

Original article

Plasma microRNA profile as a predictor of early virological response to interferon treatment in chronic hepatitis B patients

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Background: Interferon (IFN) and pegylated interferon (PEG-IFN) treatment of chronic hepatitis B leads to a sustained virological response in a limited proportion of patients and has considerable side effects. To find novel markers associated with prognosis of IFN therapy, we investigated whether a pretreatment plasma microRNA profile could be used to predict early virological response to IFN.

Methods: We performed microRNA microarray analysis of plasma samples from 94 patients with chronic hepatitis B who received IFN therapy. The microRNA profiles from 13 liver biopsy samples were also measured. The OneR feature ranking and incremental feature selection method were used to rank and optimize the number of features in the model. Support vector machine prediction engine and jack-knife cross-validation were used to generate and evaluate the prediction model.

Results: The optimized model consisting of 11 microRNAs yielded a 74.2% overall accuracy in the training group and was independently confirmed in the test group (71.4% accuracy). Univariate and multivariate logistic regression analyses confirmed its independent association with early virological response (OR=7.35; $P=2.12 \times 10^{-5}$). Combining the microRNA profile with the alanine aminotransferase level improved the overall accuracy from 73.4% to 77.3%. Co-transfection of an HBV replicative construct with microRNA mimics revealed that let-7f, miR-939 and miR-638 were functionally associated with the HBV life cycle.

Conclusions: The 11 microRNA signatures in plasma, together with basic clinical variables, might provide an accurate method to assist in medication decisions and improve the overall sustained response to IFN treatment.

Introduction

HBV is a highly prevalent infectious agent causing chronic liver disease and increasing the risk of hepatocellular carcinoma [1]. Although recombinant vaccines are widely available, there are still approximately 300 million people carrying this virus who will need antiviral medication when chronic liver disease is activated. Interferon- α (INF- α) is one of the major medications for treating patients with chronic hepatitis B

(CHB). INF- α not only activates a direct antiviral state by stimulating genes of intracellular host defence, but it also possesses immunomodulatory activity; both activities are considered important for clearance of HBV. Recent application of pegylated interferons (PEG-IFN- α 2a and PEG-IFN- α 2b) to CHB patients significantly improved the virological response rate (52% at the end of treatment for PEG-IFN- α 2a) [2].

More importantly, the hepatitis B e antigen (HBeAg; PEG-IFN- α 2a 32% and PEG-IFN- α 2b 36%) and hepatitis B surface antigen (HBsAg; PEG-IFN- α 2a 2.9%) seroconversion rates were greater than any of the nucleoside analogues available [1,2]. However, PEG-IFN treatment has been found to be effective in only some chronically infected patients: HBeAg seroconversion is only achieved in 30–40% of HBeAg-positive patients. Furthermore, treatment is often accompanied by severe side effects, like thrombocytopenia or leukopenia, and needs subcutaneous injection [3].

In light of all the pros and cons of IFN treatment, methods for prediction of treatment outcome are needed for more effective personalized medicine and optimal cost-effectiveness. Currently, prognostic parameters for sustained response after IFN treatment include high baseline alanine aminotransferase (ALT) levels, low baseline HBV DNA, female sex and on-treatment HBsAg dynamics during treatment [4,5]. In addition, different genotypes also considerably influence response rate: genotype A has the highest HBeAg clearance rate (47%), followed by genotype B (44%), C (28%) and D (25%) [6]. Over the past few years, with some success, researchers have constructed models and treatment indexes for application in clinical practice [5]. However, models based on these clinical parameters are still not sufficiently robust for high-confidence prediction [5,7]. Therefore, novel molecular markers are needed to improve the estimation of prognosis. Ideally, these molecules should be conveniently and quantitatively monitored at the onset of treatment without invasive procedures.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that exist in many organisms and suppress mRNA translation and/or induce mRNA degradation by base-pairing to partially complementary sites of mRNAs, mainly in the 3' untranslated region [8]. Recent studies indicate that miRNAs are present in the plasma and have been shown to be a sensitive marker for liver injury and cancer [9–11]. Our aim here was to investigate whether the pretreatment plasma miRNA profile could be used to predict early virological response to IFN therapy.

