Serum microRNAs as novel biomarkers for primary sclerosing cholangitis and cholangiocarcinoma

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Accepted for publication 9 February 2016
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Summary

The diagnosis of primary sclerosing cholangitis (PSC) is difficult due to the lack of sensitive and specific biomarkers, as is the early diagnosis of cholangiocarcinoma (CC), a complication of PSC. The aim of this study was to identify specific serum miRNAs as diagnostic biomarkers for PSC and CC. The levels of 667 miRNAs were evaluated in 90 human serum samples (30 PSC, 30 CC and 30 control subjects) to identify disease-associated candidate miRNAs (discovery phase). Receiver operating characteristic (ROC) curves were established and only miRNAs with an area under the curve (AUC) > 0.70 were considered useful as biomarkers. In the discovery phase we identified the following: 21 miRNAs expressed differentially in PSC versus controls, whereas miR-483-5p and miR-194 showed deregulated expression in CC compared with controls. We also demonstrate a difference in the expression of miR-222 and miR-483-5p in CC versus PSC. Combination of these specific miRNAs further improved the specificity and accuracy of diagnosis. This study provides a basis for the use of miRNAs as biomarkers for the diagnosis of PSC and CC.

Keywords: biomarkers, cholangiocarcinoma, liver cancer, miRNAs, primary sclerosing cholangitis
Introduction

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by progressive inflammation and fibrosis of intrahepatic and extrahepatic bile ducts. In a large proportion of patients, PSC progresses to biliary cirrhosis and ultimately end-stage liver disease. The diagnosis of PSC can be difficult because of the heterogeneous nature of PSC and the lack of a quantifiable diagnostic test [1]. There is an enormous unmet need in PSC, and among the autoimmune liver diseases it is the most enigmatic and includes not only the inflammation and fibrosis of bile ducts but, as discussed below, the appearance of the fatal complication, cholangiocarcinoma (CC) [2–6]. CC is a well-established and frequently fatal complication of PSC [7], with the highest incidence within the first 2-5 years following the diagnosis of PSC [8–10].

Currently, CC is the second most common primary hepatic malignancy, with recent epidemiological studies suggesting an increasing prevalence in Western countries [11]. CC is characterized by a poor prognosis with a median survival of less than 24 months [12,13] and a poor response to chemotherapy [14,15]. Surgical resection is the only effective therapeutic option for CC, but is applicable in fewer than 50% of cases because it is diagnosed mainly at late stages. In early-stage CC, the 5-year survival after radical surgery is greater than 80%. A number of different risk factors have been identified for CC, with PSC being the most commonly recognized one. However, due to the lack of biomarkers or imaging features for the early detection of CC, current guidelines do not support routine surveillance for CC.

The development of blood-based biomarker assays for malignancies such as CC is critical, in particular for predisposing conditions such as PSC. Therefore, it is important to identify effective surveillance strategies that will permit early detection of CC or, better yet, premalignant lesions in patients at increased risk. Several tumour markers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA 19-9), have been used to aid diagnosis of CC; however, these markers are not specific and have not been shown to be effective for early detection of CC in asymptomatic PSC patients [14,16–23]. Thus, there is an obvious need for accurate non-invasive serum markers for CC.

MicroRNAs (miRNAs) are endogenous non-coding RNAs involved in post-transcriptional gene regulation through base pairing to partially complementary sites located in the 3′-UTR of coding RNA [24]. There is an enormous interest in developing newer diagnostic technology and, in particular, using miRNAs to understand autoimmune and chronic inflammatory disorders more clearly [25,26].

Many miRNAs have been implicated in the control of various disease states as well as all aspects of normal physiology, including acting as tumour suppressor genes or oncogenes. Because miRNAs are protected from RNases and remain stable in plasma and serum [27], they hold potential as biomarkers for early cancer detection [28,29]. Recent studies on human CC cell lines and tissues concluded that miRNA expression is markedly different in normal cholangiocytes compared with malignant ones [30]. In this study, we aimed to determine the diagnostic utility of miRNA in differentiating PSC and CC by analysing the serum miRNAs profile of PSC, CC and control subjects.

