


# Identification of circulating microRNAs as potential biomarkers for hepatic necroinflammation in patients with autoimmune hepatitis

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## ABSTRACT

**Objective** MicroRNAs (miRNAs) are implicated in the pathogenesis of autoimmune diseases and could be biomarkers of disease activity. This study aimed to identify highly expressed circulating miRNAs in patients with autoimmune hepatitis (AIH) and to evaluate their association with clinical characteristics.

**Methods** Microarray analyses were performed, and miRNA expression profiling for AIH, primary biliary cholangitis (PBC) and overlap syndrome (OS) using the serum of patients and healthy individuals was done. Samples were divided into discovery and test sets to identify candidate miRNAs that could discriminate AIH from PBC; the former included 21 AIH and 23 PBC samples, while the latter included five AIH and eight PBC samples.

**Results** Among 11 candidate miRNAs extracted in the discovery set, 4 (miR-3196, miR-6125, miR-4725-3p and miR-4634) were specifically and highly expressed in patients with AIH in the test set. These four miRNAs discriminated AIH from PBC with high sensitivity (0.80–1.00) and specificity (0.88–1.00). In situ hybridisation analysis revealed that these miRNAs were expressed in the cytoplasm of hepatocytes in patients with AIH. Their expression levels were highest in untreated patients with AIH, followed by those in untreated patients with OS. They drastically or moderately decreased after prednisolone treatment. Histological analysis demonstrated that the expression levels of miR-3196, miR-6125 and miR-4634 in patients with AIH and OS were correlated with severe hepatic necroinflammatory activity.

**Conclusion** These circulating miRNAs are suggested to reflect hepatic necroinflammatory activity and serve as AIH-related and treatment-responsive biomarkers. These miRNAs could be beneficial in developing new therapeutic strategies for AIH.

## INTRODUCTION

Autoimmune hepatitis (AIH) is a progressive, chronic, autoimmune liver disease characterised by elevated serum transaminase, autoantibodies, hyperglobulinaemia and interface hepatitis on histology.<sup>1–3</sup> Although

## Summary box

### What is already known about this subject?

- MicroRNAs (miRNAs) are involved in the pathogenesis of autoimmune diseases and circulating miRNAs could serve as biomarkers for disease activity.
- Approximately half of patients with autoimmune hepatitis (AIH) have persistent histological activity on liver biopsy specimens, even after biochemical remission. Therefore, non-invasive biomarkers are desirable to determine disease activity for formulating therapeutic strategies.

### What are the new findings?

- Comprehensive miRNA microarray analysis identified four circulating miRNAs (miR-3196, miR-6125, miR-4725-3p and miR-4634) that were specifically and highly expressed in patients with AIH.
- These four miRNAs were strongly or moderately expressed in the cytoplasm of hepatocytes in patients with AIH.
- The expression levels of these four miRNAs drastically decreased after prednisolone treatment.
- The expression levels of miR-3196, miR-6125 and miR-4634 were correlated with severe hepatic necroinflammatory activity on histology in patients with AIH.

### How might it impact on clinical practice in the foreseeable future?

- The miRNAs identified in this study could serve as non-invasive biomarkers associated with hepatic necroinflammatory activity in patients with AIH.

environmental, genetic and epigenetic factors and T cell-mediated immune responses are considered the pathogenesis of AIH, the molecular mechanisms underlying the disease have not been clearly elucidated.<sup>4 5</sup> Therefore, diagnostic biomarkers and radical treatments specific for AIH have not yet been developed.<sup>3 5</sup>

Repeat liver biopsies are useful in assessing disease activity and treatment response; however, these are invasive, time-consuming and burdensome to patients. Therefore, in real-world clinical settings, clinical remission is assessed based on biochemical findings, such as the normalization of transaminase and IgG levels.<sup>6</sup> Of note, approximately half of AIH patients with serum parameter normalization after immunosuppressive treatment have persistent histological activity on liver biopsy specimens, which is associated with fibrosis progression and poor prognosis.<sup>7,8</sup> Therefore, to develop a novel treatment and improve the liver-related mortality in patients with AIH, it is important to better understand the molecular mechanisms underlying the disease and to identify molecules that sensitively reflect histological disease activity.<sup>8,9</sup>

