MicroRNA's in chronic hepatitis B and C virus infection
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CHAPTER 7

PLASMA MICRORNA LEVELS ARE ASSOCIATED WITH HBsAg STATUS AND TREATMENT RESPONSE IN CHRONIC HEPATITIS B PATIENTS

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*: Participated equally in the study

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ABSTRACT

Background
Hepatitis B virus (HBV) modulates miRNA expression to support viral replication. The aim of this study was to identify miRNAs associated with HBeAg-status and response to antiviral therapy in chronic hepatitis B (CHB) patients, and to assess if these miRNAs are actively secreted by hepatoma cells.

Methods
Plasma miRNA levels were measured by RT-qPCR in healthy controls (n=10), and pre-treatment samples of an identification cohort (n=24) and a confirmation cohort (n=64) of CHB patients treated with peg-interferon/nucleotide analogue combination therapy. Levels of HBV-associated miRNAs were measured in cells, extracellular vesicles, and HBsAg particles of hepatoma cell lines.

Results
HBeAg-positive patients had higher plasma levels of miR-122-5p, miR-125b-5p, miR-192-5p, miR-193b-3p, and miR-194-5p compared to HBeAg-negative patients, and levels of these miRNAs were associated with HBV-DNA and HBsAg-levels. Pre-treatment plasma levels of miR-301a-3p and miR-145-5p were higher in responders (combined response or HBsAg loss) compared to non-responders. miR-192-5p, miR-193b-3p, and miR-194-5p were present in extracellular vesicles and HBsAg particles derived from hepatoma cells.

Conclusions
We identified miRNAs that are associated with HBeAg-status, levels of HBV-DNA and HBsAg, and treatment response in CHB patients. We demonstrated that several of these miRNAs are present in extracellular vesicles and HBsAg particles secreted by hepatoma cells.
BACKGROUND

The natural course of chronic hepatitis B (CHB) infection is determined by the interplay between hepatitis B virus (HBV) replication and the host immune response. Four phases of CHB infection are identified: the immune-tolerant phase, the immune-reactive hepatitis B e antigen (HBeAg) positive phase, the inactive HBV carrier state, and HBeAg-negative chronic hepatitis. Antiviral treatment is recommend for CHB patients in the immune-reactive HBeAg-positive phase and patients with HBeAg-negative chronic hepatitis. Current treatment options include peglated-interferon (peg-IFN) and nucleos(t)ide analogues (NUCs). While NUCs efficiently inhibit viral replication, treatment rarely leads to a functional cure (defined as HBsAg loss with or without the formation of anti-HBs antibodies). In peg-IFN treated patients a functional cure is only achieved in 3-7% of patients. Therefore, it is important to identify CHB patients who will benefit from treatment before the start of peg-IFN based therapy.

miRNAs (miRNAs) are small (19-24 nucleotides), non-coding RNA molecules which are involved in various cellular processes. miRNAs regulate gene expression at a post-transcriptional level by either blocking messenger RNA (mRNA) translation or inducing its degradation. Levels of circulating miRNAs have been linked to various diseases including viral hepatitis. microRNA-122 (miR-122) is the most abundant liver-specific miRNA, which plays a central role in liver development and homeostasis, and was found to have a regulatory function in hepatitis C virus (HCV) replication. miR-122 binds to two seed sites in the 5′UTR of the HCV genome and promotes HCV RNA accumulation by stabilizing the HCV genome, protects the viral genome from degradation and increases the rate of viral RNA synthesis. This discovery led to the development of the first successful miRNA-based therapeutic strategy wherein an anti-miRNA silences miR-122.

miRNAs may also play a pivotal role in regulating HBV replication. It has been shown that miR-122 inhibits HBV replication by direct binding to the polymerase region of viral transcripts, and (13)antisense inhibition of miR-122 resulted in increased hepatitis B surface antigen (HBsAg) and HBeAg levels in vitro. In addition, targeting of HBV transcripts by other miRNAs has been demonstrated (14–19). HBV can regulate miRNA expression and therefore the expression of miRNAs supporting viral replication may increase upon infection. Plasma miRNAs can circulate in different conditions: associated with RNA-binding proteins or lipoprotein complexes, but also packaged into microparticles, or HBsAg particles. Indeed, it has been observed that various miRNAs were upregulated in plasma of CHB patients, and that miRNAs could be used as markers predicting treatment response in CHB patients. In our cohort of HBeAg-positive and -negative CHB patients treated with a combination of peg-IFN and adefovir for 48 weeks, a high rate of HBsAg loss was observed (11-17% at year 2 of treatment-free follow-up).
The aim of this study was to identify plasma miRNAs that are associated with HBeAg-status, HBV-DNA and HBsAg-levels, and therapy response to peg-IFN based treatment in CHB patients. Secondary, we aimed to assess if our identified HBV-associated miRNAs are secreted into extracellular vesicles (EV’s) and HBsAg particles by hepatoma cells.