Materials and methods

Human subjects

Patients with CC (n = 70), PSC (n = 70) and healthy control (CTR) subjects (n = 70 with similar gender and age; mean age 55 years, 60% male) were enrolled into this study between 2008 and 2012. Clinical data of the enrolled patients are summarized in Table 1. Liver biopsy was used for staging of PSC (20 patients with histological stage I, 13 with stage II, 12 with stage III and 25 with stage IV). Twenty patients with primary biliary cirrhosis (PBC) were included as a disease control group that was not predisposed to the development of CC. The PBC patients enrolled included 10 with early PBC and 10 with advanced PBC, and 96% were positive to the anti-mitochondrial antibody test (AMA). Diagnosis was conducted according to European Association for the Study of the Liver (EASL) Clinical Practice Guidelines 2009 [31]. The study protocol was approved by the Institutional Review Board of Humanitas Clinical and Research Centre, Rozzano (MI), Italy, and all subjects gave written informed consent.

Peripheral blood samples were obtained and serum was isolated by centrifugation at 1700 g for 15 min within 2 h after blood collection. Serum was recovered, dispensed into aliquots and stored at −80°C. Total RNA was extracted from serum samples using the mirVana Paris isolation kit (Ambion, Life Technologies, Carlsbad, CA, USA) specific for liquid samples, and converted to cDNA by priming with stem-looped RT primers (Human Megaplex RT primers; Life Technologies) combined with the TaqMan® microRNA reverse transcription kit (Life Technologies). miRNA quantification was performed with the microfluidic card TaqMan® Array (Life Technologies).

Study design

This study included two phases: (1) a discovery phase in which differentially expressed miRNAs were selected from profiling using high-throughput real-time polymerase chain reaction (PCR); and (2) an independent validation phase of miRNAs selected in the discovery phase. Each subject was assigned randomly to either discovery or validation phase.
In the discovery phase, we investigated the miRNA expression profile in serum from 30 patients with CC, 30 with PSC and 30 CTR with similar gender and age. Ten pools of three serum samples each (a total volume of 400 μl) were analysed for each group; 667 miRNAs were evaluated in each pool. The aim of this phase was to obtain a specific profile of miRNA expression for each pathological condition. In the validation phase all the miRNAs found to be expressed differentially in CC and PSC were validated in an independent cohort of 120 individual serum samples (400 μl each), including 40 CTR, 40 PSC, and 40 CC with similar gender and age (Table 1). A group of 20 patients with PBC was used as the disease control group of autoimmune cholestatic liver disease, which does not predispose patients to the development of CC.

**Extraction and quantification of circulating miRNAs**

Total RNA was extracted from serum samples using the mirVana Paris isolation kit (Ambion, Life Technologies) specific for liquid samples by following the manufacturer’s protocol for total RNA isolation, with minor modifications. In brief, 400 μl of serum were denatured by the addition of an equal volume of 0.1 M guanidine thiocyanate. The denatured samples were processed using BACC RNeasy Mini Kit (Qiagen) or mirVana Paris isolation kit (Ambion, Life Technologies). The RNA was eluted with 30 μl of DEPC water. The RNase-free water was used as a negative control.