Primary biliary cholangitis (PBC) is another autoimmune liver disease characterized by the destruction of small intrahepatic bile ducts, progressive cholestasis, and the presence of highly specific serum antimitochondrial antibodies (AMAs).<sup>10–12</sup> Some patients with PBC manifest the features of AIH and are commonly classified as having so-called ‘overlap syndrome (OS)’<sup>13–15</sup>; OS has a frequency of 7%–13% in patients with autoimmune liver disease.<sup>14</sup> Of note, patients with OS are more likely to have oesophageal varices, gastrointestinal bleeding, ascites, hepatic failure and/or liver transplantation compared with those with typical PBC. Therefore, the standard treatments for both PBC (such as ursodeoxycholic acid (UDCA)) and AIH (such as corticosteroids) are required to achieve a complete treatment response in most patients with OS.<sup>15,16</sup>

MicroRNAs (miRNAs) constitute a class of small noncoding RNAs that directly bind to the 3' untranslated regions of specific target genes, thereby inducing post-transcriptional inhibition.<sup>17–25</sup> miRNAs play a crucial role in various biological processes, including tissue development and differentiation, immunity, cell proliferation, apoptosis and oncogenesis.<sup>17,18</sup> Their dysregulation is associated with liver diseases, such as viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease and hepatocellular carcinoma.<sup>20,21</sup> Therefore, elucidating the mechanisms underlying miRNA dysregulation could provide new therapeutic strategies for liver diseases.<sup>23</sup> Circulating RNAs, which are protected from RNases and remain stable in blood, are attractive, non-invasive biomarkers for disease diagnosis and disease stage/activity determination.<sup>20–25</sup> However, only few studies have evaluated the expression profiles of circulating miRNAs in patients with AIH.

The aim of this study was to identify miRNAs that were highly expressed in patients with AIH by comparing the circulating miRNA profiles of untreated patients with AIH, PBC and OS and healthy controls. Furthermore, changes in the expression levels of identified miRNAs prior to and after treatment were assessed, and their association with treatment response was evaluated.

## METHODS

### Participants and samples

In this study, 57 treatment-naïve patients with AIH (n=28) or PBC (n=29) and 7 patients with OS at the Jikei University School of Medicine (Tokyo, Japan) and Fuji City General Hospital (Shizuoka, Japan) were enrolled. Twenty-seven healthy volunteers who underwent routine medical check-up at the Jikei University School of Medicine were recruited as controls. They all had normal alanine aminotransferase (ALT;  $\leq 35$  U/L) and gamma-glutamyltransferase ( $\leq 50$  U/L) levels with no history of significant diseases. Patients with other liver diseases, such as alcoholic liver disease, drug-induced liver disease, viral hepatitis and non-alcoholic steatohepatitis, were excluded.

Serum samples were collected from patients at the time of diagnosis or exacerbation of the disease and from healthy volunteers at the time of consent. In addition, paired samples were obtained from 21 of the 28 patients with AIH at least 3 months after treatment, from 23 of the 29 patients with PBC at least 12 months after treatment, and from all of the seven patients with OS at least 6 months after additional treatment. All serum samples were stored at  $-80^{\circ}\text{C}$  until analysis. Written informed consent was obtained from each patient. In cases where serum samples had already been stored in another approved study, participants were notified of the opportunity to opt out of the study through the website of each institution.