METHODS

Study population
This study was performed in a cohort of 92 CHB patients who participated in an investigator-initiated study (controlled-trials.com; ISRCTN 77073364), which has been described in detail previously. All patients gave written informed consent. In summary, patients were documented to be HBsAg-positive for longer than 6 months, were either HBeAg-positive or -negative, and had HBV-DNA levels above 17,182 IU/mL (100,000 copies/mL). Patients received a combination of peg-IFN alfa-2a 180 mg subcutaneously once a week, and adefovir dipivoxil 10 mg daily. Several biochemical and virological analyses were conducted, including measurement of alanine aminotransferase (ALT), HBsAg-levels, HBeAg-levels and HBV-DNA levels. Therapy response was determined after six months (week 72) and 2 years (week 144) of treatment-free follow-up. Combined response was defined as the combination of a virological (HBeAg-negativity, and HBV-DNA levels ≤2,000 IU/mL) and a biochemical response (persistent normal ALT levels) in both HBeAg-positive and -negative patients, according to EASL guidelines. HBsAg loss was defined as all above criteria for combined response plus HBsAg-level <0.05 IU/mL (Abbott AxSYM). Eighty-six patients completed 48 weeks of treatment and follow-up of 2 years and fulfilled the inclusion criteria for the present study. We selected 12 HBeAg-positive and 12 HBeAg-negative patients as an identification cohort, both consisting of 6 combined responders, of which 3 patients had HBsAg loss, and 6 non-responders (Supplementary Figure 1A).

Current study design
This study comprised of three steps: (i) Identification of HBV-associated miRNAs in an identification cohort of 24 CHB patients. miRNAs with >2 or 3-fold change and p<0.1 between HBeAg-positive versus negative patients, or responders versus non-responders were selected for analysis in (ii); Confirmation analysis of candidate miRNAs in confirmation cohort of 64 CHB patients (HBeAg-positive and negative, and responders and non-responders) and 10 healthy controls (n=10) (Supplementary Figure 1B); (iii) Proof-of-concept study to assess if our HBV-associated miRNAs in (i) and (ii) are actively secreted by measuring miRNA levels in hepatoma cells, culture supernatant, EV’s, and HBsAg particles.

Plasma miRNA isolation
From pre-treatment plasma samples, RNA was isolated with the miRCURY RNA isolation kit (Exiqon, Denmark) according to the manufacturer’s protocol. Synthetic cel-miR-39
PLASMA MICRORNA LEVELS ARE ASSOCIATED WITH HBEAG STATUS

(Qiagen, USA) was spiked into the lysis buffer to check RNA isolation efficiency. RNA samples were stored at -80°C.

Cell and culture conditions
miRNA levels were analysed in the hepatoma cell lines HepG2 and HepG2 2.2.15. HepG2.2.15 cells are stably transfected with the HBV genome and produce Dane particles, as well as HBeAg, HBsAg, and HBV core particles. HepG2 and HepG2.2.15 cells were cultured at 37°C and 10% CO₂. Culturing was performed in William’s medium E (Lonza, Basel, Switzerland) supplemented with 10% (v/v) heat-inactivated Fetal Calf Serum, L-Glutamine, Dexamethasone, penicillin (100 U/ml) and streptomycin (100 μg/ml). miRNA was extracted from cells and culture supernatant with the miRNeasy mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. EV’s and HBsAg isolation from culture supernatant was performed with ultracentrifugation followed by immunoprecipitation using αCD63 or αPre-S1 antibodies and protein G magnetic beads (Supplementary Methods).

cDNA synthesis and RT-qPCR
cDNA was synthesized using qScript™ microRNA cDNA synthesis kit (Quanta Biosciences, USA) and miRCURY LNA Universal RT cDNA synthesis Kit (Exiqon, Denmark). A serum/plasma focus miRNA qPCR panel (Exiqon, Denmark) was used to determine the expression level of 179 different miRNAs in the identification cohort. Individual miRNA RT-qPCR was performed using LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Swiss). To determine the absolute copy numbers of miRNAs of interest in HepG2 and HepG2.2.15 cells standard curves were constructed (Supplementary Methods).