**Table 1. Characteristics of patients with primary sclerosing cholangitis and cholangiocarcinoma**

|                      | All       | Discovery phase | Validation phase |
|----------------------|-----------|-----------------|------------------|
| **Primary sclerosing cholangitis** |           |                 |                  |
| Patients             | 70        | 30              | 40               |
| Gender (male) (%)    | 35 (50)   | 18 (60)         | 17 (43)          |
| Age (years, mean)    | 45        | 48              | 43               |
| Duration of disease (years ± SD) | 17.5 ± 5.3 | 18.5 ± 9.6     | 16.5 ± 7.2       |
| Histological stages 3–4 (number) (%) | 37 (52.9) | 15 (50)         | 22 (55)          |
| IBD (%)              | 4 (6)     | 2 (7)           | 2 (5)            |
| UDCA (%)             | 66 (94.3) | 28 (93.3)       | 38 (95)          |
| Cholangiocarcinoma (%) | 2 (3)    | 1 (3)           | 1 (2.5)          |

|                      | All       | Discovery phase | Validation phase |
|----------------------|-----------|-----------------|------------------|
| **Cholangiocarcinoma** |          |                 |                  |
| Patients             | 70        | 30              | 40               |
| Gender (male) (%)    | 45 (64)   | 18 (60)         | 27 (68)          |
| Age (years, mean)    | 64        | 64              | 65               |
| Duration of disease (years ± SD) | 1.4 ± 0.5 | 1.0 ± 0.6       | 1.8 ± 0.3        |
| Cirrhosis (number) (%) | 46 (66)   | 21 (70)         | 25 (63)          |
| Intrahepatic cholangiocarcinoma (%) | 34 (48.6) | 14 (46.7)       | 20 (50)          |
| Primary sclerosing cholangitis (%) | 7 (10)   | 6 (20)          | 1 (3)            |

Data are expressed as number (percentage) or mean ± standard deviation (s.d.). IBD = inflammatory bowel disease; UDCA = ursodeoxycholic acid.

In the discovery phase, we investigated the miRNA expression profile in serum from 30 patients with CC, 30 with PSC and 30 CTR with similar gender and age. Ten pools of three serum samples each (a total volume of 400 μl) were analysed for each group; 667 miRNAs were evaluated in each pool. The aim of this phase was to obtain a specific profile of miRNA expression for each pathological condition. In the validation phase all the miRNAs found to be expressed differentially in CC and PSC were validated in an independent cohort of 120 individual serum samples (400 μl each), including 40 CTR, 40 PSC, and 40 CC with similar gender and age (Table 1). A group of 20 patients with PBC was used as the disease control group of autoimmune cholestatic liver disease, which does not predispose patients to the development of CC.
Statistical analysis

For the discovery phase, Ct data were extracted using RQ Manager Software version 1.2.1 and Data Assist Software version 3.01, and imported into R. Quality control and statistical analysis were performed using the package HTqPCR [33]. The ΔCt was calculated using the mean of all expressed miRNAs (Ct < 40) as reference, then relative quantities were calculated as RQ = 2−ΔCt. Association with disease was evaluated with a t-test on the ΔCt value. A miRNA was considered represented differentially if its absolute log₂(fold change) > 1 and P-value < 0.05 (t-test between two populations). Only miRNAs that demonstrated an absolute log₂(fold change) > 1, mean Ct < 30 and P < 0.05 were selected for validation.

For the validation phase, RQs were calculated using as reference the average Ct value of four endogenous controls (miR-17, miR-29a, miR-30c and miR-30b), identified by bioinformatic analysis (Supporting information and Supporting information, Fig. S1). We modelled the disease outcome as a function of the quantitative level of each miRNAs independently (Supporting information, Table S1). A standard t-test was performed on log₁₀(RQ) and the difference was considered significant if P < 0.05. Odds ratio (OR) was obtained with logistic regression for each mRNA. For an alteration of a given miRNA in log scale, we obtained an OR equal to the one indicated on each row and for each column. The reference is the control group, or the PSC group when comparing CC to PSC. The OR were filtered on the basis of the corresponding P-value, considered significant if P < 0.05 (Supporting information, Table S2). For each miRNA, we fitted a logistic model, and we extracted the coefficient (beta) relative to the effect of the miRNAs. This coefficient may be interpreted as the increase of log odds per unit increase of log₁₀(RQ) of each miRNA. The OR was calculated as OR = e^β. The P-value associated with each OR was considered significant if P < 0.05. Receiver operating characteristic (ROC) curves were obtained using logistic regression, and the area under the curve (AUC) was calculated using the Epicalc package (R package version 2.15-1.0).