Methods

Patients and clinical specimens

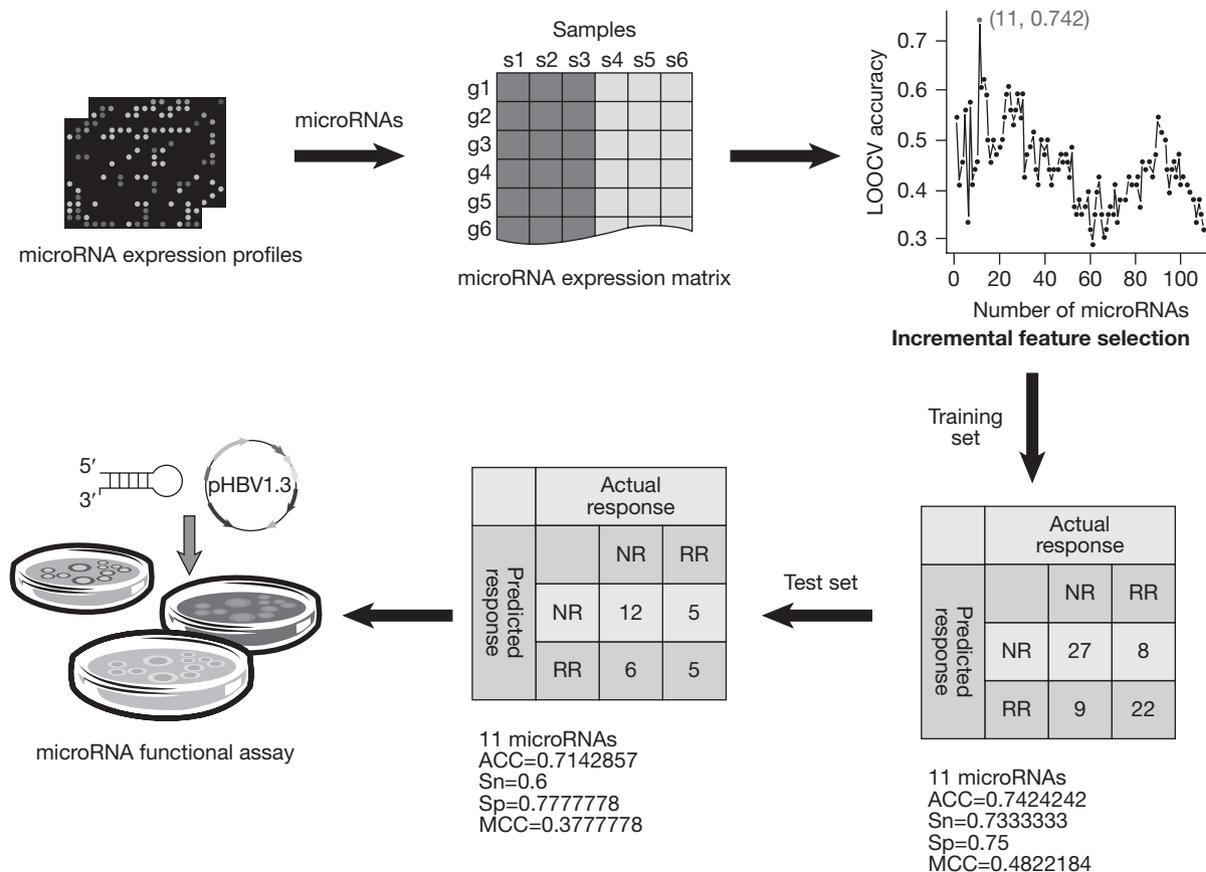
A total of 94 CHB patients who underwent PEG-IFN or conventional IFN treatment at Shanghai Public Health Clinical Center, Huashan Hospital (Fudan University, Shanghai, China) and Ruijin Hospital (Shanghai Jiao-tong University, Shanghai, China) were enrolled. These patients had not received nucleoside analogue or IFN therapy within 6 months of initiation of the study, had viral loads $>5 \times 10^4$ copies/ml and exhibited abnormal liver ALT levels (>1.0 ULN). Patients with normal ALT levels, but Scheuer scores greater than G2S2, were

also included. Exclusion criteria included coinfection with HIV or HCV and decompensated cirrhosis. All the blood samples were tested for HBsAg and HBeAg using Abbott AXSYM HBsAg (normal: 0–2S/N) and HBeAg 2.0 MEIA kit (normal: 0–1.0S/CO; Abbott Laboratories, Chicago, IL, USA). The viral load was also measured using an HBV DNA quantitative real-time PCR (qPCR) kit (Qiagen, Shenzhen, China). Plasma and liver biopsy samples were obtained with informed consent. The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of Shanghai Public Health Clinical Center.

Study design and statistical analysis

The general strategy for differentiating cases with good or poor virological response is outlined in Figure 1. The training group consisted of 66 HBV patients receiving PEG-IFN- α 2a (180 μ g/week, 60 patients) or PEG-IFN- α 2b (100 μ g/week, 6 patients) for 48 weeks. Twenty-eight patients receiving conventional IFN (IFN- α 2b, IFN- α 1b, 3–5 MU every other day) were enrolled into the test group. Patients were classified into rapid response (RR) or no response (NR) groups on the basis of whether a 2 \log_{10} decrease of viral load was achieved 12 weeks after starting treatment. In a cohort of 66 patients who received PEG-IFN- α 2a and PEG-IFN- α 2b treatment, the microarray data were first filtered based on two inclusion criteria: miRNA was present in over 20% of the samples; and at least 20% of the miRNAs had signal intensities >4 . We then employed OneR classifier in Weka [12] to rank the miRNAs, according to their significance to the IFN treatment response. The OneR method is a simple scoring method that ranks attributes according to error rate. It treats all numerical valued features as continuous and divides the range of values into disjoint intervals. The support vector machine (SVM) classifier implemented in the R packages, e1071, was used to generate classification. The parameters (that is, kernel, gamma and cost) were sigmoid, 4 and 0.25. The leave-one-out cross-validation, known as the jack-knife cross-validation method, was used to evaluate the performance of the prediction model for PEG-IFN responses. The stability of prediction was independently tested in the test case. The sensitivity, specificity, accuracy and Matthew's correlation coefficient (MCC) were calculated. Incremental feature selection (IFS) [13] was used to optimize the number of response-related miRNAs. Using N feature sets, we constructed N different SVM predictors. By means of the overall accuracy rates of all the predictors, we obtained the IFS curve with the index 'i' for the x-axis and the overall accuracy rate for the y-axis. The optimal feature set was determined when the IFS curve reached its peak.

Figure 1. Flowchart of the research strategy for optimizing a microRNA signature that can predict interferon therapy outcome



ACC, accuracy; LOOCV, leave-one-out cross-validation; MCC, Matthew's correlation coefficient; NR, non-responder; RR, rapid responder; Sn, sensitivity; Sp, specificity.