Data analysis
The amplification curves of miRNAs were analysed using the Lightcycler 480 Software (Roche Diagnostics, Swiss) for the quantification of cycles (Cq) and for the melting curve analysis. All assays were inspected for distinct melting curves. Data were pre-processed using GenEx pro software (MultiD Analyses, Sweden). Suitable reference genes (miR-93-5p and miR-425-5p) were found by applying the NormFinder and GeNorm algorithms on the plasma miRNA profile panel results. Plasma miRNAs were normalised using the ΔCq method (= Cq miRNA of interest – arithmetic mean Cq miR-93-5p and miR-425-5p). The normalised individual data points are presented as the log 10 2 ^ - ΔCq. The difference in expression level of miRNAs between study groups was calculated with the comparative Cq-method (=2 ^ - ((Cq miRNA of interest group A – arithmetic mean Cq miR-93-5p and miR-425-5p group A) – (Cq miRNA of interest group B – arithmetic mean Cq miR-93-5p and miR-425-5p group B)) and expressed as the fold change level.
We tested for difference in miRNA levels between study groups and hepatoma cell lines using the Students t-test or Mann-Whitney U test, and one-way ANOVA with post-test for linear trend using SPSS (version 23) and Graph Pad software (version 7.0). Correlations were analysed with Pearson’s correlation coefficient (r) using SPSS (version 23). Unsupervised hierarchical clustering was performed with GenePattern software.

RESULTS

Patient characteristics

Baseline characteristics of patients included in the identification cohort (n=24) and confirmation cohort (n=62) are summarized in Table 1. Patients were HBeAg-positive (n=41) or HBeAg-negative (n=45). At week 72, 31/86 (36%) patients had combined response. At week 144, 13/86 (15%) patients had HBsAg loss.

Identification of candidate miRNAs

A plasma miRNA profile of 179 miRNAs was determined in the identification cohort of 12 HBeAg-positive and 12 HBeAg-negative patients, both groups consisted of 6 responders, of which 3 patients had HBsAg loss, and 6 non-responders (Supplementary Figure 1A). Nine miRNAs (miR-122-5p, miR-125b-5p, miR-127-3p, miR-192-5p, miR-193b-3p, miR-194-5p, miR-200a-3p, miR-204-5p, and miR-29a-5p) were differentially expressed (>3-fold) between HBeAg-negative and –positive patients and met selection criteria to be analysed in the confirmation cohort (Supplementary Table 1). In HBeAg-positive patients, 3 miRNAs (miR-132-3p, miR-145-5p, miR-301a-3p) were differentially expressed (>2-fold) between responders (combined response and/or HBsAg loss) and non-responders and non-responders and non-responders and non-responders and non-responders.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Identification cohort (n=24)</th>
<th>Confirmation cohort (n=62)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBeAg positive patients</td>
<td>HBeAg negative patients</td>
</tr>
<tr>
<td>N=</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Male</td>
<td>11 (92)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Age</td>
<td>35 ± 10</td>
<td>42 ± 12</td>
</tr>
<tr>
<td>Hepatitis B virus DNA (log 10 IU/mL)</td>
<td>8.13 ± 1.28</td>
<td>4.82 ± 0.76</td>
</tr>
<tr>
<td>Hepatitis B surface antigen (log 10 IU/mL)</td>
<td>4.50 ± 0.53</td>
<td>3.31 ± 0.80</td>
</tr>
<tr>
<td>Alanine aminotransferase level (U/L)</td>
<td>287 ± 418</td>
<td>67 ± 47</td>
</tr>
<tr>
<td>Combined response week 72</td>
<td>6 (50)</td>
<td>6 (50)</td>
</tr>
<tr>
<td>Hepatitis B surface antigen loss week 144</td>
<td>3 (25)</td>
<td>3 (25)</td>
</tr>
</tbody>
</table>

Data shown as n (%) or mean ± SD.
Higher plasma levels of HBV-associated miRNAs in CHB patients versus healthy controls

