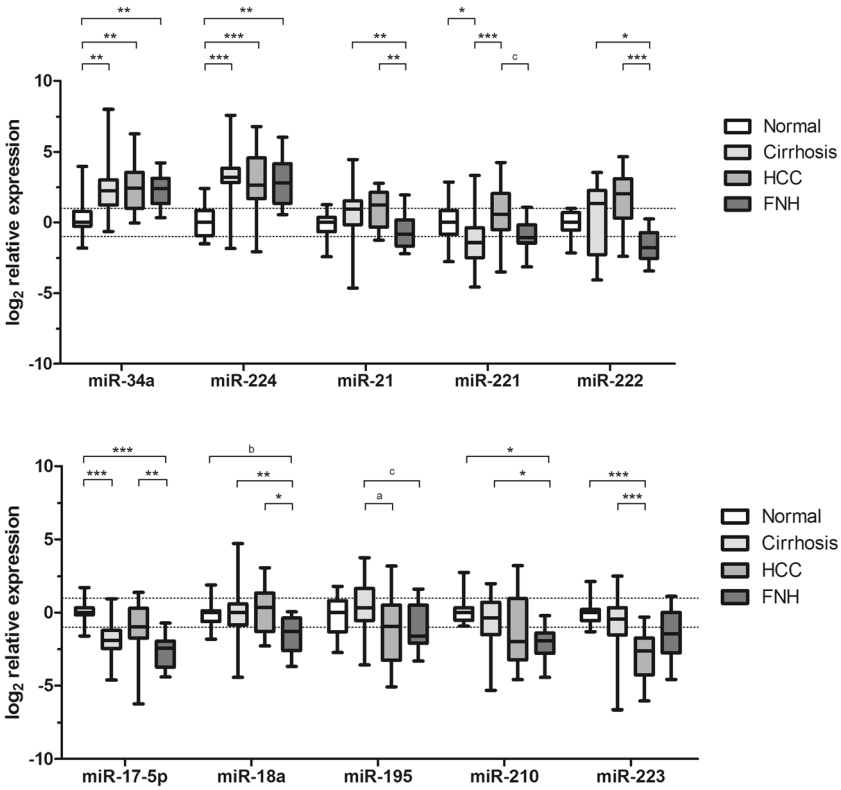

Fig. 2 Relative miRNA expression detected in normal liver, cirrhosis, hepatocellular carcinoma (HCC) and focal nodular hyperplasia (FNH). The upper dotted line indicates two-fold expressional elevation; the lower dotted line signifies a one-half reduction on expression. Thin black lines designate statistical differences analyzed using a non-parametric Kruskal-Wallis analysis of variance and median test: ***$p < 0.0001$, **$p < 0.001$, *$p < 0.01$, [a]$p < 0.02$, [b]$p < 0.03$, [c]$p < 0.04$

literature since expression of both miR-34a and miR-224 has been reported to be elevated in liver diseases including fibrosis, HCV infection, cirrhosis, alcoholic and nonalcoholic liver diseases and HCC [30–32]. One of the activators of miR-34a is TP53, resulting in apoptosis of hepatocytes and activation of hepatic stellate cells (HSC) [30]. Nevertheless, suppressed expression of miR-34a leads to decreased proliferation of cholangiocarcinoma cells [33]. Additionally, miR-34a seems to have role in liver fibrosis since it is found upregulated in HSCs and when the expression of miR-34 is silenced the levels of alpha smooth muscle actin (α-SMA), type I collagen and desmin are found to be lower [34]. Further, it has been shown that HSCs are also activated in FNH as the oxidative stress originating from the arterial hyperperfusion may activate HSCs in the central scar [35]. Thus, it seems that miR-34a helps to enhance cell survival as suggested earlier in HCC cell cultures [36], contributing to both liver tissue repair and fibrosis.

It has been found that miR-224 promotes proliferation, migration and invasion by activating AKT [37] and transforming growth factor-beta (TGF-β) [38] signaling pathways, as well as by promoting the expression of MMP-9 [39]. The fact that p65/NF-κB has been identified as a direct transcriptional regulator of miR-224 links this miRNA to inflammation and cell migration, and on that account, to HCC development and progression [15, 40]. It is therefore likely that miR-224 aids proliferation during liver injuries. Supportive of this assumption is the increasing number of data reporting on the presence of elevated miR-224 in chronic liver diseases, such as chronic viral hepatitis and liver cirrhosis [15, 23], hepatic fibrosis [19], chronic hepatitis with steatosis and hepatitis C virus negative steatosis [16]. Further, the increase of miR-224 has been found to be associated with increased progression-free and overall survival in HCC patients receiving sorafenib treatment [41].