RNA extraction, miRNA microarray and real-time quantitative RT-PCR

Total RNA was extracted from 400 μ l plasma samples by using mirVana™ PARIS™ kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). The extracted RNA was quantified using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA, USA). miRNAs from formalin-fixed paraffin-embedded (FFPE) liver biopsy samples were extracted using the Qiagen miRNeasy FFPE kit (Hilden, Germany). Human miRNA microarrays (Agilent Technologies, Santa Clara, CA, USA) were used to quantify the expression profiles of 94 plasma samples and 13 FFPE samples. The microarray contains probes for 851 human miRNAs from the Sanger database v12.0. The mean normalized signal from the biological replicates was used for comparative expression analysis. All the miRNA expression profiles were submitted to the GEO database (series number GSE29911). For qPCR, 100–200 μ l plasma was extracted as described

above, reverse transcribed with megaplex reverse transcriptase primer pools and further pre-amplified with human PreAmp pools (ABI, Foster City, CA, USA). The pre-amplified complementary DNAs were subsequently quantified with TaqMan miRNA assays using miR-92a as an internal control. HBV genotyping was performed based on genotype-specific PCR as described [14].

Cell culture and transfection

The Huh-7 cell line was maintained in Dulbecco's modified Eagle's medium supplemented with fetal calf serum, L-glutamine, penicillin and streptomycin (GIBCO BRL, Grand Island, NJ, USA). Cell viability was measured by CCK8 kit (DOJINDO Laboratory, Kumamoto, Japan). miRNA mimics and inhibitors were purchased from RiboBio (Guangzhou, China). A replicative HBV construct (pHBV3.8 or pHBV1.3) was co-transfected with miRNAs using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 48-well

Table 1. Baseline clinical characteristics and early virological response rates of enrolled patients

Clinical variable	Entire cohort (n=94)	Training group (n=66)	Testing group (n=28)	P-value ^a
Median age, years (range)	29 (18–54)	29 (18–47)	31 (18–54)	0.1694 ^b
Gender				
Male, n (%)	60 (64)	40 (61)	20 (71)	0.3179
Female, n (%)	34 (36)	26 (39)	8 (29)	–
Median ALT, ×ULN (±sd)	3.42 ±2.06	3.40 ±2.07	3.48 ±2.08	0.7552 ^b
HBV DNA, log ₁₀ copies/ml (±sd)	7.05 ±1.13	7.22 ±1.14	6.65 ±1.01	0.0664 ^b
HBeAg-positive, n (%)	77 (81.9)	58 (87.9)	19 (67.9)	0.8197
HBV genotype				
B, n (%)	25 (26.6)	19 (28.8)	6 (21.4)	–
C, n (%)	68 (72.3)	46 (69.7)	22 (78.6)	0.6108 ^c
D, n (%)	1 (1.1)	1 (1.5)	0	–
Early virological response	42.6	45.5	35.7	0.4948

^aTraining group versus test group; Fisher's exact test. ^bUnpaired Student's *t*-test. ^cOnly genotype frequency of B and C were analysed. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; ULN, upper limit of normal.

plates along with pcDNA3.1-Gluc, which encodes secreted Gaussia luciferase (Gluc). HBsAg and HBeAg in the supernatant were quantified 48 h after transfection by ELISA (Kehua Biotech, Shanghai, China). Gluc activity was measured using renilla luciferase substrate (Promega, Madison, WI, USA) and a Berthold luminometer (Lumat LB 9507). HBV core particle DNA was extracted as previously described [15]. Quantitative PCR of HBV core particle DNA was conducted using an HBV real-time PCR kit (Qia-gen, Shenzhen, China).

Results

Classification of early virological response to IFN therapy using a plasma miRNA signature

A total of 94 CHB patients receiving IFN therapy were enrolled in this study. Among them, 81.9% were HBeAg-positive and the mean HBV viral load was 7.05 log₁₀ copies/ml. Pretreatment plasma samples were subjected to miRNA expression profiling by agilent human miRNA array. A total of 66 well-defined cases were used as the training group and the miRNA predictor generated was subsequently tested in a validation group receiving conventional IFN. The baseline clinical variables (that is, age, gender, baseline ALT level and HBV DNA level) between these two groups showed no statistical difference ($P>0.05$; Table 1), which ensured their comparability. The early virological response rates (EVR) in the training and validation groups were 45.5% and 35.7%, respectively ($P=0.4948$; Fisher's exact test).

The general strategy for differentiating cases with good or poor EVR is described in detail in the *Methods* (Figure 1). We employed OneR classifier in Weka in a cohort of 66 patients, who received PEG-IFN- α 2a and PEG-IFN- α 2b treatment, to rank the miRNAs

according to their significance to the IFN treatment response. The most relevant miRNA features for prediction are exemplified by the OneR score (Table 2).

Next, we used the SVM algorithm to generate classification. Leave-one-out cross-validation was performed to confirm the performance. To determine the optimal number of miRNAs that could give the best classification outcome, IFS was used to evaluate the cross-validation accuracy (Figure 2A). It was found that the highest accuracy was achieved (74.2%, MCC=0.482) when 11 miRNA features were combined (Figure 2A); these features included hsa-let-7a, hsa-miR-30a, hsa-miR-1290, hsa-miR-106b, hsa-miR-1224-5p, hsa-miR-939, hsa-miR-1281, hsa-miR-198, hsa-let-7f, hsa-miR-22 and hsa-miR-638 (Table 2).