Of 26 previously identified HBV-associated miRNAs, relative plasma levels of 16 miRNAs were significantly higher in CHB patients (n=86, identification and confirmation cohort) compared to healthy controls (n=10) (Supplementary Table 3). CHB patients had 59-fold higher mean plasma levels of miR-122-5p compared to healthy controls (p<0.001). In addition, plasma levels were 24-fold higher in CHB patients for miR-192-5p, 20-fold higher for miR-193b-3p, 38-fold higher for miR-194-5p, and 24-fold higher for miR-215 compared to healthy controls, all p<0.001 (Supplementary Table 3). Unsupervised hierarchical clustering using the 16 highly significant (p<0.001) differentially expressed miRNAs clustered all healthy controls together (Figure 1).

Different miRNA signature in HBeAg-positive and negative CHB patients

Plasma levels of the 9 miRNAs (miR-122-5p, miR-125b-5p, miR-127-3p, miR-192-5p, miR-193b-3p, miR-194-5p, miR-200a-3p, miR-204-5p, and miR-29a-5p) with differential expression between HBeAg-positive and negative CHB patients in the identification cohort

Figure 1. Hierarchal clustering of healthy controls (white triangles), HBeAg-negative (grey triangles) CHB patients, and HBeAg-positive (black triangles) CHB patients with miRNAs differentially expressed between healthy controls and CHB patients (with p<0.001). The heatmap shows scaled expression values with highest values in red and lowest in blue.
(n=24), were measured in the confirmation cohort (n=62 CHB patients) (Supplementary Figure 1B) and compared between HBeAg-positive (n=29) and HBeAg-negative (n=33) patients. HBeAg-positive patients had significant higher plasma levels of miR-125b-5p (2.2-fold, p=0.004), miR-192-5p (2.1-fold, p=0.022), and miR-194-5p (3.4-fold, p=0.001) compared to HBeAg-negative patients (Table 2). Plasma levels of miR-122-5p and miR-193b-3p were >1.5-fold higher in HBeAg positive patients, but these differences were not statistically significant (1.8 fold, p=0.083, and 1.6-fold, p=0.089, respectively). Plasma levels of miR-127-3p, miR-200a-3p, miR-204-5p and miR-29a-5p were not different between HBeAg-positive and negative patients (fold change level >-1.5 and <1.5, and p>0.05) (Table 2). A significant increasing trend of all miRNA levels (except for miR-127-3p) was observed from healthy controls to HBeAg-negative patients to HBeAg-positive patients (Figure 2). Analysis of plasma miRNA levels of the total cohort (n=86) showed a similar pattern as seen in the confirmation cohort, with the addition of a significant higher miR-122-5p and miR-193b-3p level in HBeAg positive patients (Table 2). Next, we analysed whether plasma miRNA levels were associated with HBV-DNA level, HBsAg-level and ALT level. A correlation was found between plasma miRNA levels (miR-122-5p, miR-125b-5p, miR-192-5p, miR-193b-3p, miR-194-5p, and miR-29a-5p) and both HBV-DNA and HBsAg-levels (Figure 3). However, ALT levels were only correlated with plasma miR-193b-3p levels (Figure 3). Concluding, we observed a differential plasma miRNAs signature between HBeAg positive and negative patients, and found that various of these miRNAs are correlated with HBV-DNA and HBsAg-levels.

**Figure 2.** Relative miRNA plasma levels in healthy controls (HC), HBeAg-negative and HBeAg-positive CHB patients. The relative miRNA level is shown as the log 10 (2^- (Cq miRNA of interest – arithmetic mean Cq miR-93-5p+miR-425-5p)). Relative miRNA level < 0 : lower level of miRNA of interest compared to reference; > 0 : higher level of miRNA of interest compared to reference. The bars represent the mean and standard error of the mean. One-way ANOVA with a post-test for linear trend was used to compare the different groups. ns: not significant, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.
Table 2. miRNAs associated with HBeAg status in CHB patients

