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Serum microRNA characterization identifies *miR-885-5p* as a potential marker for detecting liver pathologies

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ABSTRACT

Circulating miRNAs (microRNAs) are emerging as promising biomarkers for several pathological conditions, and the aim of this study was to investigate the feasibility of using serum miRNAs as biomarkers for liver pathologies. Real-time qPCR (quantitative PCR)-based TaqMan MicroRNA arrays were first employed to profile miRNAs in serum pools from patients with HCC (hepatocellular carcinoma) or LC (liver cirrhosis) and from healthy controls. Five miRNAs (i.e. *miR-885-5p*, *miR-574-3p*, *miR-224*, *miR*-215 and *miR-146a*) that were up-regulated in the HCC and LC serum pools were selected and further quantified using real-time qPCR in patients with HCC, LC, CHB (chronic hepatitis B) or GC (gastric cancer) and in normal controls. The present study revealed that more than 110 miRNA species in the serum samples and wide distribution ranges of serum miRNAs were observed. The levels of *miR-885-5p* were significantly higher in sera from patients with HCC, LC and CHB than in healthy controls or GC patients. *miR-885-5p* yielded an AUC [the area under the ROC (receiver operating characteristic) curve] of 0.904 [95% CI (confidence interval), 0.837–0.951, $P < 0.0001$) with 90.53% sensitivity and 79.17% specificity in discriminating liver pathologies from healthy controls, using a cut off value of 1.06 (normalized). No correlations between increased *miR-885-5p* and liver function parameters [AFP (α-fetoprotein), ALT (alanine aminotransferase), AST (aspartate aminotransferase) and GGT (γ-glutamyl transpeptidase)] were observed in patients with liver pathologies. In summary, *miR-885-5p* is significantly elevated in the sera of patients with liver pathologies, and our data suggest that serum miRNAs could serve as novel complementary biomarkers for the detection and assessment of liver pathologies.

INTRODUCTION

Liver diseases including CHB (chronic hepatitis B), LC (liver cirrhosis) and HCC (hepatocellular carcinoma) are public health concerns and major medical challenges in the People's Republic of China [1,2]. Clinically, a panel of serological biochemical markers including aminotransferase [ALT (alanine aminotransferase)/AST

Key words: biomarker, cirrhosis, liver pathology, microRNA, *miR-885-5p*, serum.

Abbreviations: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; FNH, focal nodular hyperplasia; GC, gastric cancer; GGT, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocellular carcinoma; LC, liver cirrhosis; Mamm *U6*, mammalian *U6*; miRNAs, microRNAs; NC, normal control; qPCR, quantitative PCR; ROC, receiver operating characteristic; AUC, the area under the ROC curve; RT, reverse transcription; RT-preamp-qPCR, RT-preamplification-qPCR; snRNA, small nuclear RNA.

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(aspartate aminotransferase)] and AFP (α-fetoprotein) have been used to monitor liver pathologies for several decades, but they have limited sensitivity and specificity particularly with regard to the insidious progress of HCC [3,4].

miRNAs (microRNAs) are small non-coding RNAs of 22–25 nucleotides that play important roles in regulating gene expression by binding to and regulating the activity of their target mRNAs post-transcriptionally, contributing to diverse cellular processes such as proliferation, differentiation, apoptosis and carcinogenesis [5]. Expression of miRNAs is consistently deregulated in some physiopathological conditions including cancer [6–9]. miRNAs in plasma/serum are emerging as a new class of potential markers for minimally invasive diagnosis and monitoring of patients with several diseases [10–12]. Serum *miR-21* has been reported to be elevated in lymphoma patients [13], plasma *miR-92a* can be used to identify patients with colorectal cancer [14] and *miR-1* and *miR-208* could serve as indicators of acute myocardial infarction [15,16].

The regulatory roles of miRNAs in the development of liver-related pathologies including HCC, CHB and chronic hepatitis C have been elucidated [17–20], and circulating miRNAs including *miR-122* and *miR-192* have been reported to have diagnostic value for toxin-induced hepatocellular damage in mice [21]. However, the potential clinical value of circulating miRNAs for diagnosing and managing liver pathologies has not been evaluated. The present study investigates the hypothesis that differentially expressed miRNAs circulate in patients with liver pathologies. We first profiled serum miRNA expression in patients with HCC and LC. Next, five differently expressed miRNAs were selected and further evaluated in independent patient cohorts. Our study indicates that the various levels of specific miRNAs, especially *miR-885-5p*, could serve as markers for the preclinical detection and clinical assessment of patients with such diseases.

MATERIALS AND METHODS

Study design

The present study was performed in three phases: (i) global serum miRNA profiling using a TaqMan Human MicroRNA Array, in combination with Megaplex RT and Megaplex preamplification techniques; (ii) analysis and preliminary evaluation of candidate miRNAs; and (iii) independent validation of selected miRNAs. Each phase was conducted using independent groups of participants.

Participants

To screen miRNAs more efficiently as potential markers for liver-related diseases during the circulating miRNA profiling phase, we first generated seven pools of serum aliquots derived from individuals in the case and control groups. In brief, serum samples from 15 pathologically confirmed HCC patients with moderate histological grade and various degrees of liver cirrhosis (light to moderate) were randomly divided into three groups to generate three serum pools, and serum samples from ten patients clinically diagnosed as moderate to decompensated LC were similarly used to generate two pools. Each serum pool consisted of equal volumes of serum from all patients within the group. In addition, serum samples from ten age-matched healthy individuals were obtained to generate two NC (normal control) pools, so a total of seven serum pools were generated for miRNA profiling.

To quantify individual miRNAs, independent sets of serum samples were obtained between November 2008 and January 2010 from patients with liver pathologies [CHB, LC, HCC, ICC (intrahepatic cholangiocellular carcinoma) and FNH (focal nodular hyperplasia)] who were admitted to or hospitalized in the Department of Hepatobiliary Surgery or the Department of Gastroenterology in the Chinese PLA General Hospital (Beijing).

Collectively, none of the enrolled HCC, ICC and FNH patients had received any adjuvant therapy or other treatment before blood sampling, none of the CHB and LC patients had received treatment within 3 weeks before blood sampling and most of the LC patients were diagnosed as HBV (hepatitis B virus)-related liver cirrhosis. Additionally, age-matched healthy individuals were recruited as NCs, and serum samples from patients with newly diagnosed GC (gastric cancer) were used as disease controls. The baseline characteristics of the recruited subjects are displayed in Supplementary Table S1 (available at http://www.clinsci.org/cs/120/cs1200183add.htm) and see Table 2.

Written informed consent was obtained from each individual or their responsible guardians. This study protocol was approved by the ethics committee of the Chinese PLA General Hospital (Beijing).

Serum sample processing and RNA isolation

Peripheral blood was collected in tubes containing separating gel and clot activator and centrifuged at 3400 ***g*** for 7 min at room temperature, and the supernatants were transferred to Eppendorf tubes. The samples were centrifuged at 15 000 ***g*** for 8 min to precipitate cell debris, and the supernatants were stored at -80°C pending RNA extraction. All blood samples were processed within 24 h after they were obtained.

Total serum RNA was isolated and eluted in 100 μl of RNase-free water using a mirVana PARIS kit (#1556; Ambion) following the manufacturer's protocol for

liquid samples. For miRNA profiling, 800 μl of pooled serum was used, and for individual miRNA tests, 400 μl of serum from each participant was used. All serum RNA preparations were quantified using a DU 800 spectrophotometer (Beckman Coulter) and then pretreated with RNase-free DNase I (Promega) to eliminate potential DNA contamination.