### Diagnosis and treatment

AIH was diagnosed based on the revised International Autoimmune Hepatitis Group criteria.<sup>26</sup> The patients in this study were diagnosed as having probable or definite AIH and were treated with prednisolone alone (0.6–1.0 mg/kg/day). We defined remission of AIH as the normalization of ALT and IgG ( $\leq 1700$  mg/dL) levels.<sup>6</sup> Diagnosis of PBC was made if patients met any one of the following criteria: (1) biochemical evidence of cholestasis and histological evidence of chronic non-suppurative destructive cholangitis; (2) the presence of AMA with histologically compatible PBC features and (3) the presence of AMA with PBC clinical features and course characteristics, irrespective of histological findings.<sup>12</sup> All patients with PBC were treated with UDCA (13–15 mg/kg/day) or UDCA plus bezafibrate (400 mg/day). In this study, biochemical response to these treatments was defined according to the Barcelona criteria,<sup>27</sup> that is, an alkaline phosphatase (ALP) decrease of  $>40\%$  of baseline values or normal ALP levels ( $\leq 325$  U/L) after 12 months of treatment. OS was diagnosed according to the Paris criteria.<sup>15</sup> Six patients with OS were treated with a combination of prednisolone and UDCA, and the remaining one was treated with UDCA alone.

### Histological assessments

All patients underwent liver biopsy for diagnostic purposes. All liver specimens were histopathologically

evaluated by a single liver-specialised pathologist (YN). The severity of inflammation and fibrosis in AIH was graded according to the Ishak score.<sup>28</sup> In brief, this grading system comprises four necroinflammatory categories: (1) periportal or periseptal interface hepatitis, (2) confluent necrosis, (3) focal lytic necrosis, apoptosis and focal inflammation and (4) portal inflammation; histological grading scores, which ranged from 0 to 18, were calculated by summing the scores for each category, and were graded in this study as follows; total score 0–3, A0; score 4–8, A1; score 9–12, A2 and score 13–18, A3. Fibrosis stages ranged from 0 to 6, with stage 6 defined as liver cirrhosis.<sup>28</sup> The histological staging and grading of PBC were assessed based on the Nakanuma's classification.<sup>29 30</sup> In brief, scores for fibrosis, bile duct loss and chronic cholestasis were summed for staging as follows: total score 0, stage 1 (no progression); score 1–3, stage 2 (mild progression); score 4–6, stage 3 (moderate progression); and score 7–9, stage 4 (advanced progression). Cholangitis activity (CA) and hepatitis activity (HA) were graded as CA0–3 and HA0–3, respectively. The histological staging and grading of OS were assessed based on both the Ishak score and Nakanuma's classification.

### Detection of miRNAs in liver tissues by in situ hybridisation

To evaluate the expression of miRNAs in the liver, in situ hybridisation was performed using a RiboMap in situ hybridisation kit (Ventana Medical Systems, Tucson, AZ) on a Ventana Discovery automated in situ hybridisation instrument (Ventana Medical Systems). Digoxigenin-labelled, LNA-modified DNA probes against miR-3196, miR-6125, miR-4725–3p, miR-4634 and scramble siRNA were obtained from Exiqon (Exiqon, Vedbaek, Denmark). In situ hybridisation steps after the deparaffinisation step were performed based on the standard protocol provided in the manufacturer's RiboMap application note (<http://www.ventanamed.com>). The initial fixation step was performed by incubating the sections in a formalin-based RiboPrep reagent (Ventana Medical Systems) for 32 min at 37°C. After acid treatment using a hydrochloride-based RiboClear reagent (Ventana Medical Systems) for 12 min at 37°C, the slides were treated with a ready-to-use protease 2 reagent after an initial cell conditioning CC2 reagent (Ventana Medical Systems) for 8 min at 90°C. The initial denaturing prehybridisation step was done on the sections for 8 min at 91°C after being hybridised with an anti-sense LNA riboprobe (5 pmol/slide) using a RiboHybe hybridisation buffer (Ventana Medical Systems) for 10 hours at 55°C. After three stringency washes with 2×RiboWash (Ventana Medical Systems) for 4 min at 51°C, postprobe fixation was performed using a RiboFix reagent for 20 min at 37°C, followed by incubation of antidigoxigenin alkaline phosphatase Fab fragments 1:800 (Roche) for 30 min at 37°C. A signal was detected using a BlueMap NBT/BCIP substrate kit for 4 hours at 37°C. Finally, the sections were counterstained with Kernechtrot (nuclear fast red) as a marker stain and covered with a glass coverslip.