<table>
<thead>
<tr>
<th>N=</th>
<th>HBeAg negative patients</th>
<th>HBeAg positive patients</th>
<th>Fold change in HBeAg pos</th>
<th>p-value</th>
<th>HBeAg negative patients</th>
<th>HBeAg positive patients</th>
<th>Fold change in HBeAg pos</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Confirmation cohort (n=62)</td>
<td></td>
<td></td>
<td></td>
<td>Total cohort (n=86)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>hsa-miR-122-5p</td>
<td>33</td>
<td>0.80 ± 0.46</td>
<td>1.06 ± 0.65</td>
<td>1.80</td>
<td>0.083</td>
<td>0.80 ± 0.45</td>
<td>1.16 ± 0.63</td>
<td>2.29*</td>
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<tr>
<td>hsa-miR-125b-5p</td>
<td>-0.55 ± 0.32</td>
<td>-0.21 ± 0.53</td>
<td>2.19*</td>
<td>0.004*</td>
<td></td>
<td>-0.56 ± 0.33</td>
<td>-0.16 ± 0.49</td>
<td>2.54*</td>
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<tr>
<td>hsa-miR-127-3p</td>
<td>-1.17 ± 0.36</td>
<td>-1.22 ± 0.45</td>
<td>-1.13</td>
<td>0.611</td>
<td></td>
<td>-1.13 ± 0.39</td>
<td>-1.21 ± 0.41</td>
<td>-1.20</td>
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<tr>
<td>hsa-miR-192-5p</td>
<td>-0.01 ± 0.53</td>
<td>0.32 ± 0.58</td>
<td>2.14*</td>
<td>0.022*</td>
<td></td>
<td>-0.04 ± 0.50</td>
<td>0.37 ± 0.51</td>
<td>2.55*</td>
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<tr>
<td>hsa-miR-193b-3p</td>
<td>-0.67 ± 0.46</td>
<td>-0.45 ± 0.50</td>
<td>1.63</td>
<td>0.089</td>
<td></td>
<td>-0.68 ± 0.46</td>
<td>-0.41 ± 0.47</td>
<td>1.87*</td>
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<td>hsa-miR-194-5p</td>
<td>-0.73 ± 0.48</td>
<td>-0.20 ± 0.65</td>
<td>3.37*</td>
<td>0.001*</td>
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<td>-0.70 ± 0.47</td>
<td>-0.13 ± 0.63</td>
<td>3.71*</td>
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<tr>
<td>hsa-miR-200a-3p</td>
<td>-1.83 ± 0.37</td>
<td>-1.86 ± 0.48</td>
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<td>0.760</td>
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<td>-1.82 ± 0.42</td>
<td>-1.79 ± 0.45</td>
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<td>hsa-miR-204-5p</td>
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<td>-0.65 ± 0.39</td>
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<td>0.896</td>
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<td>-0.64 ± 0.33</td>
<td>-0.64 ± 0.36</td>
<td>1.00</td>
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<tr>
<td>hsa-miR-29a-5p</td>
<td>-1.77 ± 0.28</td>
<td>-1.77 ± 0.59</td>
<td>-1.02</td>
<td>0.945</td>
<td></td>
<td>-1.82 ± 0.39</td>
<td>-1.71 ± 0.51</td>
<td>1.30</td>
</tr>
</tbody>
</table>

Relative miRNA level (=log 10 (2^ - Cq miRNA of interest – arithmetic mean Cq miR-93-5p+miR-425-5p)) shown as mean ± SD; relative miRNA level < 0: lower level of miRNA of interest compared to reference miRNA; > 0: higher level of miRNA of interest compared to reference miRNA; fold change level (= 2^ - ∆∆Cq) shown as mean; fold change level < 1: fold decrease, > 1: fold increase; #: p < 0.05 and fold change level < -1.5 or > 1.5.
Higher baseline plasma level of miR-301a-3p and miR-145-5p in responders versus non-responders

Baseline plasma levels of miR-132-3p, miR-145-5p, and miR-301a-3p were measured in all HBeAg-positive patients (n=41) and compared between patients with combined response (n=14) and non-response (n=27) at week 72, and between patients with HBsAg loss (n=5) and non-response (n=29) at week 144. Plasma miR-301a-3p levels were 1.8-fold (p=0.002) and 1.6-fold (p=0.088) higher in HBeAg-positive patients with combined response and HBsAg loss compared to non-responders, respectively (Table 3). Plasma miR-132-3p and miR-145-5p levels were lower in HBeAg-positive responders versus non-responders, but this difference was not significant (Table 3). In total, 18 candidate miRNAs were measured in all HBeAg-negative patients (n=45) and compared between responders (combined response and/or HBsAg loss) and non-responders. miR-145-5p was 1.5-fold (p=0.028) higher in HBeAg-negative patients with HBsAg loss compared to non-responders at week 144 (Table 3). All other miRNA levels (n=17) were not different (fold change level >-1.5 and <1.5, and p>0.05) between HBeAg-negative responders and non-responders (Table 3). Taken together, we showed that pre-treatment plasma miR-301a-3p level was higher in HBeAg positive patients with combined response compared to non-responders, and that plasma miR-145-5p level was higher in HBeAg negative patients with HBsAg loss.
Table 3. miRNAs associated with treatment response