Serum miRNA profiling and data analysis

In our study, a three-step procedure was performed to profile the miRNAs in the pooled serum samples. First, for cDNA synthesis from the miRNAs, 10 ng of total RNA from the pooled serum was subjected to RT (reverse transcription) using a TaqMan® microRNA Reverse Transcription Kit (#4366596; Applied Biosystems) and Megaplex RT primers (Human Pool A, #4399966; Applied Biosystems) following the manufacturer's protocol, allowing simultaneous reverse transcription of 380 mature human miRNAs to generate an miRNA cDNA library corresponding to each serum pool. RT was performed on a Mastercycler Epgradient thermocycler (Eppendorf) with the following cycling conditions: 40 cycles of 16 °C for 2 min, 42 °C for 1 min and 50 °C for 1 s, followed by a final step of 80 °C for 5 min to inactivate the reverse transcriptase.

Thereafter, to generate enough miRNA cDNA template for the following real-time PCR, the cDNA libraries were pre-amplified using Megaplex PreAmp primer (Humam Pool A, #4399233; Applied Biosystems) and PreAmp Master Mix (#4384266; Applied Biosystems) following the manufacturer's instructions. The PreAmp primer pool used here consisted of forward primers specific for each of the 380 human miRNAs and a universal reverse primer. The preamplification cycling conditions were as follows: 95 °C for 10 min, 55 °C for 2 min, 72 °C for 2 min followed by 12 cycles of 95 °C for 30 s and 60 °C for 4 min; the samples were then held at 99.9 °C for 10 min.

After the preamplification step, the products were diluted with RNase-free water, combined with TaqMan gene expression Master Mix and then loaded into TaqMan Human MicroRNA Array A (#4398965; Applied Biosystems), which is a 384-well formatted plate and real-time PCR-based microfluidic card with embedded TaqMan primers and probes in each well for the 380 different mature human miRNAs; the Mamm *U6* (mammalian U6) transcript was used as a normalization signal. qPCR (quantitative PCR) was performed according to the manufacturer's instructions. Real-time PCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems) with the following cycling conditions: 50 °C for 2 min, 94.5 °C for 10 min followed by 40 cycles of 95 °C for 30 s and 59.7 °C for 1 min. The C_t (cycle threshold) was automatically given by SDS 2.3 software (Applied Biosystems) and is defined as the fractional cycle number at which the fluorescence passes the fixed threshold of 0.2. Mamm *U6* embedded in the TaqMan Human MicroRNA Arrays was used as an endogenous control.

The relative expression levels of miRNAs were calculated using the comparative $\Delta\Delta C_t$ method as described previously [22,23]. The fold changes in miRNAs were calculated by the equation $2^{-\Delta\Delta C_t}$. Cluster 3.0 software was used to perform unsupervised hierarchical clustering using Pearson's correlation metrics and average linkage methods. Java Treeview 1.1.3 was used to visualize the clustering results.

Measurement of liver function parameters

The concentrations/activities of serum aminotransferases (ALT and AST) and AFP in each serum sample were measured using a Hitachi 7600 Modular Analytic system.

TaqMan miRNA assay for individual miRNAs

Individual miRNA tests were performed on independent sets of serum samples and a two-step procedure was used. First, 5 ng of total RNA isolated from 400 μl of each serum sample was subjected to RT using an miRNA-specific primer from the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) as described previously [10]. Briefly, RT was conducted in a scaled-down RT reaction volume of 7.5 μl, which contained 2.08 μl of water, 0.75 μl of 10× RT buffer, 0.095 μl of RNase inhibitor, 0.075 μl of dNTPs with dTTP, 0.5 μl of multiscribe reverse transcriptase, 1.5 μl of miRNA-specific stem-loop RT primer (Applied Biosystems) and 2.5 μl (5 ng) of digested RNA preparations. RT was carried out on a Mastercycler Epgradient at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min.

Thereafter, real-time qPCR was performed using a TaqMan MicroRNA assay (Applied Biosystems) to quantify individual miRNAs as described previously [10]. In brief, 4.5 μl of 5:28 diluted RT product was combined with 5.0 μl of TaqMan gene expression Master Mix and 0.5 μl of Taqman miRNA assay mix. qPCR was carried out on an ABI PRISM 7300 sequence detection system at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCR reactions were performed in triplicate and the C_t values greater than 35 from the real-time PCR assays were treated as 35. The PCR products were analysed by electrophoresis on 3.0 % agarose gels to validate the specific generation of the expected product. The expression levels ($2^{-\Delta\Delta C_t}$) of miRNAs were calculated as described previously [22].

Statistical analysis

The Mann–Whitney test or Kruskal–Wallis test was performed to determine the significance of serum miRNA

levels. MedCalc 9.6.4 software was used to construct ROC (receiver operating characteristic) curves and calculate the AUC (area under the ROC curve). Linear regression analysis was used to examine correlations between the levels of the miRNAs and liver function parameters. P values <0.05 were considered statistically significant. All statistical calculations were performed using SPSS software (version 11.0).

RESULTS

Serum miRNA profiling and data analysis

The first aim of the present study was to investigate whether aberrantly expressed miRNAs are present in patients with liver pathologies. To screen differentially expressed miRNAs in serum samples efficiently, we generated seven serum pools of serum aliquots derived from individuals in the NC, LC and HCC groups (two NC, two LC and three HCC pools). The circulating miRNAs in the seven serum pools were profiled separately using RT-preamp-qPCR (RT-preamplification-qPCR) as described in the Materials and methods section.

The data indicated that a total of 115 miRNAs could be detected (assays giving C_t values <33 in at least four pools were classed as detectable) (results not shown). By comparing the miRNA profiles between HCC and NC and LC and NC, two miRNA expression patterns were obtained (Table 1). This process generated a list of more than 20 up-regulated candidate miRNAs. As shown in Table 1, the average levels of 26 miRNAs in the HCC serum pools and 22 miRNAs in the LC serum pools were higher than in NC.

To investigate the relative abundances of the miRNAs detected, they were normalized in each serum pool (NC, LC and HCC) to Mamm *U6* in the corresponding pool. As shown in Figure 1, miRNAs such as *miR-223*, miR-16 and *miR-146a* were present in relatively high abundance in the serum samples, while *miR-99a*, *miR-224*, *miR*-192, *miR*-128 and *miR*-100 were present at significantly lower abundance compared with the corresponding Mamm *U6* (28.1 in NC, 29.4 in LC and 30.0 in HCC pool, respectively). In addition, the abundances of different miRNA species in a specified serum pool varied widely. For example, the relative abundances (normalized to Mamm *U6*) of *miR-223* and *miR-100* in the NC serum pool were 295.09 and 0.00026, respectively.

Furthermore, unsupervised hierarchical clustering analysis demonstrated that different serum pools had different miRNA spectra (Figure 2A), and a small panel of miRNAs including *miR-146a*, *miR-224*, *miR-574-3p* and *miR-885-5p* were clustered together because of their similarly up-regulated expression in HCC serum pools compared with NC serum pools (Figure 2B).