We then confirmed whether the miRNA signature could discriminate EVR in the testing group. As shown in Table 1, the basal clinical variables in the test group were comparable with those in the training group, except that conventional IFN was administered. Nevertheless, the model still yielded an overall accuracy of 71.4% in the test group (MCC=0.378).

To validate the microarray data, TaqMan miRNA qPCR assays (Applied Biosystems, Foster City, CA, USA) were performed on the top two miRNAs (that is, let-7a and miR-30a). Correlations analysis showed that the microarray signals could correlate well with the Δ Ct values of the TaqMan real-time PCR assays (let-7a, $r=0.559$, $P=3.24\times 10^{-4}$; miR-30a, $r=0.634$, $P=2.94\times 10^{-4}$; see Additional file 1).

Comparison of the miRNA predictor with prognosis-associated clinical variables

To compare the significance of the miRNA predictor with other prognosis-associated clinical parameters, we performed univariate logistic regression analysis on independent factors associated with EVR. It was

Table 2. Summary of response-related microRNAs

Order	microRNA	OneR score	Mean intensity in RR	Mean intensity in NR	Validated targets	Reported functions
1	hsa-let-7a	66.6667	2.9	5.4	TRIM71, NF2, STAT3	Down-regulated by HBx and targets STAT3, modulates IL6-dependent survival signalling
2	hsa-miR-30a	65.1515	16.2	12.0	ZNF294, WNT5A, TNFRSF10B, TNFAIP2, B-myb	Inhibits senescence, differentially expressed in the PBMCs of patients with chronic hepatitis C
3	hsa-miR-1290	65.1515	22.9	22.2	NA	NA
4	hsa-miR-106b	62.1212	31.3	28.9	CDKN1A/p21, E2F1, TβR II	Promotes cellular proliferation, impairs TGF-β signalling
5	hsa-miR-1224-5p	62.1212	14.5	10.3	Sp1	Regulates LPS-induced TNF-α production; hypermethylated in urothelial cell carcinoma
6	hsa-miR-939	62.1212	48.7	36.7	NA	NA
7	hsa-miR-1281	62.1212	14.8	14.2	NA	NA
8	hsa-miR-198	62.1212	5.7	4.5	CyclinT1	Inhibits HIV-1 gene expression, down-regulated in HCV-induced HCC
9	hsa-let-7f	62.1212	2.4	3.8	Myc, cyclinD1, STAT3	Inhibits thyroid cancer cell growth, down-regulated by HBx and targets STAT3
10	hsa-miR-22	60.6061	432.9	390.7	PTEN, HDAC4, MYCBP	Regulates PTEN/AKT pathway, inhibits HCC proliferation and anchorage-independent growth
11	hsa-miR-638	60.6061	468.5	450.4	NA	Involved in HCV entry, up-regulated in replicative senescence

HBx, HBV x protein; HCC, hepatocellular carcinoma; IL-6, interleukin-6; LPS, lipopolysaccharide; NA, not applicable; NR, non-responder; PBMC, peripheral blood mononuclear cell; PTEN/AKT, phosphatase and tensin homologue/protein kinase B; RR, rapid responder; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor β; TNF-α, tumour necrosis factor α.

observed that the miRNA signature was highly associated with EVR ($P=2.12 \times 10^{-5}$, OR=7.35, 95% CI 2.93–18.52; Table 3). In addition, serum ALT was an important prognostic factor ($P=0.002$, OR=1.47, 95% CI 1.15–1.88), whereas female sex ($P=0.127$), HBV DNA ($<5 \times 10^7$ versus $\geq 5 \times 10^7$, $P=0.078$) and HBV genotype (B versus C, $P=0.290$) did not reach statistical significance in this study (Figure 2B; Table 3). Indeed, elevated ALT level was observed in the RR group compared with the NR group ($P=4.65 \times 10^{-4}$; Mann–Whitney U test; see Additional file 2), whereas HBV DNA level was not significantly different ($P=0.069$; Additional file 2). Furthermore, multivariate analysis demonstrated that the miRNA predictor was an independent factor with a 6.62-fold increase in the likelihood of EVR ($P=2.00 \times 10^{-4}$, 95% CI 2.44–17.86; Table 3). Serum ALT remained significant in the multivariate analysis (OR=1.37, $P=0.029$, 95% CI 1.07–1.76). Stratification of HBeAg-positive cases in the cohort showed similar results, as the miRNA predictor was still strongly associated with EVR ($P=4.74 \times 10^{-4}$, OR=5.95, 95% CI 2.19–16.13; see Additional file 3). However, in HBeAg-positive patients, female sex showed a significant association with EVR ($P=0.034$, OR=2.78, 95% CI 1.08–7.14), whereas HBV DNA was still statistically irrelevant ($P=0.136$; see

Additional file 3). It seems that gender might influence treatment outcome in an HBeAg-dependent manner.