### Analysis of miRNA expression by microarray

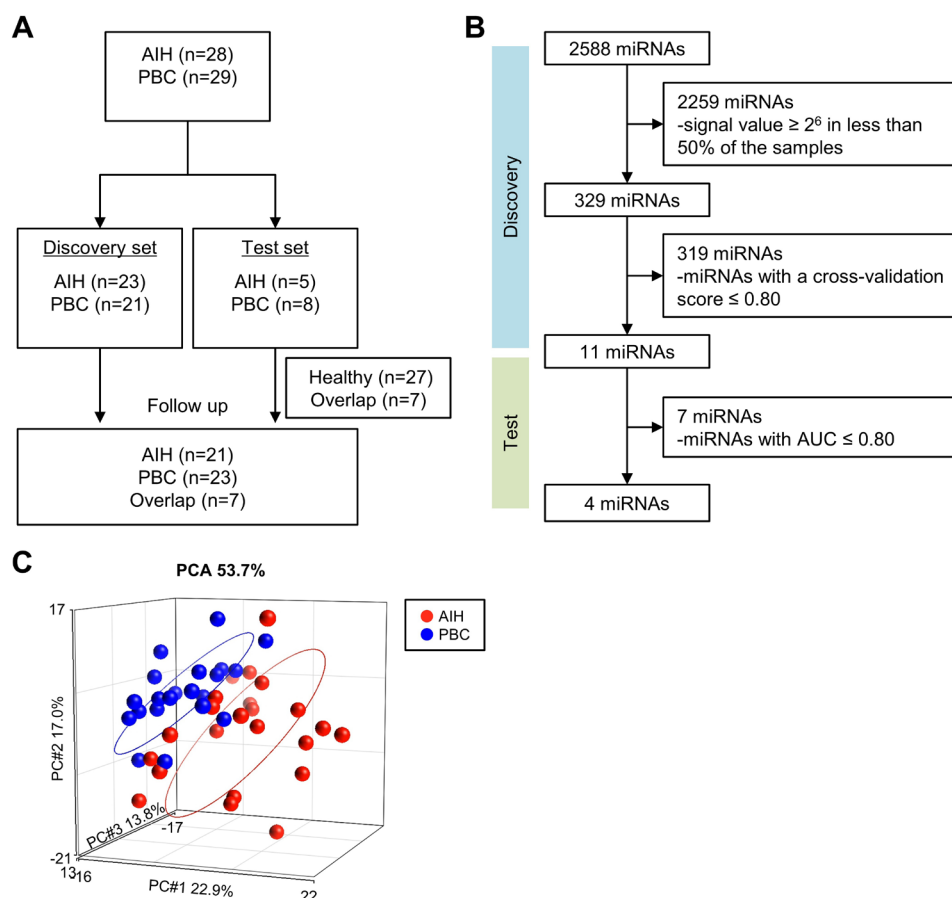
Our standard operating procedures for miRNA microarray analysis have been previously published.<sup>31–34</sup> In Brief, total RNA was extracted from 300 µL serum samples obtained from patients with AIH, PBC and OS, and from healthy individuals using a 3D-Gene RNA extraction reagent (Toray Industries, Tokyo, Japan). Comprehensive miRNA expression analysis was performed using the 3D-Gene miRNA labelling kit and the 3D-Gene Human miRNA Oligo Chip (Toray Industries), designed to detect 2588 miRNAs registered in the miRBase release 21 database (<http://www.mirbase.org/>). To normalise microarray signals, three preselected internal control miRNAs (miR-149–3p, miR-2861, and miR-4463) were used. All microarray data from this study are publicly available in the GEO database (accession #GSE-140249) (<https://www.ncbi.nlm.nih.gov/geo/>).