Table 1 Up-regulated miRNAs in serum pools of HCC and LC

Fold change was calculated as described previously [14] and is presented as $2^{-\Delta\Delta C_t}$. miRNAs with mean fold change >2 in HCC serum pools ($n = 3$) and >1.5 in LC serum pools ($n = 2$) when compared with NC ($n = 2$) are listed respectively. miRNAs underlined are common miRNAs with up-regulated levels in both HCC and LC when compared with NC. The five miRNAs highlighted in bold represent HCC/LC-related candidate miRNAs selected for subsequent validation.

miRNAs in serum of HCC compared with NC		miRNAs in serum of LC compared with NC	
miRNA	Average fold change	miRNA	Average fold change
miR-99a	191.3	*miR-99a*	328.4
miR-224	87.1	*miR-100*	59.5
miR-100	82.5	*miR-125b*	29.7
miR-122	16.3	miR-128	14.7
miR-885-5p	**13.5**	***miR-215***	**14.4**
miR-125b	8.5	*miR-224*	14.1
miR-95	7.9	*miR-192*	8.9
miR-215	7.8	*miR-194*	8.2
miR-134	7.3	*miR-133a*	7.7
miR-194	6.3	*miR-210*	7.0
miR-192	5.7	*miR-375*	6.6
miR-193b	5.7	*miR-423-5p*	5.7
let-7b	5.2	*miR-185*	5.3
miR-133a	5.1	*miR-141*	5.2
miR-483–5p	5.0	*miR-122*	5.2
miR-886–3p	4.7	*miR-19b*	4.3
miR-574-3p	**4.6**	***miR-885-5p***	**2.7**
let-7d	3.7	*miR-16*	2.7
miR-146a	**3.4**	***miR-574-3p***	**1.9**
miR-18a	3.1	*miR-30b*	1.8
miR-141	2.9	***miR-146a***	**1.6**
miR-486-5p	2.8	*miR-26a*	1.5
miR-222	2.7		
miR-181a	2.5		
miR-92a	2.2		
miR-16	2.0		

Candidate miRNAs selection and preliminary evaluation

In this phase, preliminary tests of candidate miRNAs were carried out on a second set of serum samples including NC ($n = 16$) and patients with LC ($n = 12$) or HCC ($n = 20$) (Supplementary Table S1).

Initially, the expression of *U6* snRNA (small nuclear RNA) and *miR-16* (both proposed as the most commonly used internal controls in the literature [14,24–26]) in sera from NC, LC and HCC was determined using real-time qPCR in order to select suitable internal controls. Both *U6* snRNA and *miR-16* were specifically and consistently detected in all sera (Supplementary Figures 1A and 1B available at

Table 2 Clinical characteristics of participants in validation

Ages are given as means (S.D.), and AFP, ALT, AST and TB are given as medians (range). No-HCC, patients with liver diseases including nine with ICC and six with FNH. In the LC group, 18 patients had decompensated liver function. HBsAg, hepatitis B surface antigen; ND, not detected; TB, total bilirubin.

Group	HCC	Non-HCC	LC	CHB	GC	NC
Individuals (*n*)	46	15	26	23	17	24
Gender (*n*)						
Male	39	12	23	17	15	17
Female	7	3	3	6	2	7
Age (years)	54.2 (9.2)	45.7 (8.6)	61.2 (10.2)	41.3 (8.9)	58 (7.5)	50.6 (5.6)
AFP (ng/ml)	38.8 (1.62–20000)	2.97 (1.1–422.3)	4.22 (0.61–365.9)	3.6 (1.22–23.7)	1.81 (1.02–6.87)	2.6 (1.23–6.38)
Abnormal (AFP >20 ng/ml) (*n*)	24	3	3	2	0	0
Normal (AFP<20 ng/ml) (*n*)	22	12	19	11	12	24
ND (*n*)	0	0	4	10	5	0
ALT (units/l)	34.1 (17.8–421.9)	29.8 (9.1–281.8)	28.9 (14.5–101.9)	27.4 (5.8–137.2)	13.8 (7.6–50.1)	17.2 (13.1–32.6)
AST (units/l)	31.1 (9.1–281.8)	26.3 (9.4–540.5)	33.7 (14.7–85.1)	25.1 (10.2–296)	15.8 (10.6–24.1)	15.1 (8.2–29.6)
GGT (units/l)	61.2 (11.5–908.0)	62.3 (15.7–1310)	59.3 (24.8–199.3)	26.1 (12.3–118.5)	18.6 (7.6–135.2)	ND
TB (μmol/l)	14.2 (6.0–227.3)	11.4 (5.4–377.2)	19.6 (7.5–47.7)	10.3 (2.8–32.8)	9.6 (2.3–56.9)	ND
HbsAg (*n*)						
Positive	33	2	20	23	0	0
Negative	13	12	5	0	16	24
Not known	0	1	1	0	1	0
Cirrhosis (*n*)						
Normal	0	13	0	Unknown	–	–
Low	17	2	0	Unknown	–	–
Moderate	29	0	12	Unknown	–	–
High	0	0	14	Unknown	–	–
Histological grade (*n*)						
Good	6	–	–	–	–	–
Moderate	32	–	–	–	–	–
Poor	8	–	–	–	–	–

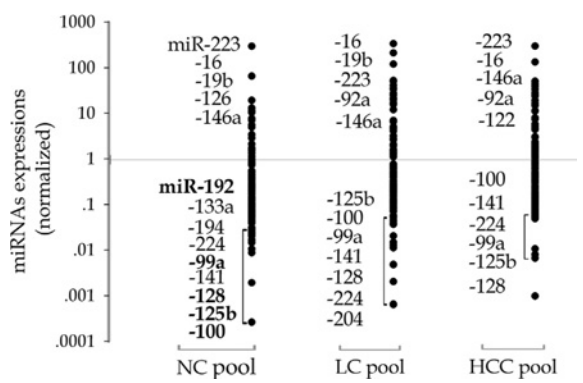

Figure 1 Expression of 115 miRNAs in representative serum pools from the NC, LC and HCC groups

miRNAs were quantified using RT-preamp-qPCR as described in the Materials and methods section. The relative abundance of each miRNA is presented as $2^{-\Delta C_t}$, where ΔC_t was calculated by subtracting the C_t values of Mamm *U6* (28.1 in NC, 29.4 in LC and 30.0 in HCC pool, respectively) from the *C*t of each miRNA detected within the corresponding groups. The levels of various miRNAs are shown (normalized) in representative NC, LC and HCC serum pools.

http://www.clinsci.org/cs/120/cs1200183add.htm), with an average raw C_t of 32.8 for *U6* snRNA and 24.7 for *miR-16*. No significant differences in raw C_t values of *U6* snRNA were detected among the three groups ($P = 0.128$, Kruskal–Wallis test), but there was variability among the raw C_t values within the respective groups. *miR-16* was significantly higher in HCC sera than in NC and LC ($P = 0.014$, Kruskal–Wallis test). Therefore *U6* snRNA was selected as the internal normalization control for the subsequent miRNA qPCR.

Additionally, miRNAs such as *miR-99a*, *miR-100* and *miR-125b* were excluded from further analysis because their expression levels were significantly lower than Mamm *U6* in global miRNA profiling (Figure 1). As a pilot study, the potentially increased expressions of a subset of miRNAs (i.e. *miR-146a*, *miR-215*, *miR-224*, *miR-574-3p* and *miR-885-5p*) were assessed using a TaqMan microRNA assay (Figure 2 and Table 1). Both *miR-885-5p* and *miR-146a* were consistently and specifically detected in all 48 serum samples (i.e. all with average C_t values <35) (Supplementary Figure 1C).