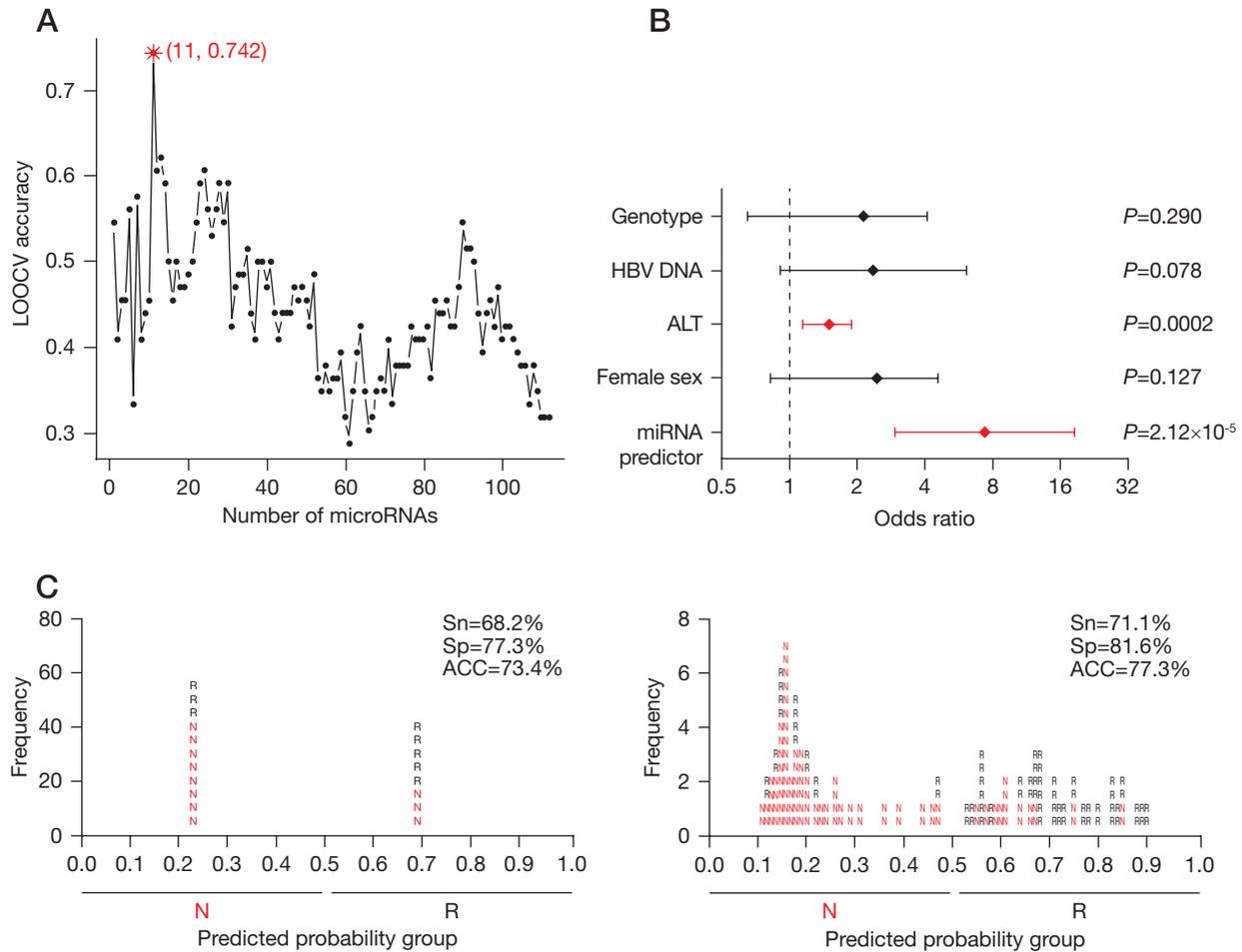
Combining the miRNA profile and ALT level improved overall accuracy

As the logistic regression analysis found miRNA and ALT level to be the most important factors for EVR, we examined whether the prediction accuracy could be improved if the ALT level was also considered. As shown in Figure 2C, the logistic regression model yielded a moderate increase in the overall accuracy from 73.4% to 77.3% when both factors were combined, as illustrated by the classification plot. It is worth noting that both sensitivity (68.2–71.7%) and specificity (77.3–81.6%) were improved by this approach, suggesting the synergism of the combined prediction.

High degree of correlation between liver and plasma miRNA profiles

miRNAs in the plasma fluid are presumed to be secreted products from various organs in the human body. The liver is a vital organ for detoxification and plasma protein synthesis; the liver has been reported to release miRNAs into circulation in normal and pathological conditions [10]. Previous reports have shown

Figure 2. Evaluation and optimization of the prediction model



(A) Incremental feature selection curve of microRNA (miRNA) features. The leave-one-out cross-validation (LOOCV) accuracy is plotted against increasing number of miRNAs. (B) Odds ratio plot of various clinical parameters and miRNA predictor. (C) Frequency plot of logistic regression analysis using the miRNA predictor only (left-hand panel) and the miRNA plus alanine aminotransferase (ALT; right-hand panel). ACC, accuracy; N, non-response; R, rapid response; Sn, sensitivity; Sp, specificity.

Table 3. Univariate and multivariate logistic regression analyses of factors associated with EVR^a

Clinical variable	Odds ratio	95% CI	P-value
Univariate analysis			
miRNA predictor	7.35	2.93–18.52	2.12×10⁻⁵
Age in years	0.99	0.94–1.05	0.84
Female sex	1.94	0.83–4.57	0.127
Serum ALT (×ULN)	1.47	1.15–1.88	0.002
HBV DNA<5×10 ⁷ versus ≥5×10 ⁷	2.35	0.91–6.09	0.078
HBV genotype (B versus C)	1.64	0.65–4.14	0.290
Multivariate analysis			
Serum ALT (×ULN)	1.37	1.07–1.76	0.029
miRNA predictor	6.62	2.44–17.86	2.00×10⁻⁴

Factors that have a P-value <0.05 are presented in bold. ^aAnalysis was performed on the entire cohort (n=94). ALT, alanine aminotransferase; CI, confidence interval; ULN, upper limit of normal.