To evaluate the discriminating effect of serum miRNAs, ROC curves were established for each validated miRNA. The AUC values were calculated from the logistic regression (Supporting information, Table S3). The results revealed some miRNAs with large areas under the ROC curve (AUC > 0.70) that are useful as biomarkers.

Results

Discovery phase: identification of disease-associated miRNA candidates

In this study we analysed the miRNA profiling results with the aim of identifying circulating miRNAs specific for PSC and CC. A total of 667 miRNAs were evaluated in 10 pools of three subjects for each group: PSC (n = 30), CC (n = 30) and CTR subjects (n = 30). An average 219 of 667 miRNAs were detected per sample (Supporting information, Fig. S2). There were no differences detected among different stages, consistent with the small number of samples at each stage.

We performed the following comparisons to obtain miRNAs signatures: (a) CC and PSC samples compared to CTR samples to identify disease-correlated candidate miRNAs deregulated in PSC and CC, (b) PSC compared to CTR samples to identify PSC-associated candidate miRNAs, (c) CC compared to CTR samples to extract CC-associated candidate miRNAs and (d) CC compared to PSC samples.

Using the criteria illustrated in the Methods section (Supporting information, Figs S3 and S4), we obtained a list of candidate miRNAs (Fig. 1) which were carried forward to the validation phase.

Validation phase: disease-associated miRNAs

Serum miRNAs identified in the discovery phase were validated using an independent cohort of PSC (n = 40), CC (n = 40) and CTR subjects (n = 40). As stated above, we used a single-subject serum sample for validation.

Similarly to the discovery phase, we compared (a) CC and PSC (combined) to CTR samples, (b) PSC to CTR samples, (c) CC to CTR samples and (d) CC to PSC.

Supporting information, Table S1 illustrates the miRNAs included in the validation phase and corresponding P-value.

Using the criteria stated in the Methods section, we found that miR-200c was down-regulated specifically in PSC (P = 0.0001) (Fig. 2), whereas miR-483-5p and miR-194 were up-regulated in CC when compared with CTR (P < 0.0001 and P = 0.0099, respectively) (Fig. 3). Importantly, we also found that miR-222 and miR-483-5p were up-regulated specifically in CC when compared with PSC (P < 0.0011 and P = 0.0013, respectively) (Fig. 4). The combined ROC analysis of identified miRNAs from each disease group increases the AUC value significantly, suggesting that the combination of these markers makes the diagnosis even more specific and reliable.

We also observed up-regulation of miR-193b, miR-122 and mir-885-5p in PSC and CC compared with a healthy control group (Supporting information, Fig. S5); these miRNAs have been reported to be altered in other liver diseases and can be considered non-specific [34,35]. These results were also confirmed by the analysis of PBC patients, an autoimmune cholestatic liver disease that does not predispose patients to the development of CC, because the expression of the three miRNAs is also significantly different from that seen in the CTR group (Supporting information, Table S4).