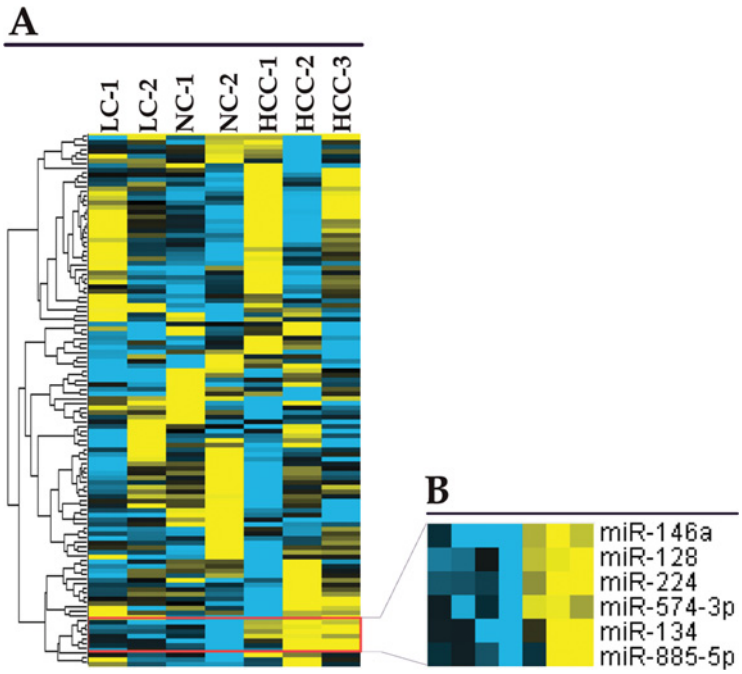

Figure 2 Serum miRNAs profiled using real-time qPCR-based arrays

(A) miRNA expression profiles in two NC, two LC and three HCC serum pools. Assays with C_t values <33 in at least four pools were classed as detectable, and a total of 115 miRNAs were detected. The expression levels of miRNAs were normalized to Mamm *U6* and data are presented as $2^{-\Delta C_t}$. miRNA expression was mean-centred, and a heat map was generated using Pearson's correlation metrics and average linkage methods of Cluster 3.0 software. Samples are shown in columns and miRNAs in rows. (B) *miR-146a*, *miR-128*, *miR-224*, *miR-574-3p*, *miR-134* and *miR-885-5p* were clustered together. The colour of each cell represents the expression level of the corresponding miRNA in the corresponding sample. The higher intensities of yellow relate to higher expression levels, and the increasing intensities of blue reflect lower expression of miRNAs.

However, regarding mean C_t <35 as a positive signal, the detection rates for *miR-574-3p* and *miR-224* were 71 % and 23 %, respectively.

Using *U6* snRNA as the normalization control, we demonstrated that *miR-885-5p* was significantly higher in HCC and LC serum samples than in NC ($P < 0.0001$), with a 6.5-fold increase in HCC and an 8.8-fold in LC (Figure 3A). In addition, increased amounts of serum *miR-146a* and *miR-224* were observed in the HCC and LC groups (Figures 3B and 3C). However, no significant differences in the levels of *miR-574-3p* were observed among the NC, LC and HCC groups (Figure 3D), and the low level of *miR-215* made it difficult to quantify its abundance in the sera accurately using real-time qPCR (most mean C_t values >35).

Validation of *miR-885-5p* as a potential liver pathology-associated marker

Considering that the level of *miR-885-5p* was consistently increased in serum samples from the HCC and LC groups (Figure 3A), the abundance of this miRNA was further measured in another independent cohort of serum samples consisting of LC ($n = 26$), HCC ($n = 46$), CHB ($n = 23$), ICC ($n = 9$), FNH ($n = 6$) and NC ($n = 24$). In addition, 17 patients with GC were included as disease controls to investigate the specificity of *miR-885-5p* as a liver pathology-associated molecule. Information regarding the disease control individuals in terms of demographics, biochemical testing, virological investigation and histological grade is summarized in Table 2.

The data demonstrated that patients with HCC, LC or CHB had significantly ($P < 0.0001$) higher serum levels of *miR-885-5p* than normal controls and GC patients (Figure 4A and Supplementary Table S2 at http://www.clinsci.org/cs/120/cs1200183add.htm). There was no statistically significant difference in the serum levels of *miR-885-5p* between normal controls and patients with GC ($P = 0.19$, Mann–Whitney test). Furthermore, ROC analysis indicated that using a cut off value of 1.06 ($2^{-\Delta C_t}$ in comparison with *U6* snRNA), *miR-885-5p* yielded an AUC of 0.904 (95 % CI: 0.837–0.951, $P < 0.0001$) that could be used to discriminate patients with liver pathologies (LC, $n = 26$; HCC, $n = 46$; and CHB, $n = 23$) from normal controls ($n = 24$), with specificity and sensitivity of 90.5 and 79.2 %, respectively (Figure 4B). For comparison, ALT was used to distinguish patients from healthy controls and yielded an AUC of

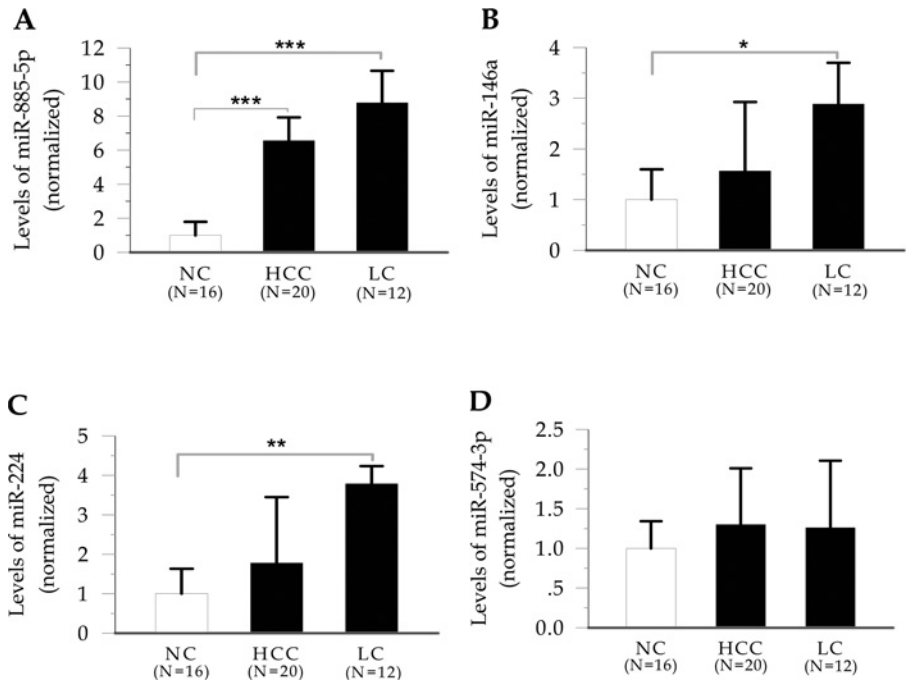

Figure 3 Comparisons of levels of serum miRNAs in NC, HCC and LC groups

Serum abundances of *miR-885-5p* (A), *miR-146a* (B), *miR-224* (C) and *miR-574-3p* (D) in an independent set of serum samples from NC ($n = 16$), HCC ($n = 20$) and LC ($n = 12$) were quantified using real-time qPCR. Each qPCR was carried out in triplicate in 96-well plates. Expression levels of selected miRNAs were normalized to *U6* snRNA and are presented as fold changes ($2^{-\Delta\Delta C_t}$) above NC. Data are shown as the mean fold changes compared with the mean value in NC ($\pm$ S.E.M.). A Mann–Whitney or Kruskal–Wallis test was used to determine statistical significance. ***$P < 0.0001$, **$P < 0.001$, *$P < 0.01$.