that the quantification of miRNAs in FFPE samples highly correlated with that in fresh frozen samples [16]. Therefore, we performed miRNA profiling on 13 FFPE liver biopsy samples from the enrolled patients. The basic clinical characteristics are shown in Additional file 4. We evaluated whether the plasma miRNA profile could, to some extent, reflect that in the liver. As expected, we observed a close relationship between the liver and plasma profiles in every individual ($r=0.26-0.57$, $P=0.001-4.06\times 10^{-11}$, Pearson correlation) and in the overall analysis ($r=0.39$, $P<10^{-250}$; Figure 3). When only considering the 11 miRNAs from the predictor, a significant correlation was also evident ($r=0.36$, $P=6.64\times 10^{-6}$). These results suggest that the circulating miRNA profile might at least partially reflect that in the liver.

Potential role of selected miRNAs in the life cycle of HBV

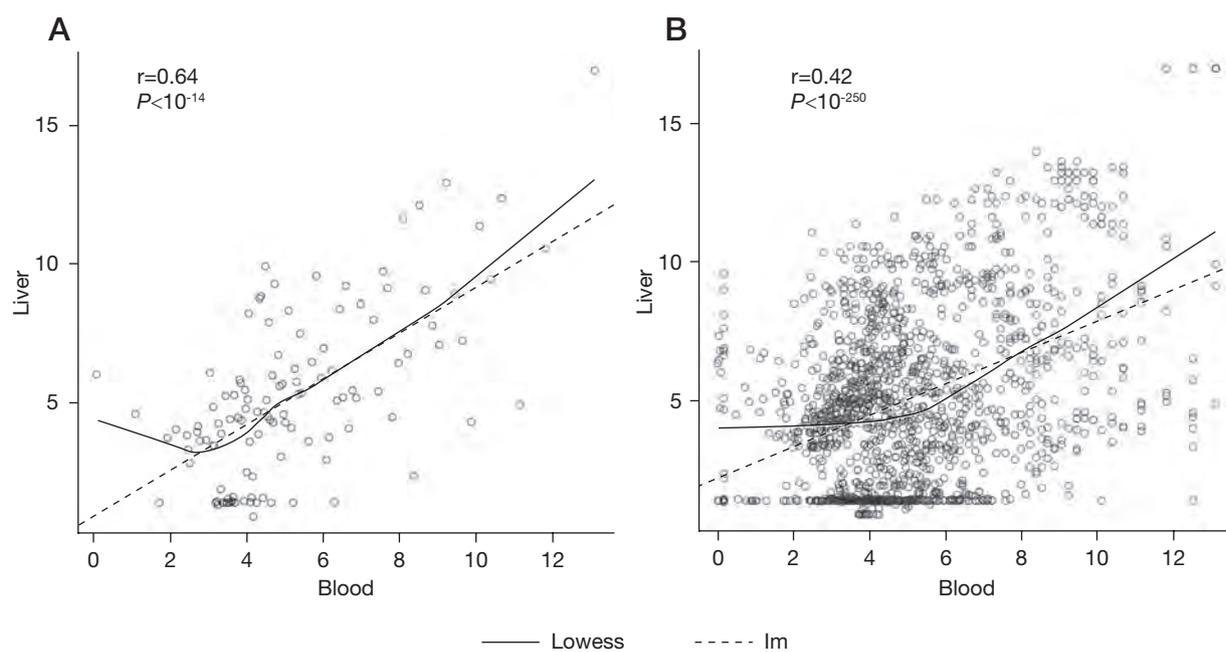
To further investigate whether the selected miRNAs played a functional role in regulating HBV replication, we performed co-transfection of miRNA mimics or inhibitors with two HBV replicative constructs (pHBV3.8 with a 1.2 copy genome [subtype adr, genotype C] [15] and pHBV1.3, a 1.3 copy HBV construct [subtype adw, genotype B] [17]). miRNAs that altered HBsAg/HBeAg secretion in both the pHBV1.3 and

pHBV3.8 systems were considered to possess HBV modulatory activity. The initial screening showed that let-7f, miR-939 and miR-638 mimics can significantly inhibit viral replication ($P<0.01$; Figure 4A). The miR-1281 inhibitor showed the highest potency in inhibiting HBV replication ($P<0.001$; Figure 4B), whereas its mimic had no effect. However, the miR-1281 inhibitor could non-specifically inhibit gene expression, as the Gluc control was also significantly suppressed (data not shown), suggesting that miR-1281 inhibitor might interfere with other cellular processes. Dose-dependent response experiments indicated that let-7f had a significant effect when 20 nM of mimic was transfected (Figure 4C). miR-939 and miR-638 also inhibited HBV viral antigen expression in a dose-dependent manner (Figure 4C). It is worth noting that these miRNAs did not show significant cell toxicity effects, as illustrated in the CCK-8 assay (Figure 4D). Quantification of core particle DNA suggested that miR-939, miR-638 and let-7f could inhibit viral DNA synthesis ($P<0.0001$), whereas a negative control, miR-1290, had no significant effect (Figure 4E).