### Statistical analysis

Serum samples from patients with AIH and PBC were divided into the discovery and test cohorts. The discovery cohort was used to select candidate miRNAs that could discriminate between AIH and PBC, and the test cohort was used to confirm the results obtained from the discovery cohort. In this study, miRNAs with median signal values  $\geq 2^6$  were defined as abundant miRNAs in serum and were included in subsequent analyses. To identify robust biomarkers that could discriminate between AIH and PBC, a cross-validation score was calculated for each miRNA in the discovery set. This score was based on leave-one-out cross-validation and obtained by summing the results (hit or not) of each leave-out sample and dividing it by the number of repeats (the same as total sample number), as previously described.<sup>34</sup> miRNAs with cross-validation scores  $>0.8$  were selected for further analyses. The diagnostic sensitivity, specificity and area under the curve (AUC) were calculated for the selected miRNAs. In the test set, we determined the performances of the selected miRNAs in discriminating between AIH and PBC, and miRNAs with AUC  $>0.8$  were considered to be AIH-specific.

Statistical analyses were performed using R V.3.1.2 (R Foundation for Statistical Computing, <http://www.R-project.org/>), compute.es package V.0.2–4, hash package V.2.2.6, MASS package V.7.3–45, mutoss package V.0.1–10, pROC package V.1.8 and IBM SPSS Statistics V.22 (IBM Japan, Tokyo, Japan). Principal component analysis (PCA) was performed using the Partek Genomics Suite version 6.6. Continuous variables were presented as mean  $\pm$  SD. Unpaired and paired t-tests were used to evaluate the differences in the distribution of continuous variables between the two groups. The Fisher's exact test was used to evaluate the significance of differences in the distribution of categorical variables between the two groups. Correlations between variables were analysed using the Pearson's correlation test.





**Figure 1** The strategy used to identify highly expressed microRNAs (miRNAs) in autoimmune hepatitis (AIH). (A) Serum samples were obtained from 28 untreated patients with AIH and 29 untreated patients with primary biliary cholangitis (PBC). these samples were divided into two sets: the discovery set and the test set. paired serum samples were obtained from 21 treated patients with AIH, 23 treated patients with PBC, 7 patients with overlap syndrome, and 27 healthy individuals (controls). (B) Flow diagram for selecting candidate miRNAs. Of the total 2588 miRNAs, 2259 were excluded due to their low expression levels. Eleven candidate miRNAs with cross-validation scores  $>0.8$  were selected in the discovery set; of these, four miRNAs, with area under the curve (AUC) $>0.8$ , were selected in the test set. (C) Principal component analysis (PCA) for the 23 untreated AIH and 21 untreated PBC samples in discovery set using 329 highly expressed miRNAs. The axes show the first three principal components, with their respective fractions of explained variance. The first three principal components explain 53.7% of variance. miRNA profiles of AIH and pBC were well segregated.

## RESULTS

### Characteristics of the study participants

A total of 142 samples consisting of 49 samples from 28 patients with AIH (including 21 paired samples), 52 samples from 29 patients with PBC (including 23 paired samples), 14 samples from 7 patients with OS (including 7 paired samples), and 27 samples from 27 healthy controls (online supplemental table S1) were subjected to miRNA microarray analysis. First, untreated AIH and PBC samples were classified into the discovery and test cohorts to identify circulating miRNAs that could discriminate AIH from PBC (figure 1A, online supplemental table S2). The discovery set included 23 untreated AIH and 21 untreated PBC samples. The test set included five untreated AIH and eight untreated PBC samples. Regarding treatment response, all patients with AIH (n=21) met the remission criteria, and all patients with PBC (n=23) were biochemical responders. Among the seven patients with OS, four had already been receiving

treatment for either PBC or OS and developed acute exacerbation of liver disease when the first samples were collected. For the management of disease exacerbation, prednisolone was added to the preceding UDCA monotherapy in three patients, and its dose was increased in one patient who was on UDCA and prednisolone combination therapy. The remaining three patients were newly diagnosed with OS and were treatment-naïve. All patients with OS had normalised ALT and ALP levels after the treatment, and only one had elevated IgG levels despite the treatment.