Discussion

The discovery of non-invasive specific biomarkers that can be measured routinely in easily accessible samples and help to diagnose PSC or CC is an unmet clinical need. In fact, to
Fig. 1. Deregulated miRNAs identified in the discovery phase. (a) Deregulated miRNAs in primary sclerosing cholangitis (PSC) and cholangiocarcinoma (CC) relative to healthy controls (CTR) were identified using a \( t \)-test between PSC-CC and CTR \((P < 0.05)\). The 183 miRNAs with \( \text{Ct} < 40 \) in > 50% samples per group; (b) deregulated miRNAs in PSC relative to CTR \( t \)-test between 10 PSC and 10 CTR \((P < 0.05)\). The 172 miRNAs with \( \text{Ct} < 40 \) in > 50% samples per group; (c) deregulated miRNAs in CC relative to CTR \( t \)-test between 10 CC and 10 CTR \((P < 0.05)\). The 184 miRNAs with \( \text{Ct} < 40 \) in > 50% samples per group; (d) deregulated miRNAs in CC relative to PSC \( t \)-test between 10 CC and 10 PSC \((P < 0.05)\). The 177 miRNAs with \( \text{Ct} < 40 \) in > 50% samples per group). The miRNAs that did not reach a \( \log_2(\text{fold change}) > 1 \) are represented with a lighter bar.
date such diagnoses are not only difficult to achieve but may remain uncertain, even after imaging and cytological sampling have been attempted. The goal of this study was to identify a panel of deregulated miRNAs in the serum samples from these patients and to investigate their potential as biomarkers for detection and staging of disease; indeed, the present work shows that specific patterns of deregulated miRNAs are able to support each specific diagnosis.

Cell-free miRNAs in body fluids are stable under harsh conditions, including boiling, low/high pH, extended storage and multiple freeze-thaw cycles [27,30,36]. Recently, advances in studies of circulating miRNAs have led to the concept that tissue- or organ-specific intracellular miRNAs may be released into the circulation during cell death or apoptosis owing to cell turnover, cellular destruction or pathological injury. Therefore, miRNA profiles in serum may, potentially, be used as biomarkers that could improve diagnosis and the clinical management of diseases.

It is clear that chronic inflammatory common in cholestatic diseases are predisposing factors for CC [37,38]. It has been found that miRNAs are involved in the pathogenesis of CC, as specific miRNAs are either up-regulated [39] or down-regulated [40,41]. The miRNAs in human bile have been investigated for the purpose of identifying potential CC biomarkers; however, bile samples were obtained by endoscopic retrograde cholangiopancreatography, an invasive procedure with associated complications [42].

To date, miRNA expression in serum and bile in PSC and CC has been reported only in small cohorts, due perhaps to the rarity of CC and PSC [29,43]. Voigtlander et al. described a specific serum miRNA profile in a single pool of six sera from patients with PSC and a single pool of six sera from CC patients. Unfortunately, their small study is also limited by the lack of data from any disease control groups. In our study, we analysed PSC and CC as a single pathological group with the aim of defining a specific biomarker to help in the diagnosis of PSC and CC. In addition, the use of PBC as a pathological control group allowed us to confirm some well-known liver-specific miRNAs, such as miR-122.

In this study, by profiling the serum miRNA expression in PSC and CC, we showed a number of miRNAs deregulated. In particular we have identified: (1) a PSC-associated serum miRNA that can supplement the current diagnosis of this disease; (2) a group of serum miRNAs associated with CC that can be used for the diagnosis of this cancer; (3) a serum miRNA that characterizes the evolution of PSC to CC and, therefore, might allow a very early diagnosis in individuals at risk of developing this dangerous cancer; and finally, (4) a group of serum miRNAs altered in all diseases studied, that can be considered non-specific. The analyses of sera from patients with PBC suggest strongly the disease-specificity of the identified serum miRNAs.

Some of the liver disease-associated miRNAs, miR-193b, miR-122 and miR-885-5p, have already been described in
the literature as expressed in the liver at the tissue and serum level. These miRNAs emphasize an ongoing hepatic pathological condition [34,35]. miR-885-5p and miR-122 are also up-regulated in hepatocellular carcinoma (HCC), non-alcoholic fatty liver disease (NAFLD) and cirrhosis. Their levels are correlated with fibrosis, steatosis and inflammatory activity. In agreement with these previous reports, our results showed an up-regulation of miR-122 and miR-885-5p. In fact, serum concentrations of these miRNAs, together with miR-193b, were increased significantly in all diseases studied including PSC, CC and PBC.