0.742 (Figure 4C). In addition, *miR-885-5p* levels in the sera of patients with ICC and FNH were also increased (Figure 4D).

Relationships between serum *miR-885-5p* and liver pathology parameters

To examine whether elevated serum *miR-885-5p* was correlated with traditional liver disease markers such as ALT and AST, linear regression analysis was used to investigate the relationships between *miR-885-5p* and AFP, ALT, AST and GGT in patients with liver pathologies. There was no correlation between serum *miR-885-5p* and ALT in 95 patients with liver pathologies (LC, $n = 26$, HCC, $n = 46$, CHB, $n = 23$) (Supplementary Figure 2A available at http://www.clinsci.org/cs/120/cs1200183add.htm). Similar results were obtained for *miR-885-5p* compared with AST and *miR-885-5p* compared with GGT (results not shown). There was an increase in AFP concentrations in 46 patients with pathologically confirmed HCC (closed circles), but the levels of serum *miR-885-5p* (open circles) did not increase (Supplementary Figure 2B).

Multiple factors including age, gender, *miR-885-5p*, AFP, ALT, AST and GGT in patients with liver pathologies were used to establish a logistic regression model. This model revealed that *miR-885-5p* was a potential marker for the detection of liver pathologies ($P < 0.0001$), and the odds ratio for an individual with *miR-885-5p* >1.12 being associated with liver pathologies was 16.3 (95 % CI: 6.43–25.37).

DISCUSSION

Recent findings have exposed circulating miRNAs as potential biomarkers for several disease conditions including human cancer [10–16,20–21,24–27]. To our knowledge, this report is the first to investigate the feasibility of using serum miRNAs as indicators of liver pathologies. We found that individuals with HCC, LC and CHB had significantly elevated levels of serum *miR-885-5p*. *miR-885-5p* yielded an AUC of 0.904 (95 % CI: 0.837–0.951, $P < 0.0001$) with 90.53 % sensitivity and 79.17 % specificity in terms of discriminating patients with liver pathologies from healthy controls and, more importantly, had a better diagnostic performance than ALT (74.2 %). The odds ratio for individuals with *miR-885-5p* >1.012 having liver pathologies was 16.3 (95 % CI: 6.43–25.37). However, there was no correlation between circulating *miR-885-5p* and AFP, ALT, AST and GGT, and the level of serum *miR-885-5p* did not increase despite an increase of AFP in the HCC group. There was no significant difference in serum levels of *miR-885-5p* between normal controls and patients with GC. These

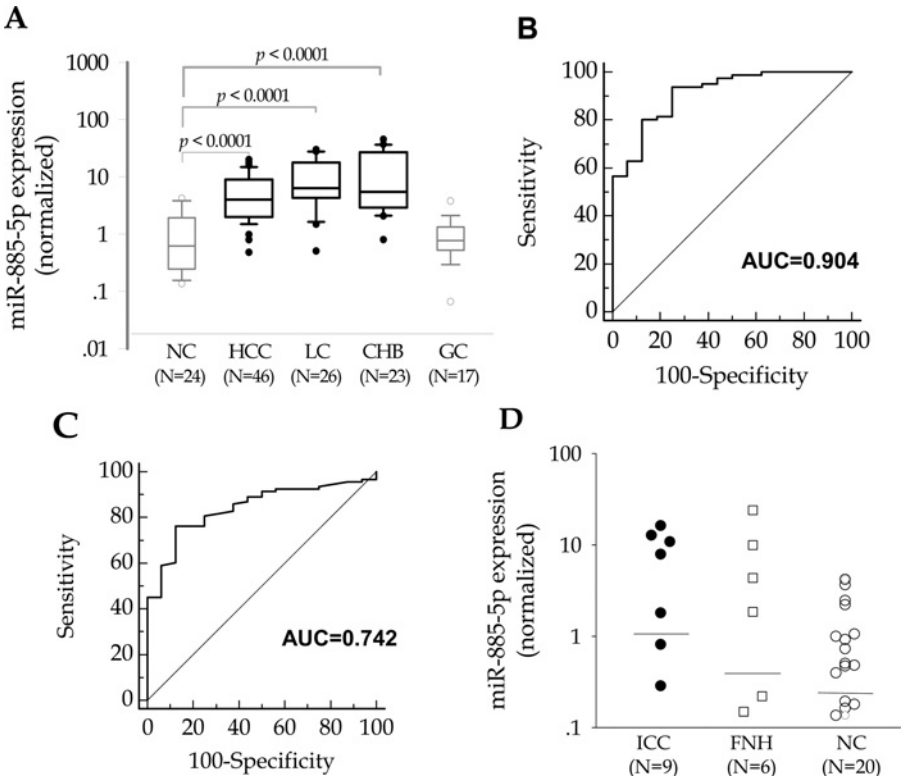

Figure 4 Validation of *miR-885-5p* in an independent set of serum samples

(A) Box plots of serum levels ($2^{-\Delta C_t}$) of *miR-885-5p* in NC ($n = 24$), HCC ($n = 46$), LC ($n = 26$), CHB ($n = 23$) and GC ($n = 17$). Expression levels of miRNAs were normalized to *U6* snRNA (Log10 scale on *y*-axis). The upper and lower borders of the boxes indicate the 75th and 25th percentiles, respectively. The whisker caps indicate the 90th and 10th percentiles. The horizontal lines in the boxes indicate the median values. Outliers are illustrated as circles. A Mann–Whitney or Kruskal–Wallis test was used to determine statistical significance. (B) ROC curve analysis of serum *miR-885-5p* to differentiate patients with liver pathologies ($n = 95$) from NC ($n = 24$). *miR-885-5p* yielded an AUC of 0.904 with 93.8 % sensitivity and 75.0 % specificity in discriminating liver pathologies. (C) ROC curve analysis indicated that ALT yielded an AUC of 0.742 in distinguishing patients with liver pathologies ($n = 95$) from NC. (D) Scatter plots indicate that the expression of *miR-885-5p* in serum samples from patients with ICC and FNH is greater than in controls (Log_{10} scale on *y*-axis). The horizontal lines across the data points indicate the median values.

results suggest that serum *miR-885-5p* could potentially be an independent and complementary liver pathology-associated biomarker.

Clinically, new biomarkers are necessary to improve the diagnosis and management of patients with liver pathologies, particularly insidious liver impairment and early HCC [1–2,28]. Recent advances in genomics and proteomics have accelerated the discovery of many potential markers for clinical use. The present study comprises a preliminary investigation of the expression of circulating miRNAs in clinical serum samples of patients with liver pathologies. Using RT-preamp-qPCR [12,29,30], more than 110 miRNA species were detected in clinical samples. In agreement with the findings of Mitchell et al. [10], miRNAs such as *miR-223* and *miR-16* were present in the circulation with relatively high abundance (Figure 1). In addition, a wide dynamic range of distribution of serum miRNA species was observed; for example, the relative abundances of *miR-223* and *miR-100* were 295.09 and 0.00026 in the NC serum pool, respectively (Figure 1), which may reflect the different origins and metabolic characteristics of different circulating miRNA species *in vivo*. Furthermore, miRNAs in the pooled serum samples from patients with HCC and LC were differentially expressed. As shown in Figure 1, which shows the relative abundance of each miRNA in three representative serum pools, similar trends were observed when the miRNAs were ranged according to their relative abundances, e.g. with consistently relatively high copy numbers of *miR-16* and relatively low abundance of *miR-100* in each pool. Additionally, some miRNAs showed up-regulated levels in both the HCC and LC (Table 1) serum pools, which may reflect the fact that, in most cases, HCC develops in association with liver cirrhosis. Furthermore, unsupervised hierarchical clustering analysis revealed that different serum pools had different miRNA spectra (Figure 2A), which besides some possible technical variations in miRNAs profiling (discussed below) might indicate that the circulating miRNAs in serum pools

derived from different patients reveal different metabolic characteristics. Additionally, Figure 2(B) shows that *miR-146a*, *miR-224*, *miR-574-3p* and *miR-885-5p* were clustered together because of their similarly up-regulated expression in the HCC serum pools (Figure 2B).