Discussion

Successful treatment of CHB significantly decreases the development of cirrhosis, liver failure and hepatocellular

Figure 3. Correlation analysis of liver-plasma microRNA expression profile

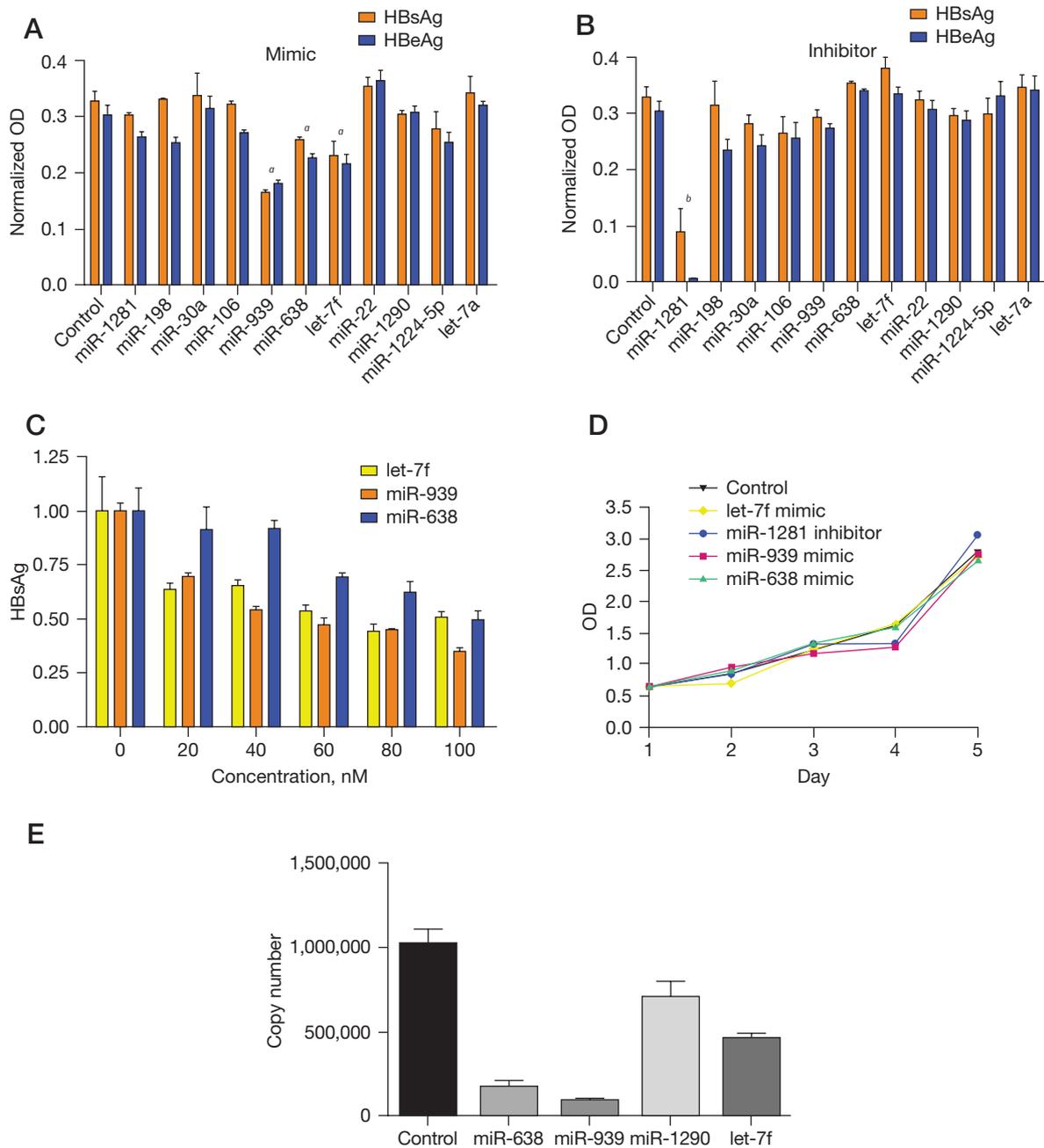


The plasma and corresponding hepatic microRNA profile was compared in (A) patient No. 4622 and (B) in all the 13 patients. lm, least square regression; Lowess, locally weighted regression.

carcinoma [18]. Conventional interferon, which has suboptimal pharmacokinetics and an inconvenient dosing schedule, can only clear HBeAg and HBV DNA in 19% of patients [19]. The introduction of PEG-IFN to

HBV-infected individuals improves the seroconversion rate and facilitates the clearance of viral DNA [2,20]. However, the partial responses, high cost and debilitating side effects associated with IFN therapy often

Figure 4. Potential role of selected microRNAs in regulation of HBV replication



(A) microRNA (miRNA) mimics or (B) inhibitors were co-transfected with pHBV1.3 and pcDNA3.1-Gluc into Huh-7 cells. Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the supernatant 48 h after transfection were measured by ELISA. Gaussia luciferase (Gluc) expression was measured as internal control. (C) Dose-dependent response of selected miRNAs on HBsAg expression. (D) A CCK-8 cell proliferation assay was conducted after transfection of selected miRNAs. (E) Quantification of core particle DNA after transfection of selected miRNAs. OD, optical density.

restrain patients from taking this option. A reliable prediction model is needed to selectively enrol patients who have an increased likelihood of responding to this therapy and to direct those who have a lower chance of response towards nucleoside analogue-based therapy.