Importantly, in this study we identify PSC- and CC-associated miRNAs as potential biomarkers. Indeed, miR-200c may be used for diagnostic purposes, because there are no PSC-specific serum markers available; similarly, miR-194 and miR-483-5p can allow the diagnosis of CC. Particular attention should be paid to miR-222 and miR-483-5p, which were both up-regulated in CC compared with PSC. The combined use of miR-222 and miR-483-5p could be used to monitor the course of PSC patients for early CC detection. The validation as possible biomarkers of miR-483-5p, up-regulated in CC compared with CTR and PSC is very important. In contrast, miR-200c was down-regulated in PSC compared with CTR and then up-regulated in CC compared with PSC. Taken together, these results could be important in diagnosis and patient monitoring, and could identify an early development of the tumour.

Some limits of our work, however, need to be mentioned. First, due to the rarity of this disease, only a few of the CC samples included in the study actually come from
PSC patients. A prospective study with a large cohort of PSC patients is needed to validate the clinical use of these markers. Secondly, some of the CC samples included were intrahepatic CC developed in cirrhotic liver; even though it is not clear if intra- and extrahepatic CC have a different aetiology, it is known that liver cirrhosis itself predisposes to cancer development. Finally, as a reflection of specific characteristics of our tertiary referral centre, few PSC patients in the current study have concomitant inflammatory bowel diseases (IBD). This assures a more homogeneous study population but necessitates future studies on patients with both PSC and IBD.

It is known that an inactivation of miR-200c results in an induction of epithelial–mesenchymal transition, whereas activation of miR-200c led to a reduction of epithelial–mesenchymal transition, including a reduced cell migration. Neural cell adhesion molecule 1 (NCAM1), a known hepatic stem/progenitor cell marker, was demonstrated to be a direct target of miR-200c [44]. Moreover, Loo et al. described miR-483-5p as a robust endogenous suppressor of liver colonization and metastasis [45]. It has been shown that miR-194 targeted the 3'-UTRs of several genes that were involved in epithelial–mesenchymal transition and cancer metastasis [46]. These results support a role for miR-194, which is expressed specifically in liver parenchymal cells, in preventing liver cancer cell metastasis. In addition, p53 activation also induces a significant increase of miR-194 expression in tumour cell lines.

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**Fig. 4.** Cholangiocarcinoma (CC)-associated miRNAs in primary sclerosing cholangitis (PSC). (a) P-value relative to box-plot (b) and odds ratio of CC-associated miRNA in PSC. (b) miRNA levels. The box-plot shows the minimum, median and maximum of miRNA levels. A standard t-test was performed and miRNA were considered represented differently if absolute log10(RQ) > 1 and P-value < 0.05 (P-values are shown in the table). Two miRNAs were identified: miR-222 and miR-483-5p. (c) Receiver operating characteristic (ROC) curve analysis of CC-associated miRNAs and ROC curve analysis combined with the altered miRNAs. Fraction of true positive results (sensitivity) and false positive results (1-specificity) for levels of miRNA are shown. ROC curves were obtained using logistic regression, and the area under the curve (AUC) was calculated using the Epicalc package. AUC was considered significant if > 0.70.
Considering the prominent role of p53 in preventing metastasis, the induction of miR-194 by p53 may represent a protective or self-controlling mechanism in tumour progression. Multiple miRNAs are also involved in the nuclear factor kappa B (NF-κB) signalling pathway; for example, miR-222 plays important inhibitory roles in cancer progression and may be a potential therapeutic target [47]. Special attention should be given to miRNAs deregulated in PSC and in CC in the pathogenesis of these diseases. The miRNAs directly involved in PSC progression and CC development could serve not only as specific biomarkers but also as novel therapeutic targets.