In our study, the miRNAs showing decreased expression in FNH included miR-17-5p, miR-18a, miR-21, miR-195, miR-210, miR-221 and miR-222 as compared with cirrhosis, HCC and/or normal liver. Regarding FNH, the decreased levels of miR-21, miR-222, miR-17-5p, miR-18a are intriguing findings since these miRNAs target genes that inhibit cell proliferation and cell cycle progression (Supplementary Table 1). Elevated miR-21 and miR-222 have been found in various types of cancers, including HCC [21, 42, 43]; whereas statistically lower miR-21 and miR-222 expression has been detected in benign liver tumors, such as hepatocellular adenoma and FNH [22]. miR-21 targets phosphatase and tensin homolog (PTEN), the negative regulator of phosphatidylinositol 3-kinase (PI3K)/Akt pathway [44], leading to cellular proliferation, migration and tumor growth [45]. miR-222 targets cyclin-dependent kinase inhibitor 1B (CDKN1B, p27Kip1) [46] and protein phosphatase 2 regulatory subunit B alpha (PPP2R2A, an inhibitor of AKT phosphorylation) [43], which are two negative controls of cell cycle and cell growth. Our observations that miR-21 and miR-222 are elevated in cirrhosis and HCC but not in FNH seem to support the involvement of these miRNAs in hepatocarcinogenesis. Indeed, the targets of miR-21 affect major processes of cancer biology [47] and miR-21 in conjunction with NF-κB helps maintain a transformed state [48]. The level of miR-222 correlates with the level of collagen type 1 alpha 1 (Col1A1), smooth muscle actin alfa 2 (ACTA2, α-SMA) and matrix metalloproteinase 2 (MMP-2) in HCV infection-related advanced fibrosis [49]. By contrast, a low level of miR-21 may also initiate proliferation through activating the inhibitor of the Hippo pathway, allowing therefore the transcriptional co-activator YAP to initiate gene expression [50, 51]. This might explain our observation according to which proliferation in FNH is also enabled even if the levels of miR-21 are low, but is certainly manifested under a regulation different from the situation when the expression of miR-21 is high.

The targets of miR-17-5p and miR-18a are also cell cycle inhibitors [52, 53]. Nevertheless, it has been reported that both high and low levels of miR-17-5p could promote proliferation. In a dominantly proliferation-promoting system, miR-17-5p may stabilize the pro-proliferative signal by removing the proliferation-inhibitors; however, in a dominantly proliferation-inhibiting system, decreased expression of miR-17-5p may lead to the increase of proliferation-promoters [52]. Thus, low levels of miR-17-5p detected in FNH and cirrhosis may support proliferation but under different circumstances than when miR-17-5p expression is high.

In contrast to the previous oncogenic miRNAs, miR-195 has a tumor suppressor function since it inhibits proliferation and suppresses angiogenesis [54]. This might account for the low miR-195 expression observed in FNH and HCC in comparison to cirrhosis, indicating ongoing proliferation. Nevertheless, miR-195 may activate HSCs, resulting in elevated α-SMA and reduced Smad7 levels [55], which might explain why miR-195 is elevated in cirrhosis since the TGF-β/Smad pathway is pivotal in promoting liver fibrosis, where Smad7 is the negative regulator. miR-210 is a multifaceted miRNA, which is predominantly involved in surviving hypoxia [48]. Nevertheless, it has been reported that overexpression of miR-210 leads to cell cycle arrest and inhibition of miR-210 accelerates cell cycle progression [56]. This might elucidate the observed low levels of miR-210 in FNH and HCC as compared with cirrhosis.

In summary, our study revealed elevated expression of miR-21 and miR-222 in cirrhosis and HCC but not in FNH,

which may be related to malignant processes of the liver. Nevertheless, miR-34a and miR-224 were increased not only in cirrhosis and HCC but also in FNH, indicating that elevation of these miRNAs may be associated with both benign and malignant proliferative processes of the liver. The decreased expression of miR-18a, miR-195 and miR-210 may further differentiate FNH from cirrhosis, which might reflect the different pathogenesis of these two entities contrary to their histologically similar features.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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