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RAPID COMMUNICATION

A pilot study of serum microRNA signatures as a novel biomarker for occult hepatitis B virus infection

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Abstract The implementation of hepatitis B surface antigen (HBsAg) screening tests has significantly enhanced blood transfusion safety. However, the transmission of HBsAg-negative blood components can still occur in the acute phase of infection during the seronegative window period or during chronic stages of infection such as occult hepatitis virus B infection (OBI). OBI, characterized by the presence of HBV infection without detectable HBsAg, is capable to elude the routine detection with HBV serologic markers and harbor a potential risk of HBV transmission through blood transfusion or organ transplantation. Here, we test the hypothesis that OBI patients have a differentially expressed profile of microRNA (miRNA) in serum, and this unique serum miRNA signature can serve as a biomarker to detect OBI. Employing TaqMan probe-based quantitative reverse transcription polymerase chain reaction (qRT-PCR), we assessed the expression level of miRNAs in serum samples. To control for miRNA quantitation, we added an exogenous plant miRNA, MIR156a, into the samples before RNA extraction and used it as an internal control. After screening 13 previously identified HBV-specific serum miRNAs, we obtained four miRNAs, let-7c, miR-23b, miR-122, and miR-150, which are differentially expressed in OBI sera compared to healthy control sera. This 4-serum miRNA signature shows a high level of accuracy in discriminating both OBI (AUC = 0.999) and HBV (AUC = 0.989) cases from the non-infected controls. Cluster analysis also demonstrates that this 4-miRNA signature can clearly separate OBI patients from the control group. Our results demonstrate for the first time that a profile of serum miRNAs can serve as a sensitive and accurate biomarker for OBI detection.

Keywords OBI · HBV · Serum miRNA · Diagnosis · RT-qPCR

Abbreviations

HBV	Hepatitis B virus
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
OBI	Occult HBV infection
HBsAg	Hepatitis B surface antigen
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AUC	Area under curve
ROC	Receiver operating characteristic

Introduction

Although the implementation of hepatitis B surface antigen (HBsAg) in blood screening in the 1970s significantly increased transfusion safety, transmission by HBsAg-negative blood components can still occur in the acute phase of infection during the seronegative window period or during chronic stages of infection, such as occult hepatitis virus B

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infection (OBI) [1–5]. Patients with OBI generally contain HBV DNA in blood or liver tissues, but are negative for HBsAg. Some OBI maybe anti-HBc positive; therefore, anti-HBc was often included in blood donor screening to detect occult hepatitis B infections in blood donors. However, more than 20% of the occult carriers are negative for all serum markers of HBV infection [6]. These occult carriers may evade blood screening and thus serve as the source of HBV transmission because current blood screening practices largely depend on HBsAg detection.

Accurately detecting occult HBV infection cases is crucial to eliminate potential infection during blood transfusion processes involving HBV-infected populations. Compared to chronic HBV cases, OBI patients generally have a lower serum viral load that is usually less than 200 IU/ml [5, 7]. Previous studies show different mechanisms for controlling viral replication in seropositive and seronegative occult infections [8–10]. Although the mechanism causing OBI remains undefined, several factors have been suggested to play a potential role, including mutations of the HBV DNA sequence, integration of HBV DNA into host's chromosomes, infection of peripheral blood mononuclear cells by HBV, formation of HBV-containing immune complexes, altered host immune response, and interference of HBV by other viruses. The clinical implications of occult HBV infection are varied, but it is clear that OBI can harbor HBV transmission through blood transfusion, hemodialysis, and organ transplantation. OBI may also serve as the cause of cryptogenic liver diseases and contribute to acute exacerbation of chronic hepatitis B and development of hepatocellular carcinoma. In addition, OBI may affect the disease progression and treatment response of chronic hepatitis C. Since some OBI patients are HBsAg negative with or without serologic markers of previous viral exposure, the diagnosis of these infected cases is difficult and usually requires a highly sensitive HBV DNA PCR assay.