Acknowledgements

This study was supported by grants from the Fondazione Umberto Veronesi, Associazione Italiana per la Ricerca sul Cancro (AIRC) and Programma Ricerca Indipendente – Regione Lombardia, and partly by Dr Nicholas C. Hightower, Centennial Chair of Gastroenterology from the Scott and White Hospital, and the NIH grant DK07698 to G. A. The supporting parties had no influence on the study design, data collection and analyses, writing of the manuscript, or the decision to submit the manuscript for publication.

Disclosure

All authors have no disclosures to declare.

Role of each author

F. B. designed the experiments, wrote and edited the manuscript and conducted statistical analysis. F. M. designed the experiments, wrote and edited the manuscript and conducted statistical analysis. A.L. performed the experiments, wrote and edited the manuscript and conducted statistical analysis. M. C., M. M., G. A., D. A., K. M. B., M. L. and G.-X. Y. conducted statistical analysis. C. L. B. wrote and edited the manuscript. M. E. G. and P. I. designed the experiments and wrote and edited the manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Cumulative distribution of the coefficient of variation shows the reduction of the variability after normalization. The coefficient of variation (CV) was calculated for miRNAs detected in all samples. Data presented in the plot are either not normalized (RQ, grey line), normalized with the arithmetic mean (NRQ_mean, yellow line), geometric mean (NRQ_geomean, blue line) or with four selected reference miRNA (NRQ_ref red line).

Fig. S2. (a) The miRNA population consists of more than 200 expressed miRNAs for each sample. On average, 219 miRNAs were detected per sample. (b) The mean and median of Ct value for each pool before normalization.

Fig. S3. Analysis of pairwise raw Ct correlation. These results indicate a good correlation among the data because all the coefficients are higher than 80%, demonstrating the reproducibility of the data. A grouping of all the data along the straight line of slope equal to 1 and passing through the origin in a graph showing the raw values in Ct of the two samples being compared is consistent with the high reproducibility of results. The more the points are grouped along the bisector, the better is the reproducibility. The distribution can be evaluated through the assignment of an average correlation coefficient of the compared data: the closer the coefficient is to the ideal value of 1, the better is the correlation. This pairwise correlation analysis demonstrated overall satisfactory quality and good reproducibility before normalization and statistical testing.

Fig. S4. Data normalization. The mean Ct value of all expressed miRNAs (Ct < 40) was used as the normalization factor. The normalized data were properly centred, confirming that part of the technical variability between samples had been removed.

Fig. S5. Liver disease-associated miRNAs. (a) P-value relative to box-plot and odds ratio of liver disease-specific miRNAs. (b) Liver disease-specific miRNA levels in healthy controls (CTR), primary sclerosing cholangitis (PSC) and cholangiocarcinoma (CC). Box-plots show the minimum, median and maximum of miRNA levels. A standard t-test was performed and a miRNA was considered differently represented if absolute log_{10} (RQ) > 1 and P-value < 0.05 (P-values are shown in the table). Three liver disease-specific miRNAs were identified: miR-193b,
miR-122 and miR-885-5p, all up-regulated in PSC and CC. (c) Receiver operating characteristic (ROC) curve analysis by combination of the three miRNAs. Fraction of true positive results (sensitivity) and false positive results (1-specificity) for levels of miRNA are shown. ROC curves were obtained using logistic regression and the area under the curve (AUC) was calculated using the package Epicalc. AUC was considered significant if $AUC > 0.70$.

**Table S1.** Deregulated miRNAs included in the validation phase and corresponding $P$-value. A standard $t$-test was performed and data were considered significant if $P < 0.05$.

**Table S2.** Odds ratio (OR) and $P$-value associated with each miRNA. OR was obtained with logistic regression for each miRNA. $P$-value was considered statistically significant if $P < 0.05$.

**Table S3.** Area under curve (AUC) of each miRNA.

**Table S4.** Deregulated miRNAs analysed in sera from 20 PBC patients (10 early and 10 advanced) compared to healthy control (CTR) subjects.