As a pilot study, five miRNAs, *miR-885-5p*, *miR-574-3p*, *miR-224*, *miR*-215 and *miR*-146a, that are up-regulated in the HCC and LC serum pools (Table 1) were selected and further quantified using real-time PCR in independent sets of patients with HCC, LC, CHB, ICC and FNH. Our case control study confirmed that serum *miR-885-5p* is consistently and significantly up-regulated in individuals with HCC, LC and CHB (Figure 4A). The median values of *miR-885-5p* in limited ICC and FNH cases were also elevated (Figure 4D). Nevertheless, although four other miRNAs (*miR-574-3p*, *miR-224*, *miR-215* and *miR-146a*) were increased in the global miRNAs profiling experiment (Table 1), we failed to validate their elevation in the second groups of HCC and LC patients (Figures 3B–3D). A probable reason for the discrepancies is that although the TaqMan microRNA array is promising for simultaneously measuring miRNAs expression in a high-throughput manner [31,32], and the introduction of an miRNA cDNA preamplification step significantly reduces the amount of RNA needed [30], potential preamplification bias [30] may be responsible for some variation in the C_t values and may thus diminish the reproducibility with which miRNAs of low abundance such as miR-*574-3p*, *miR-224* and *miR-215* can be quantified in the serum.

Recently, advances in circulating miRNAs research [10–16] have generated the concept that tissue or organ-specific intracellular miRNAs may be released and/or leaked into the circulation during processes accompanying cell death and/or apoptosis owing to cell turnover, cellular destruction or pathological injury. It has been suggested that comprehensive investigations aimed at elucidating the associations between circulating miRNAs and various physiopathological conditions may provide new opportunities to use plasma/serum miRNAs as indicators in a clinical setting [10,11]. In view of their relatively low complexity and excellent stability and the availability of high throughput approaches such as 'deep sequencing', it is possible to use a panel of circulating miRNAs as markers to complement and/or improve disease monitoring and management [10,33].

Furthermore, our 'proof-of-principle' observation supports the idea that 'normal' compositions and levels of circulating miRNAs can be deregulated under certain disease conditions, and the finding that *miR-885-5p* is up-regulated in the sera of patients with liver pathologies could reflect a metabolic imbalance of this miRNA *in vivo*. Additionally, other miRNAs such as *miR-122* and *miR*-192 were elevated in patients with both HCC and LC (Table 1). Interestingly, *miR-122*, a liver-associated miRNA, was recently shown to be increased significantly in the circulation of toxicant-treated mice [21]. Therefore the fact that *miR-122* is elevated in the sera of patients with liver pathologies merits further investigation. However, it should be noted that the conclusion that serum *miR-885-5p* is a potential marker for liver pathologies is based on results from a relatively small sample, and we could not analyse the relationship between the elevated levels of serum *miR-885-5p* and the severity and aetiology of liver pathology. Therefore it might be interesting to enlarge this study by prospective follow-up and investigate the possible correlation between aberrant circulating *miR-885-5p* and the progression of hepatic cirrhosis/fibrosis. In addition, given that the metabolism and function of circulating miRNAs remain unknown, it is unclear whether serum *miR-885-5p* is liver-restricted/derived or originates from immune cells involved in the antiviral responses that accompany the development of liver diseases. In a newly published paper [34], *miR-885-5p* was found to be a relatively liver tissue-enriched miRNA (Supplementary Figure S3 available at http://www.clinsci.org/cs/120/cs1200183add.htm) and our bioinformatics analysis indicates that ABCA1 (ATP-binding cassette subfamily A member 1), which functions as the gatekeeper of CRT (cholesterol reverse transport) and is a key modulator in phosphatide metabolism, is a potential target of *miR-885-5p* (Supplementary Figure S4 and Supplementary Table S3 available at http://www.clinsci.org/cs/120/cs1200183add.htm). That might mean that this miRNA has some important cellular function in the liver. Further investigation into the expression of *miR-885-5p* in a variety of clinical liver biopsies and related cell lines might help to elucidate its biological role in the progression of chronic liver diseases. Additionally, although our present observation that the circulating *miR-885-5p* is significantly up-regulated in patients with HCC, LC and CHB, future studies should include stratification analysis of a large sample to eliminate sample selection biases and to clarify the pathological role of circulating *miR-885-5p* at different stages of HCC and its correlation with the degree of hepatic chronic inflammation and the severity of liver impairment. Furthermore, as mentioned earlier, since no correlation was found between circulating *miR-885-5p* and traditional liver parameters such as ALT, it will be interesting to investigate whether overexpressed circulating *miR-885-5p* correlates with other liver function parameters and hepatic histopathological indicators such as platelets, serum albumin and Scheuer grading system in patients with liver pathologies.

In summary, *miR-885-5p* was significantly elevated in sera from individuals with liver pathologies. The detection method was sensitive and could be utilized for minimally invasive and complementary detection of

liver pathologies in a clinical setting. Although the clinical significance of these findings needs further investigation, miRNAs could serve as novel screening and diagnostic tools for detecting individuals with liver pathologies in routine clinical practice.

AUTHOR CONTRIBUTION

Junhao Gui, Yaping Tian and Xinyu Wen designed the study. Junhao Gui, Wenhui Zhang, Pengjun Zhang, Wei Run and Liyuan Tian recruited the patients and provided the samples. Jing Gao and Xingwang Jia determined the liver function parameters, which was supervised by Yanhong Gao. Junhao Gui and Wenhui Zhang collected and assembled the data. Junhao Gui, Wenhui Zhang and Yaping Tian performed data analysis and interpretation; and Junhao Gui and Yaping Tian wrote the manuscript.

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SUPPLEMENTARY ONLINE DATA

Serum microRNA characterization identifies *miR-885-5p* as a potential marker for detecting liver pathologies

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Table S1 Patients' information

Ages are given as means (S.D.), and ALT is given as the median. LC group included seven patients with decompensated liver function.