The differential expression of microRNAs (miRNAs) in both human and animal models has recently been linked to many forms of disease, especially cancer [11–15]. As a class of non-coding RNAs whose processed products are ~22 nucleotides in length, miRNAs regulate gene expression at the post-transcriptional level in plants and animals [16]. Accumulating evidence suggests an association between HBV infection and miRNA expression [17–19]. Recent studies by us [20–23] and other investigators [24–28] further demonstrated that miRNAs stably expressed in serum, plasma, synovial fluid, and urine could be used as molecular fingerprints to detect various types of diseases. These findings may open a new avenue for the study of molecular mechanisms underlying hepatitis virus B infection and the diagnosis of OBI.

The aim of the present study is to test the hypothesis that OBI patients have a unique serum miRNA profile and that this miRNA signature can be used to discern OBI patients from healthy controls. Our previous study had identified 13 miRNAs that were differentially expressed in serum of HBV patients compared to normal and HCV patients [23]. Here, we employed a quantitative RT-PCR (qRT-PCR) assay to analyze the level of 13 HBV-associated miRNAs [23] in serum samples from 11 OBI patients, 30 HBV infection cases, and 29 healthy controls. The results demonstrate that the expression pattern of four serum miRNAs, let-7c, miR-23b, miR-122, and miR-150, can serve as sensitive and specific non-invasive biomarker for OBI diagnosis.

Materials and methods

Patient characteristics and clinical features

The present study was approved by the Institutional Review Board of Nanjing University and Nanjing Medical University, Nanjing, China, and written informed consent was obtained from each participant. As shown in Table 1, we recruited 30 healthy controls (age, mean $\pm$ SD: 35.60 $\pm$ 15.27 years; range: 20–61 years; 14 men and 16 women), 29 people with chronic HBV infection (age, mean $\pm$ SD: 39.24 $\pm$ 11.53 years; range: 17–61 years; 17 men and 12 women), and 11 OBI patients (age, mean $\pm$ SD: 42.82 $\pm$ 15.96 years; range: 23–60 years; 5 men and 6 women) from the First Affiliated Hospital of Nanjing Medical University and the Nanjing Second Hospital, Nanjing, China. As shown in Table 1, OBI patients are negative for HBV surface antigens and have normal ALT and AST activities, while chronic HBV patients are positive in HBV surface antigens and have high ALT and AST activities. OBI patients have a lower viral DNA load than chronic HBV patients. Of the 11 OBI patients, 6 are anti-HBc positive.

HBsAg was tested with Diagnostic Kit for Antibody to Hepatitis B Surface Antigen (ELISA) (Shanghai Kehua Bio-Engineering Co., Shanghai, China). The detection wavelength was 450 nm, and reference wavelength was 630 nm. The minimum detectable amount by this assay is 10 IU/ml. For various controls, we obtained OD values in various ranges: a blank control (OD $\leq$0.015), a negative control (OD $\leq$0.05), and a positive control (OD $\geq$1.000). HBV DNA was tested with "Quantitative Diagnostic Kit for Hepatitis B Virus DNA (PCR-Fluorescent Probing)" (QIAGEN, Valencia, CA). The minimum detectable amount of DNA is 5×10^2 IU/ml. The linear working range is 1×10^3–5×10^7 IU/ml. The amounts of DNA in various controls are the following: negative control—0 IU/ml;

Table 1 Clinical features and classification of three serum sample groups

Variable	CTL (30)	HBV (29)	OBI (11)	Reference range
Age ± SD (year)	35.60 ± 15.27	39.24 ± 12.83	42.82 ± 17.96	
	Range: 20–61	Range: 14–67	Range: 23–80	
Gender	Male: 14	Male: 17	Male: 5	
	Female: 16	Female: 12	Female: 6	
HBsAg ± SD (IU/ml)	0.03 ± 0.01 (Negative)	237.53 ± 49.56 (Positive)	0.03 ± 0.01 (Negative)	0.00–0.05
	Range: 0.01–0.04	Range: 23.90–250.00	Range: 0.01–0.04	
Anti-HBs (mIU/ml)	Positive: 18	Positive: 3	Positive: 5	0.00–10.00
	Negative: 12	Negative: 26	Negative: 6	
HBeAg ± SD (S/CO)	0.42 ± 0.09 (Negative)	442.85 ± 427.36 (Positive)	0.4 ± 0.12 (Negative)	0.00–1.00
	Range: 0.29–0.54	Range: 2.94–1,144	Range: 0.25–0.67	
Anti-HBe (S/CO)	20.70 ± 24.42 (Negative)	Positive: 8	Positive: 4	1.00–999.00
	Range: 1.46–61.29	Negative: 21	Negative: 7	
Anti-HBc ± SD (S/CO)	0.29 ± 0.13	9.64 ± 2.91 (Positive)	Positive: 6	0.00–1.00
	Range: 0.15–0.53	Range: 4.18–14.31	Negative: 5	
ALT ± SD (IU/l)	22.1 ± 7.4	278.1 ± 315.3	20.4 ± 10.9	0.0–40.0
	Range: 6.9 ± 35.6	Range: 48.3–1,170.1	Range: 8.5–39.7	
AST ± SD (IU/l)	19.5 ± 9.1	183.1 ± 228.5	26.3 ± 10.4	0.0–40.0
	Range: 7.4 ± 38.7	Range: 49.5–952.5	Range: 12.5–40.7	
HBV–DNA ± SD (copies/ml)	$<5.0 \times 10^2$	Range: 1.1×10^5–5.0×10^9	Range: 2.4×10^3–3.3×10^7	$<5.0 \times 10^2$
Genotype		Type B (13)	Type B (2)	
		Type C (16)	Type C (4)	
			ND[a] (5)	