<table>
<thead>
<tr>
<th>miRNA</th>
<th>HBeAg pos (n=41)</th>
<th>HBeAg neg (n=45)</th>
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<tr>
<td></td>
<td>NR week 72</td>
<td>CR week 72</td>
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<tr>
<td>hsa-miR-132-3p</td>
<td>-0.79 ± 0.68</td>
<td>-0.90 ± 0.36</td>
</tr>
<tr>
<td>hsa-miR-145-5p</td>
<td>-0.97 ± 0.54</td>
<td>-1.05 ± 0.15</td>
</tr>
<tr>
<td>hsa-miR-301a-3p</td>
<td>-1.45 ± 0.24</td>
<td>-1.20 ± 0.20</td>
</tr>
<tr>
<td>hsa-let-7d-3p</td>
<td>-0.60 ± 0.34</td>
<td>-0.68 ± 0.15</td>
</tr>
<tr>
<td>hsa-let-7e-3p</td>
<td>0.23 ± 0.20</td>
<td>0.18 ± 0.29</td>
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<tr>
<td>hsa-miR-106b-3p</td>
<td>-1.00 ± 0.25</td>
<td>-0.92 ± 0.31</td>
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<td>hsa-miR-10b-5p</td>
<td>-0.66 ± 0.25</td>
<td>-0.59 ± 0.23</td>
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<td>hsa-miR-125b-5p</td>
<td>-0.53 ± 0.31</td>
<td>-0.62 ± 0.37</td>
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<td>hsa-miR-141-3p</td>
<td>-2.15 ± 0.64</td>
<td>-1.97 ± 0.34</td>
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<td>hsa-miR-143-3p</td>
<td>-0.94 ± 0.18</td>
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<td>-0.97 ± 0.17</td>
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<td>hsa-miR-146b-5p</td>
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<td>hsa-miR-193b-3p</td>
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<td>hsa-miR-194-5p</td>
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<td>hsa-miR-215</td>
<td>-1.01 ± 0.31</td>
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<td>hsa-miR-326</td>
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<td>hsa-miR-361-3p</td>
<td>-1.56 ± 0.19</td>
<td>-1.72 ± 0.40</td>
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<tr>
<td>hsa-miR-485-3p</td>
<td>-1.01 ± 0.31</td>
<td>-0.97 ± 0.34</td>
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</tbody>
</table>

Relative miRNA level (=log 10 (2 ^ - Cq miRNA of interest – arithmetic mean Cq miR-93-5p+miR-425-5p)) shown as mean ± SD; relative miRNA level < 0: lower level of miRNA of interest compared to reference miRNA; > 0: higher level of miRNA of interest compared to reference miRNA; fold change level (= 2 - ∆∆Cq) shown as mean; fold change level < 1: fold decrease, > 1: fold increase; #: p < 0.05 and fold change level < -1.5 or > 1.5
Identification and secretion of HBV-associated miRNAs in hepatoma cell lines

As a proof-of-concept study, levels of our identified HBV-associated miRNAs were measured in HepG2 and HepG2.2.15 cells, culture supernatant, EV’s and HBsAg particles. For this analysis, we selected 5 miRNAs (miR-122-5p, miR-125b-5p, miR-192-5p, miR-193b-3p and miR-194-5p) which were all significant up-regulated in CHB patients versus HC, and in HBeAg-positive versus negative patients (Table 2, Supplementary Table 3). In HepG2.2.15 cells, mean HBV-DNA level was 4.3 log 10 copies in intracellular viral capsids, and 2.72 log 10 copies in culture supernatant, demonstrating active HBV replication (Supplementary Figure 2). miR-122-5p and miR-125b-5p were low expressed in both HepG2 and HepG2.2.15 cells (Supplementary Table 4, Figure 4A).30 miR-192-5p, miR-193b-3p and miR-194-5p were expressed in both HepG2 and HepG2.2.15 cell lines (Supplementary Table 4, Figure 4B+C+D), with significant higher levels of miR-192 and miR-194 in HepG2.2.15 cells (with HBV) compared to HepG2 cells (without HBV). In addition, absolute levels of miR-192 and miR-194 were significant higher in culture supernatant of HepG2.2.15 cells compared to HepG2 cells (Figure 4B+D).