Until now various clinical parameters, such as gender, ALT levels, HBV DNA, HBV genotype and dynamics of HBsAg titre during therapy, have been examined to predict the outcome of IFN therapy on CHB. However, these factors are still of limited value as their prediction accuracy is often marginal [5,7]. There is a dearth of reports on predicting therapy outcome in CHB patients using genomics-based technology. By contrast, there is plenty of evidence to suggest that genome-wide mRNA and/or miRNA expression profiles are valuable indicators of clinical response in chronic HCV infection. Chen *et al.* [21] discovered that baseline expression of some IFN-stimulated genes can significantly discriminate non-responders from rapid responders, this phenomenon was subsequently confirmed by other groups [22,23]. Genome-wide association studies in multiple cohorts also provided unexpected markers, two single-nucleotide polymorphisms of the IL28B gene were recently identified as highly associated with spontaneous resolution as well as outcome of PEG-IFN therapy [24,25]. For miRNAs, there is also a report suggesting that the hepatic miRNA profile is associated with response to IFN treatment in chronic hepatitis C [26].

Circulating miRNAs have been established recently as novel biomarkers for various cancers and other diseases [9,10]. In this study, we attempted to use the plasma miRNA profile to predict the outcome of IFN therapy in CHB patients. Although standard IFN differs from PEG-IFN in its pharmacokinetics and virological/serological response rate, they both activate very similar short-term antiviral and long-term immunoregulatory pathways in the human body. Molecular markers that can predict response to IFN therapy would be most desirable for clinical application. Hence, we developed the prediction model in patients receiving PEG-IFN and then tested it in an independent group receiving conventional IFN. A similar association was found between the miRNA profile and the EVR in the testing group, suggesting its general applicability.

SVM, a supervised machine-learning methodology, can perform well with noisy data in which no simple linear separation is possible. When using an appropriate kernel, a non-linear mapping to a feature space is automatically generated. SVM has been successfully used to address a wide variety of biological problems, such as evaluation of microarray data and predicting protein fold. On the basis of this methodology we obtained an optimized model (overall accuracy 74.2%) composed of 11 response-associated miRNAs, which was further validated in a test group. In addition, the inclusion of

ALT data improved the accuracy to 77.3%. This finding suggests the complementarity of the miRNA profile with existing prognosis-associated parameters. The clinical variables (HBV DNA, genotype, and so on) that are reported to be associated with response could not be used to distinguish the outcome. The insignificance of these parameters is possibly caused by the limited size of our cohort, but it might also suggest that their predictive power is relatively low.

Plasma or serum samples are the best material in terms of feasibility and ease of handling for clinical practice. Recent reports suggest that miRNA can be actively secreted in the form of exosome into the circulation, which provided a mechanistic association between plasma miRNA level and specific organ dysfunction. For example, let-7 family members are selectively secreted from a metastatic gastric cancer cell line [27]. Exosome secretion by hepatocytes has also been characterized [28] and is postulated as a cell-to-cell communication mechanism in various physiological or pathological conditions. In our study, we found that the liver is a major contributor of circulating miRNAs, and plasma miRNA levels partially reflect the dynamics of miRNAs in hepatocytes. These findings support the possibility of using circulating microRNAs as prediction biomarkers in personalized therapy of viral hepatitis and other diseases.

Interestingly, some of the selected miRNAs associated with EVR are also reported to be modulated by HBV *in vitro*. For example, let-7a,c and f were reported to be down-regulated in HepG2.2.15 cells which harbour the complete HBV genome and replicate actively [29]. A recent report suggested that let-7 family members were down-regulated by HBx protein [30]. We observed that over-expression of let-7f had an inhibitory effect on viral replication. In addition, miR-939 and miR-638 can also modulate HBV replication. By analysing their putative target genes in multiple databases and filtering through the HBV-Human Interaction Map database, a putative interaction map was developed (Additional file 5). Genes such as cyclinD1, cyclinA2 and CDKN1A(p21), which are key regulators of cell cycles, are enriched in this analysis. In addition, NFKB2, STAT3 and STAT5B, which are involved in diverse biological processes, might also account for the inhibition. The exact mechanism of action of the selected miRNAs is worthy of further investigation.

In conclusion, our study shows that a plasma miRNA profile, made up of 11 miRNAs, can be used to predict the initial response of HBV patients receiving IFN injections. When the ALT and miRNA predictor are combined in the logistic regression model, the overall sensitivity and specificity can be further improved. Co-transfection with HBV replicative constructs suggested that let-7f, miR-939 and miR-638 can modulate HBV

replication. Therefore, we propose that the miRNA profile constitutes a novel circulating marker that can help to predict response to IFN treatment in CHB patients. We believe that when combined with basic clinical parameters and other genetic (for example, IL28B SNP [31]) and epigenetic markers, more reliable treatment indexes can be developed and ultimately applied to the clinic. A larger confirmatory study is being conducted for further validation and improvement of this model. Further mechanistic investigation of the selected miRNAs is underway and might yield new information about the miRNA signalling network and HBV life cycle. In turn, this knowledge might help us to develop novel antiviral therapies for CHB patients.

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Disclosure statement

The authors declare no competing interests.

Additional files

Additional file 1: A supplementary figure showing a correlation analysis of the let-7a and miR-30a expression in microarray and TaqMan qPCR experiments can be found at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2322_Zhang_Add_file_1v2.pdf

Additional file 2: A supplementary figure showing the Mann–Whitney U test of ALT level and HBV DNA between the rapid response and no response groups can be found at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2322_Zhang_Add_file_2.pdf

Additional file 3: A supplementary table showing factors associated with EVR in HBeAg-positive patients can be found at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2322_Zhang_Add_file_3.pdf

Additional file 4: A supplementary table showing clinical variables of patients undergoing FFPE analysis can be found at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2322_Zhang_Add_file_4.pdf

Additional file 5: A supplementary figure showing the network analysis of miR-939, miR-638 and let-7f can

be found at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2322_Zhang_Add_file_5.pdf

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