[a] *ND* not detected

strong positive control—1×10^5–1×10^7 IU/ml; and weak positive control—1×10^3–9×10^4 IU/ml.

Five milliliter of venous blood was collected from each participant during his/her first admission to the hospital. To harvest cell-free serum, each coagulated blood sample was immediately centrifuged at 3,000 rpm for 10 min, and the supernatant serum was recovered and then stored at −80°C until analysis.

RNA isolation and qRT-PCR assay

RNA isolation and Solexa sequencing were performed as described previously [20]. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). A synthetic, non-human miRNA, plant MIR-168, was equally spiked into serum samples prior to RNA extraction. The level of plant MIR-168 served as an internal control in serum miRNA quantitative analysis. TaqMan probe-based qRT-PCR assays were conducted to quantify the serum levels of miRNAs as previously described [20, 22, 23]. In each step from serum purification to qRT-PCR, equal volumes of serum were processed. The qRT-PCR assays were performed in triplicate using the same ABI 7300 machine. The expression levels of miRNAs were calculated using the C_T values. The ratio of the two groups of serum miRNAs was calculated with the equation $2^{-\Delta G}$, in which $\Delta G = C_{T\ group1} - C_{T\ group2}$. All primers used were obtained from Applied Biosystem Company (Carlsbad, CA). The assay IDs are the following: hsa-let-7c (000379), hsa-miR-23b (000400), hsa-miR-122 (002245), and hsa-miR-150 (000473). Based on the standard curve, the linear range of serum miRNA in our qRT-PCR assay is 2–2×10^7 fmol/l. One hundred fmol/l of synthesized miRNA was added into the serum to serve as an internal control.

Statistical analysis

To compare the average fold change of miRNAs in HBV patient sera and normal control sera, a paired *t* test was used. Values of $P < 0.01$ were considered statistically significant. In addition to controlling variations throughout the steps, we also standardized all of the data to the mean of zero and the SD of 1 before clustering. We used hierarchical clustering in Cluster 3.0 with the complete linkage method. For risk scoring, the 5 or 95% (relative to the reference group) reference interval of each miRNA, denoted as *t*, was set as the threshold to code the expression level of the corresponding miRNA for each sample. The risk score of each miRNA, denoted as *s*, was calculated as:

$$95\%\ \text{threshold},\ s_{ij} = \begin{cases} 0 & \text{if } r_{ij} < t_j \\ 1 & \text{otherwise} \end{cases} \quad (1)$$

$$5\%\ \text{threshold},\ s_{ij} = \begin{cases} 1 & \text{if } r_{ij} < t_j \\ 9 & \text{otherwise} \end{cases} \quad (2)$$

In this formula, we used i to denote the ith sample and j to denote the jth miRNA. We then presented frequency tables and receiver operating characteristic (ROC) curves to evaluate the diagnostic effects of the profiles. The statistical analyses were performed with Statistical Analysis System software (v.9.1.3; SAS Institute, Cary, NC).