	Group		
Parameter	HCC	LC	NC
Individuals (*n*)	20	12	16
Gender (*n*)			
Male	17	11	14
Female	3	1	2
Age (years)	58.3 (6.2)	61.2 (7.5)	55.6 (4.3)
AFP			
>20 ng/ml (*n*)	9	2	0
<20 ng/ml (*n*)	11	10	16
ALT (units/l)	32.7	24.5	17.4

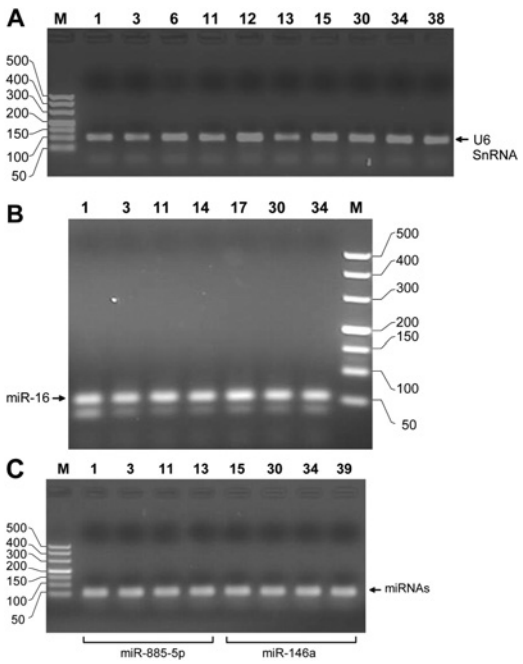

Figure S1 Representative PCR products of *U6* and miRNAs in sera

The expected PCR products of *U6* snRNA (A), *miR-16* (B) and *miR-885-5p* and *miR-146a* (C) in serum samples were generated. Single bands in each RNA preparation demonstrate the production of corresponding PCR product. M, DL-500 DNA marker (bp).

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Table S2 Raw and normalized data of *miR-885-5p* in independent groups of 136 serum samples including LC ($n = 26$), HCC ($n = 46$), CHB ($n = 23$), NC ($n = 24$) and GC ($n = 17$)

Each sample was run in triplicate. The expression levels of *miR-885-5p* were normalized to *U6* snRNA.

Group	Number	Average C_t of *miR-885-5p*	Average C_t of *U6* snRNA	*miR-885-5p* levels
NC	NC-1	32.1599	34.2276333	4.192275
	NC-2	33.67397	30.7961333	0.136046
	NC-3	33.57357	30.9563	0.162976
	NC-4	32.86893	31.5361	0.396989
	NC-5	34.91237	34.8511	0.95842
	NC-6	32.46287	32.5458333	1.059191
	NC-7	33.43427	31.0678667	0.193928
	NC-8	32.82667	31.7420667	0.471522
	NC-9	31.52813	32.8263	2.459167
	NC-10	33.0165	32.5614667	0.729493
	NC-11	33.17187	30.6956	0.179708
	NC-12	33.76207	33.6406	0.919251
	NC-13	34.23343	34.225	0.994174
	NC-14	33.63983	32.5824	0.480487
	NC-15	31.675	30.3933333	0.41132
	NC-16	31.66773	29.0564333	0.163652
	NC-17	32.1874	31.1854	0.499307
	NC-18	32.5456	30.7805	0.29421
	NC-19	32.12083	29.3367	0.14518
	NC-20	32.91237	34.0595	2.21473
	NC-21	32.39513	34.26407	3.65262
	NC-22	31.98293	32.03833	1.03915
	NC-23	33.84713	31.1042333	0.14938
	NC-24	31.574	32.3941	1.76553
LC	LC-1	33.8519	32.86523	0.504642
	LC-2	29.89	32.60567	6.568816
	LC-3	29.5953	33.75953	17.92871
	LC-4	31.17277	31.70423	5.781591
	LC-5	28.9101	32.7759	14.57914
	LC-6	31.8153	32.6132	1.738569
	LC-7	30.55037	32.79297	4.732492
	LC-8	33.8876	35.1087	3.78703
	LC-9	30.20783	34.85347	25.03081
	LC-10	31.7782	32.83893	4.171983
	LC-11	30.70237	33.32663	6.165708
	LC-12	31.99237	34.21587	4.670251
	LC-13	30.54093	34.268	13.24216
	LC-14	32.1442	33.0567	1.882348
	LC-15	29.56203	33.89423	20.14291
	LC-16	32.284	32.3891	1.075519
	LC-17	30.3293	34.2244	14.87825
	LC-18	32.58497	34.81817	4.701757
	LC-19	32.214	33.78757	2.976396
	LC-20	33.72003	34.29103	1.485553
	LC-21	29.13983	30.81847	3.201246
	LC-22	28.91017	31.17233	4.797114
	LC-23	29.57373	31.22837	3.148432
	LC-24	29.80063	31.39323	3.015924

Table S2 Continued

Group	Number	Average C_t of *miR-885-5p*	Average C_t of *U6* snRNA	*miR-885-5p* levels
	LC-25	29.71727	30.01577	1.229865
	LC-26	30.77273	30.7567	0.988948
HCC	HCC-1	30.84197	32.3715	2.886918
	HCC-2	31.7599	32.6890667	1.904176
	HCC-3	29.79517	31.2246667	2.693527
	HCC-4	30.4622	33.6257	8.960008
	HCC-5	29.98013	31.9065333	3.801064
	HCC-6	29.64737	33.3839	13.32931
	HCC-7	28.7177	31.891	9.021079
	HCC-8	29.0095	32.3431667	10.0817
	HCC-9	29.9224	31.8601667	3.831121
	HCC-10	30.26987	31.8788333	3.050326
	HCC-11	32.80137	32.4628333	0.790843
	HCC-12	32.74657	32.7132333	0.977158
	HCC-13	28.76793	32.7439	15.73571
	HCC-14	31.87567	32.8606333	1.979263
	HCC-15	29.782	33.2526	11.08549
	HCC-16	29.21323	33.5275	19.89412
	HCC-17	30.8785	34.1069	9.37228
	HCC-18	32.4533	35.3911	7.662419
	HCC-19	29.4815	31.8434667	5.140707
	HCC-20	30.91573	33.4428667	5.764265
	HCC-21	31.78673	33.0726	2.43829
	HCC-22	31.27717	32.0824	1.747424
	HCC-23	30.34453	31.7258333	2.605036
	HCC-24	31.33337	31.9600333	1.54399
	HCC-25	31.8361	32.4900667	1.573489
	HCC-26	32.85017	34.4231333	2.975152
	HCC-27	31.33333	34.106	6.833715
	HCC-28	33.30637	37.1948	14.80928
	HCC-29	31.67763	33.5156333	3.575149
	HCC-30	32.26983	34.6443667	5.185693
	HCC-31	31.14843	32.1360333	1.982888
	HCC-32	32.548	33.1246333	1.491365
	HCC-33	30.00793	32.5442333	5.801007
	HCC-34	29.27783	31.539	4.793801
	HCC-35	29.94617	31.9494333	4.009058
	HCC-36	30.17673	32.3858667	4.623985
	HCC-37	33.7755	32.7146333	0.479344
	HCC-38	27.60693	30.4339667	7.096151
	HCC-39	28.33053	32.4747	17.68152
	HCC-40	31.835	29.9329667	0.267566
	HCC-41	29.8067	29.3254	0.716332
	HCC-42	30.01933	31.2975667	2.425424
	HCC-43	30.54367	30.2766667	0.831044
	HCC-44	29.90017	31.2251333	2.505265
	HCC-45	29.8667	30.3007	1.350974
	HCC-46	30.3879	31.3652667	1.968868
CHB	CHB-1	31.5161	32.6277333	2.160901
	CHB-2	32.51433	33.9452	2.696093
	CHB-3	25.51897	33.7916333	309.2572
	CHB-4	29.3154	31.164	3.601505