Next, we investigated if miRNAs detected in the culture supernatant were secreted by hepatoma cells into EV’s(for HepG2 and HepG2.2.15) and/or HBsAg particles (for HepG2.2.15 only). We observed that HepG2 and HepG2.2.15 cells packaged and secreted comparable levels of miR-192-5p and miR-193b-3p in EV’s(Supplementary Table 4, Figure 4B+4C). In contrast, miR-194-5p level was significantly higher in EV’s secreted by HepG2.215 cells compared to HepG2 cells (1.66 log 10 copies/total RNA versus 0.57 log 10 copies/total RNA, p=0.02). miR-192-5p, miR-193b-3p, and miR-194-5p levels were all present in HBsAg particles secreted by HepG2.2.15 cells (Figure 4B-D).

DISCUSSION

In this study, we showed that HBeAg-positive patients have higher plasma levels of five miRNAs (miR-122-5p, miR-125b-5p, miR-192-5p, miR-193b-3p and miR-194-5p) compared to HBeAg-negative patients and that levels of these miRNAs were associated with HBV-DNA and HBsAg-levels, indicating a relation with viral replication. Furthermore, we found that pre-treatment plasma levels of miR-301a-3p and miR-145-5p were higher in CHB patients who achieved combined response or HBsAg loss following peg-IFN/nucleotide analogue therapy compared to non-responders. As a proof-of-concept study, we showed that miR-192-5p, miR-193b-3p, and miR-194-5p levels were present in EV’s and HBsAg particles of hepatoma cell line with and without active HBV replication which might suggest active packaging and secretion of these miRNAs.

Similar to previous studies, we showed that plasma miR-122 levels were ~60-fold higher in CHB patients compared to healthy controls, and ~2-fold higher in HBeAg-positive patients...
versus HBeAg-negative patients \(^{31-33}\). Surrogate parameters for HBV replication and translation, namely HBV-DNA and HBsAg-levels, were positively correlated with plasma miR-122 levels. Increases in circulating miR-122 levels in plasma might be caused by HBV induced upregulation of miR-122 expression and thus increased secretion from the liver. Variations of serum miRNA profiles between CHB patients and healthy controls, and across the different phases of CHB infection were observed in previous studies and partly confirm our findings \(^{20,34,35}\). We showed that plasma miR-125b-5p levels are ~5-fold higher in CHB patients versus healthy controls, and ~2-fold higher in HBeAg-positive versus negative patients. miR-125b-5p was previously associated with direct and indirect downregulation of HBV \(^{34,36}\). In addition, miR-125b-5p has been shown to suppress other virus infections as well as tumorigenesis \(^{37,38}\). This might indicate that miR-125b-5p may be part of the innate protection of the host against viral infection. We observed that plasma levels of miR-192-5p, miR-193b-3p, miR-194-5p and miR-215 were all highly upregulated (≥20-fold) in CHB patients compared to healthy controls. Interestingly, three of these miRNAs are encoded in clusters: the miR-215/miR-194-1 cluster on chromosome 1 (1q41) and the miR-192/miR-194-2 cluster on chromosome 11 (11q13.1) \(^{39}\). miRNA members of the same cluster are usually co-expressed and co-regulated and may target the same molecule or hit several molecules in the same biological pathways. Downregulation of these three miRNAs was previously reported in various types of cancer \(^{39,40}\). In addition, an upregulation of miR-194-