Results

Our recent report demonstrated that patients with HBV infection had a unique serum miRNA profile. The expression profile of these HBV-associated miRNAs in serum could be used as a biomarker in the detection of HBV and HBV-positive HCC [23]. To test whether the expression profile of HBV-associated miRNAs in serum can be used as a signature to discern OBI, we employed a TaqMan probe-based qRT-PCR assay to measure the level of all HBV-associated miRNAs. The miRNAs we investigated in the serum samples from OBI, HBV, and control groups include the following: miR-375, miR-92a, miR-10a, miR-223, miR-423, miR-23b/a, miR-342-3p, miR-99a, miR-122a, miR-125b, miR-150, and let-7c. To warrant the consistency in the experimental procedures that involved RNA extraction from serum samples and qRT-PCR, we spiked exogenous plant MIR156 into the serum samples before RNA extraction. The MIR156 RNA was then used as an internal control for normalizing miRNA expression levels. As shown in Table 1, OBI patients are negative in HBV surface antigens and have normal ALT and AST activities and a low viral DNA loading compared to chronic HBV patients. We identified four miRNAs, let-7c, miR-23b, miR-122, and miR-150, with significantly increased expression levels in OBI serum compared to control serum. All four miRNAs have been reported to be associated with chronic HBV infection [23]. These miRNAs were also significantly altered in the chronic HBV group compared to the control group.

Next, we analyzed whether the expression of the four serum miRNAs correlates with the genotype of HBV and OBI cases. According to the method described by Naito et al [29], we designed primers for HBV genotyping of the 29 chronic hepatitis B infections as well as the 11 OBI patients and performed the genotype assays. As shown in Table 1, of the 29 HBV cases, 16 cases are C type and 13 cases are B type. There is no significant difference in the expression of the four serum miRNAs between B and C type (let-7c, $P = 0.28449$; miR-23b, $P = 0.457055$; miR-122, $P = 0.356391$; miR-150, $P = 0.335812$). Among 11 OBI cases, 4 cases are C type, 2 cases are B type, and 5 cases are undefined. Undetectable PCR signals using genotyping primers in these OBI cases are likely due to the low amount of HBV DNA in OBI serum. Since the 11 OBI cases are found both anti-HBc positive (6 of 11) and anti-HBc negative (5 of 11), we further analyzed these samples separately. This analysis indicates that the expression of three miRNAs, let-7c, miR-122, and miR-150, is not significantly different between the anti-HBc-positive and anti-HBc-negative OBI cases. However, the expression of miR-23b in anti-HBc-negative cases is 3.35-fold higher than in anti-HBc-positive cases ($P = 0.023$).

The differential expression of the 4-miRNAs in OBI, HBV, and control groups is detailed in Table 2. Compared to non-infected controls, OBI and HBV cases had significantly elevated levels of let-7c (11.74 ± 2.12- and 7.49 ± 1.87-fold, respectively), miR-23b (95.88 ± 26.24-

Table 2 C_T values, relative concentrations, and fold changes of four serum miRNAs in the three groups

miRNA	Sample/no	Mean $C_T \pm$ SD	Median relative concentration (fM/l)	Fold change	P*
let-7c	CTL/30	31.03 ± 1.24	0.337	1.00	Ref.
	HBV/29	29.10 ± 1.16	1.907	5.65	0.000761[a]
	OBI/11	28.23 ± 0.56	4.691	13.90	0.000177[b]
miR-23b	CTL/30	30.87 ± 0.61	0.266	1.00	Ref.
	HBV/29	28.85 ± 1.15	1.666	6.26	0.004893[a]
	OBI/11	26.79 ± 1.34	22.842	85.78	0.001763[b]
miR-122	CTL/30	30.84 ± 1.62	0.306	1.00	Ref.
	HBV/29	26.13 ± 1.14	24.655	80.55	0.002568[a]
	OBI/11	26.26 ± 1.25	32.701	106.83	0.004950[b]
miR-150	CTL/30	27.75 ± 1.48	6.549	1.00	Ref.
	HBV/29	25.96 ± 0.99	47.626	7.27	0.000381[a]
	OBI/11	25.25 ± 1.11	88.394	13.50	0.006278[b]

* All P values are calculated by Student's t test

[a] "HBV" versus "CTL"

[b] "OBI" versus "CTL"