Table S2 Continued

Group	Number	Average C_t of *miR-885-5p*	Average C_t of *U6* snRNA	*miR-885-5p* levels
	CHB-5	28.85253	32.7692667	15.10272
	CHB-6	28.69013	30.3378667	3.133417
	CHB-7	31.5974	31.2667333	0.795169
	CHB-8	29.42473	32.1433	6.582201
	CHB-9	28.66833	33.9994	40.25427
	CHB-10	30.22393	33.8725	12.54091
	CHB-11	28.49853	31.7207667	9.332326
	CHB-12	29.06453	33.4399333	20.75523
	CHB-13	27.39193	33.7456667	81.78343
	CHB-14	28.2652	31.9182333	12.57977
	CHB-15	28.2643	32.4806667	18.58886
	CHB-16	32.44817	34.0811	3.101422
	CHB-17	29.03807	32.8067	14.14902
	CHB-18	30.19323	31.7042333	2.850082
	CHB-19	28.45323	30.5798	4.36678
	CHB-20	30.11127	31.8844333	3.418026
	CHB-21	27.23567	32.3738	35.21529
	CHB-22	30.77673	32.3135667	2.901576
	CHB-23	30.91903	31.9664333	2.066806
GC	GC-1	35.20143	34.4075667	0.576797
	GC-2	33.84457	34.0378	1.143321
	GC-3	33.2822	32.3433333	0.521642
	GC-4	32.38527	32.5210667	1.098699
	GC-5	35.24717	31.3213667	0.065798
	GC-6	33.0854	34.9977	3.764087
	GC-7	33.03173	31.3279	0.30697
	GC-8	33.41497	32.997	0.748477
	GC-9	33.41317	33.0260333	0.764646
	GC-10	32.84237	31.0731333	0.293364
	GC-11	34.34217	33.2585333	0.471838
	GC-12	31.27697	31.4482333	1.126044
	GC-13	32.58207	33.4471	1.821378
	GC-14	33.58183	33.9913333	1.328228
	GC-15	32.20793	33.2848333	2.109503
	GC-16	34.1714	33.7893333	0.767338
	GC-17	33.57597	32.9598	0.652401

Table S3 The 18 potential targets of *miR-885-5p* predicted by TargetScan, Microrna and miRanda respectively.

Genes	Full name of predicted gene
ABCA1	ATP-binding cassette, subfamily A, member 1 (ABC1)
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2
C12orf41	Chromosome 12 open reading frame 41
CAMK4	Calcium/calmodulin-dependent protein kinase IV
CTNNB1	Catenin (cadherin-associated protein), β1, 88kDa
EIF4G2	Eukaryotic translation initiation factor 4 γ2
FBN1	Fibrillin 1
FCHSD1	FCH and double SH3 domains 1
GRM8	Glutamate receptor, metabotropic 8
HTRA2	HtrA serine peptidase 2
KTN1	Kinectin 1 (kinesin receptor)
LIP1	Lymphocyte cytosolic protein 1 (L-plastin)
MAN1C1	Mannosidase α, class 1C, member 1
MRPS10	Mitochondrial ribosomal protein S10
RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1
RFX1	Regulatory factor X, 1 (influences HLA class II expression)
TSC22D2	TSC22 domain family, member 2
UBE2N	Ubiquitin-conjugating enzyme E2N (UBC13 homologue, yeast)

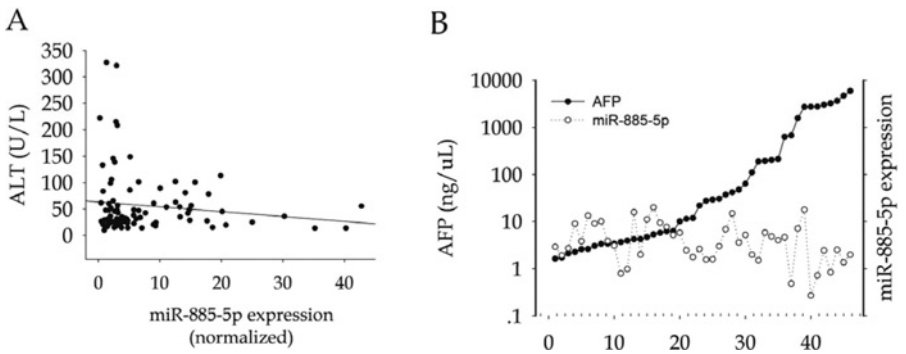

Figure S2 Correlations between serum *miR-885-5p* and ALT and AFP in patients with liver pathologies

(A) The correlation of *miR-885-5p* and ALT in patients with liver pathologies ($n = 95$). (B) The changes in AFP concentrations and relative levels of serum *miR-885-5p* in 46 pathologically confirmed HCC patients (log10 scale of AFP concentration). The expression level of serum *miR-885-5p* in each patient with liver pathologies was determined in triplicate and normalized to *U6*. U/L, units/l.

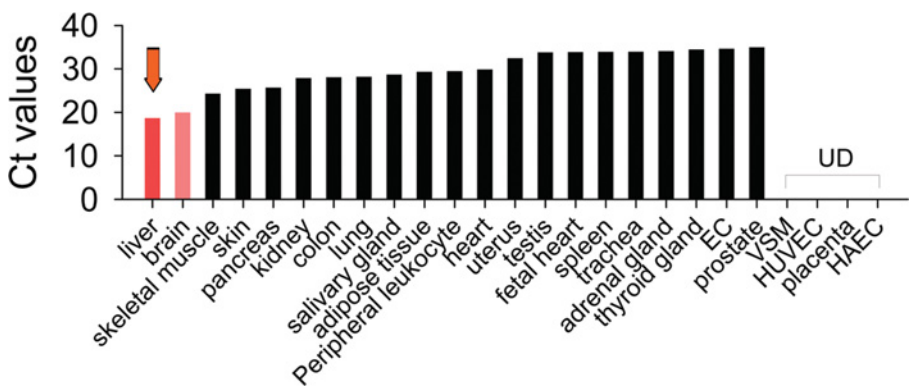

Figure S3 Expression of *miR-885-5p* in different human tissues and several cell lines

This result indicated that *miR-885-5p* is a relatively liver-tissue-restricted miRNA (shown with an arrow). The raw data were derived from Supplementary Table S1 in [1]. UD, undetected; EC, endothelial cell; VSM, vascular smooth muscle; HUVEC, human umbilical vein endothelial cell; HAEC, human aortic endothelial cell.

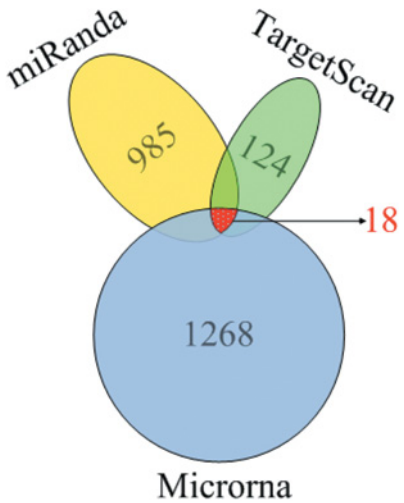

Figure S4 **Potential targets of *miR-885-5p***

Bioinformatics softwares including TargetScan (http://www.targetscan.org/vert_50/), Microrna (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and miRanda (http://www.microrna.org/microrna/home.do) were used to predict the potential targets of *miR-885-5p*. The result shows that 124, 985 and 1268 targets of *miR-885-5p* are predicted by TargetScan, Microrna and miRanda respectively, and 18 targets are predicted by all three softwares (highlighted in red).

REFERENCE

1 Adachi, T., Nakanishi, M., Otsuka, Y., Nishimura, K., Hirokawa, G., Goto, Y., Nonogi, H. and Iwai, N. (2010) Plasma microRNA 499 as a biomarker of acute myocardial infarction. Clin. Chem. **56**, 1183–1185

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