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Figure 4. Absolute levels of miR-122 and miR-125b (4A), miR-192 (4B), miR-193b (4C) and miR-194 (4D) in HepG2 and HepG2.2.15 cells, culture supernatant, extracellular vesicles (EV’s), and HBsAg particles (HepG2.2.15 only). All figures show the mean with SD of three independent experiments. *: p<0.05, ****: p<0.0001.
5p levels in HBeAg-positive patients compared to healthy controls and HBeAg-negative patients was previously described. In this study, we observed higher pre-treatment plasma miR-301a-3p levels in HBeAg-positive patients who received combination therapy and who developed combined response at week 72 compared to non-responders. Also, we showed that high pre-treatment plasma miR-145-5p level was significantly associated with HBsAg loss in HBeAg-negative patients. Although miR-301a-3p and miR-145-5p have been associated with other liver diseases such as non-alcoholic fatty liver disease and HBV induced hepatocellular carcinoma, our study is the first to identify miR-301a-3p and miR-145-5p as potential pre-treatment response markers for treatment outcome in CHB patients. Previously, it was demonstrated that baseline miR-122 levels were significantly lower in HBV/HCV co-infected patients treated with peg-IFN and ribavirin who cleared HBsAg than in patients who did not. Although we did observe a more than 3-fold lower baseline miR-122 level in CHB patients with HBsAg loss in the identification cohort, this difference was not significant and therefore not further assessed in the confirmation cohort.

It was shown that nearly 90% of the total miRNAs in the liver are comprised of approximately 10 miRNAs (of which miR-122 is the major contributor with 50-70% of the miRNA profile). Following miR-122, miR-192-5p was the most abundantly expressed miRNA in the human liver, and except for miR-301a-3p, all our other identified HBV-associated miRNAs were found to be expressed in liver tissue. A broad miRNA expression profiling study revealed miR-301a-3p expression in various tumor tissues, hepatic stellate cells, and immune cells. In addition, it was shown that miR-301a-3p was expressed in HepG2.2.15 cells and not in HepG2 cells, suggesting a possible relation with HBV. In this study we have measured circulating miRNA levels instead of hepatic miRNA levels, and the functions of plasma miRNAs have been debated. Several studies suggest that plasma miRNAs do not have any biological function and are by-products of dying cells. However, miRNAs can also be actively secreted and used for inter-cell communication, and it was shown that viruses can actively package miRNAs into EV’s and transfer them to uninfected cells and thereby support viral replication and/or induce an innate immune response.

In CHB patients, it was previously demonstrated that circulating subviral HBsAg particles carry host cellular miRNAs. In cell lines, we investigated in a proof-of-concept study if our plasma HBV-associated miRNAs were actively packaged and secreted. We measured miRNA levels in two hepatoma cell lines including a cell line which is stably expressing HBV infection (HepG2.2.15). The presence of miRNAs in the culture supernatant could suggest that miRNAs are actively secreted by hepatoma cells. We could not assess secretion of miR-122-5p and miR-125b-5p in HepG2 and HepG2.2.15 cells since these miRNAs were expressed at very low levels. This can be explained by translational repression of the miR-122 gene by reduced accessibility of chromatin of hepatocyte nuclear factor-4-
alpha (HNF4α) in the HepG2 promoter. We found that miR-192-5p and miR-194-5p were higher expressed in HepG2.2.15 cells compared to HepG2 cells. The higher expression of miR-192-5p may be caused by the HBx induced expression of growth factor beta-1 which in turn could lead to the upregulation of miR-192. The secretion and active packaging of miR-194-5p into EV’s was significantly higher for HepG2.2.15 (with HBV) compared to HepG2 (without HBV) cells. This finding was in line with our previous observation that CHB patients had higher plasma miR-194-5p levels compared to healthy controls.

Since current treatment options for CHB patients rarely achieve a functional cure, there is a need for new therapeutic options. Our identified HBV-associated miRNAs may play a functional role in HBV replication by direct targeting of viral transcripts or cellular factors required for HBV replication, or be related to immune functions. Future studies should aim to identify links between miRNAs, their putative targets and HBV replication. If miRNAs are shown to have a direct or indirect role in the regulation of HBV replication, these miRNAs would be attractive novel targets for CHB treatment. In addition, circulating miRNAs could serve as potential biomarkers for CHB disease stage or to predict response to antiviral treatment.

To conclude, we have identified miRNAs that are associated with HBeAg-status, HBV-DNA and HBsAg-levels, and treatment response in CHB patients. We demonstrated that various of these HBV-associated miRNAs are secreted into EV’s and HBsAg particles derived from cells stably transfected with a complete HBV genome.
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CHAPTER 7

FUNDING
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SUPPLEMENTARY DATA
Available upon request.