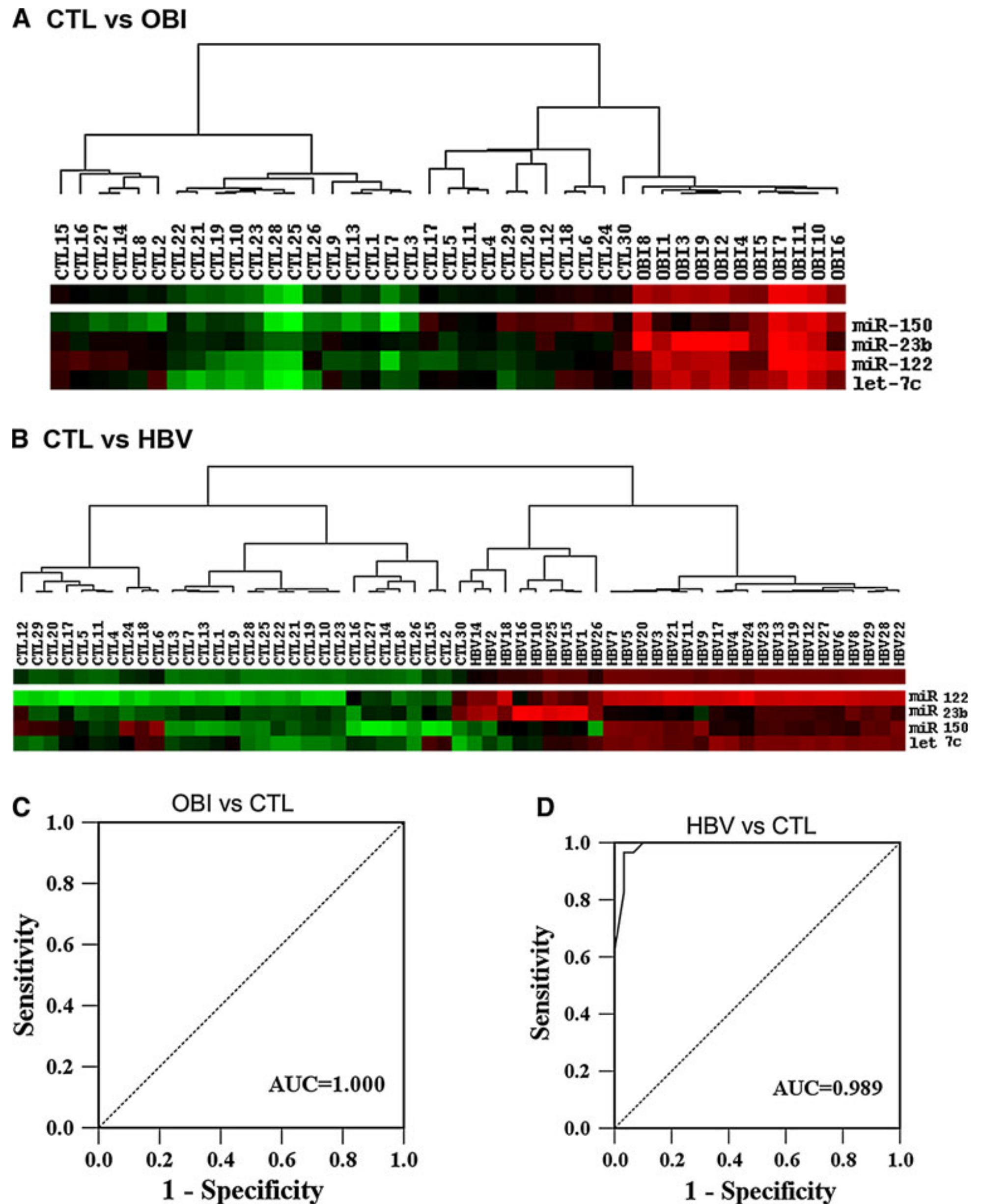

Fig. 1 Separation of OBI and HBV serum samples from controls by the 4-serum miRNA-based biomarker. **a** Clustering analysis based on the 4-serum miRNA profile was performed using samples from OBI, HBV, and control groups. **c** and **d** ROC curves between control and OBI groups (**c**), control and HBV groups (**d**)

and 15.01 ± 5.14-fold, respectively), miR-122 (71.02 ± 23.13- and 92.93 ± 30.81-fold, respectively), and miR-150 (8.70 ± 2.65- and 3.84 ± 0.74-fold, respectively). Interestingly, when we used the profile of these four miRNAs as a biomarker to classify the serum samples from OBI, HBV, and control group, we found that it correctly separated OBI and HBV patients from the controls (Fig. 1a, b).

To evaluate the diagnostic value for this 4-serum miRNA profile in discriminating OBI and HBV cases from the controls, we further performed a risk scoring procedure on these data sets. Using the 5 or 95% reference interval of each miRNA expression value as risk scores, we constructed ROC curves and estimated the sensitivity and specificity for prediction. As shown in Fig. 1c, when the 4-miRNA biomarker was used to separate control and OBI patients, the AUC was 99.9 ± 0.0% (sensitivity: 99.9%, specificity: 99.8%). As expected, the 4-miRNA biomarker could separate the HBV group from the controls with a high specificity and sensitivity (AUC: 98.9 ± 1.0%, sensitivity: 99.1%, specificity: 98.8%) (Fig. 1d).

Discussion

Although the precise prevalence of OBI is unknown, it is clear that OBI can harbor potential risks of HBV transmission through blood transfusion, hemodialysis, and organ transplantation. OBI can also cause cryptogenic liver

disease, contribute to acute exacerbation of chronic hepatitis B, alter the disease progression and treatment response of chronic hepatitis C, or lead to the development of hepatocellular carcinoma. Because OBI patients do not have detectable hepatitis B surface antigens (HBsAg) and generally have low HBV levels (<10^4 IU/ml, often below 100 IU/ml) [5], normal assays using HBsAg are likely to be ineffective in detecting OBI. Some OBI cases are anti-HBc positive, and anti-HBc can be included as a serology parameter to detect OBI infection. However, there is still a considerable portion of OBI patients who are negative for all serum markers of HBV infection [6]. Therefore, a novel approach to detect and study OBI is required.

In the present study, we identified four miRNAs, let-7c, miR-23b, miR-122, and miR-150, which were differentially expressed in OBI serum compared to control serum. The profile of these four miRNAs has been shown to be a highly sensitive and accurate biomarker for OBI detection. These four miRNAs have been widely reported to be involved in hepatocyte dysfunction, viral infection, and immune response. Shimizu et al. [30] found that the overexpression of let-7c or let-7g led to a clear decrease of Bcl-xL expression in Huh7 and HepG2 cell lines, suggesting that let-7 miRNAs negatively regulate Bcl-xL expression in human hepatocellular carcinomas and induces apoptosis. Up-regulation of let-7 also leads to the reversal of epithelial to mesenchymal transition in gemcitabine-resistant cancer cells [31]. MiR-122 is a well-established liver-specific miRNA and accounts for over half of the total amount of miRNAs in liver tissues [32–34]. Jopling et al. [32] demonstrated that miR-122 could modulate the abundance of hepatitis C virus RNA in infected liver tissues. Using in vivo delivery of anti-miR-122 antisense oligonucleotide (ASO), Esau et al. [34] reported a regulation of lipid metabolism by miR-122. The miRNA miR-23b has been shown to regulate transforming growth factor-β (TGF-β) signaling and liver stem cell differentiation by targeting Smad [35]. Although not directly related to liver, the immune response-related miRNA [36] miR-150 plays a critical role in regulating various aspects of cellular functions, including the control of cell fate [37], early B-cell development [38], and B-cell differentiation [39]. These previous studies using cell and tissue samples indicate that a dysregulated expression of the four miRNAs may reflect a specific pathophysiological status. Here, we show that elevation of four miRNAs in serum can be used as a signature for OBI detection. In addition, because the levels of these four serum miRNAs are not directly dependent on serologic biomarkers such as antigen or antibody, but are related to whole-body immune response, the serum miRNA profile may be an effective biomarker for detecting OBI cases. In other words, the 4-serum miRNA signature can detect HBV infection cases regardless of whether the patient is HBsAg positive or negative.

To establish the expression profile of serum miRNAs as a practical indicator in clinical diagnosis of OBI, a large number of OBI cases collected from different regions and different age groups must be tested. However, although limited to only 11 OBI cases in this pilot study, the high accuracy of the 4-miRNA signature in discriminating OBI from control group strongly suggests that it has a great potential to emerge as a novel blood-based biomarker in OBI detection.

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Conflict of interest The authors have no conflict of interest to declare.

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