### Identification of highly expressed miRNAs in AIH

The expression levels of 2588 miRNAs were comprehensively evaluated in the miRNA microarray analysis. First, 2259 miRNAs with a median signal value  $<2^6$  were excluded. The remaining 329 miRNAs were retained for further analyses (figure 1B). PCA mapping was performed to assess the miRNA expression patterns in

**Table 1** Selected serum miRNAs for discrimination between AIH and PBC

	Discovery set			Test set	
	CV score	AUC	95% CI	AUC	95% CI
Elevated in AIH					
miR-3665	0.91	0.94	0.88 to 1.00	0.38	0.02 to 0.73
miR-8072	0.86	0.95	0.89 to 1.00	0.53	0.18 to 0.87
miR-3196	0.84	0.95	0.89 to 1.00	<b>0.90</b>	<b>0.70 to 1.00</b>
miR-6125	0.84	0.91	0.82 to 1.00	<b>0.90</b>	<b>0.73 to 1.00</b>
miR-6836-3p	0.82	0.90	0.80 to 0.99	0.38	0.03 to 0.73
miR-1908-5p	0.82	0.86	0.73 to 0.98	0.70	0.35 to 1.00
miR-6729-5p	0.82	0.90	0.81 to 0.99	0.80	0.52 to 1.00
miR-4725-3p	0.82	0.91	0.83 to 1.00	<b>0.88</b>	<b>0.65 to 1.00</b>
miR-6789-5p	0.82	0.92	0.84 to 1.00	0.78	0.51 to 1.00
miR-4634	0.82	0.91	0.82 to 1.00	<b>0.98</b>	<b>0.90 to 1.00</b>
Elevated in PBC					
miR-4655-5p	0.82	0.82	0.68 to 0.95	0.80	0.45 to 1.00

Bold indicates the AUC values of miRNAs extracted in the test set.

AIH, autoimmune hepatitis; AUC, area under the curve; CV, cross-validation; PBC, primary biliary cholangitis; .

the discovery set (figure 1C), indicating that there were distinct differences in the miRNA expression profile between AIH and PBC.

Subsequently, miRNAs that could discriminate AIH from PBC were investigated based on leave-one-out cross-validation, and 11 miRNAs (10 elevated in AIH and one elevated in PBC) with cross-validation scores >0.8 in the discovery set were selected (figure 1B, table 1). Finally, the diagnostic performance of these 11 miRNAs was assessed in the test set, and 4 miRNAs (miR-3196, miR-6125, miR-4725-3p and miR-4634) were highly accurate in discriminating AIH from PBC, with AUCs >0.8 (figure 1B, table 1). The AUC, sensitivity and specificity values for the four miRNAs were determined in the test set: miR-3196 had 0.90, 0.80 and 1.00, respectively; miR-6125 had 0.90, 0.80 and 0.88, respectively; miR-4725-3p had 0.88, 1.00 and 0.88, respectively; and miR-4634 had 0.98, 1.00 and 0.88, respectively (figure 2A).

### Changes in circulating miRNA expression levels after treatment

Changes in the expression levels of the four miRNAs prior to and after treatment in patients with AIH, PBC and OS and healthy controls were evaluated using microarray data (figure 2B). The median expression levels of the four miRNAs were highest in pretreatment AIH samples, followed by those in OS samples collected at disease diagnosis or exacerbation. More importantly, the expression levels of these miRNAs (except miR-4725-3p in OS patients) significantly decreased in posttreatment AIH and OS samples. These findings suggest that these miRNAs reflect the disease activity of AIH and that OS may dominantly involve AIH features.

### Correlation between circulating miRNA expression and liver function parameter levels

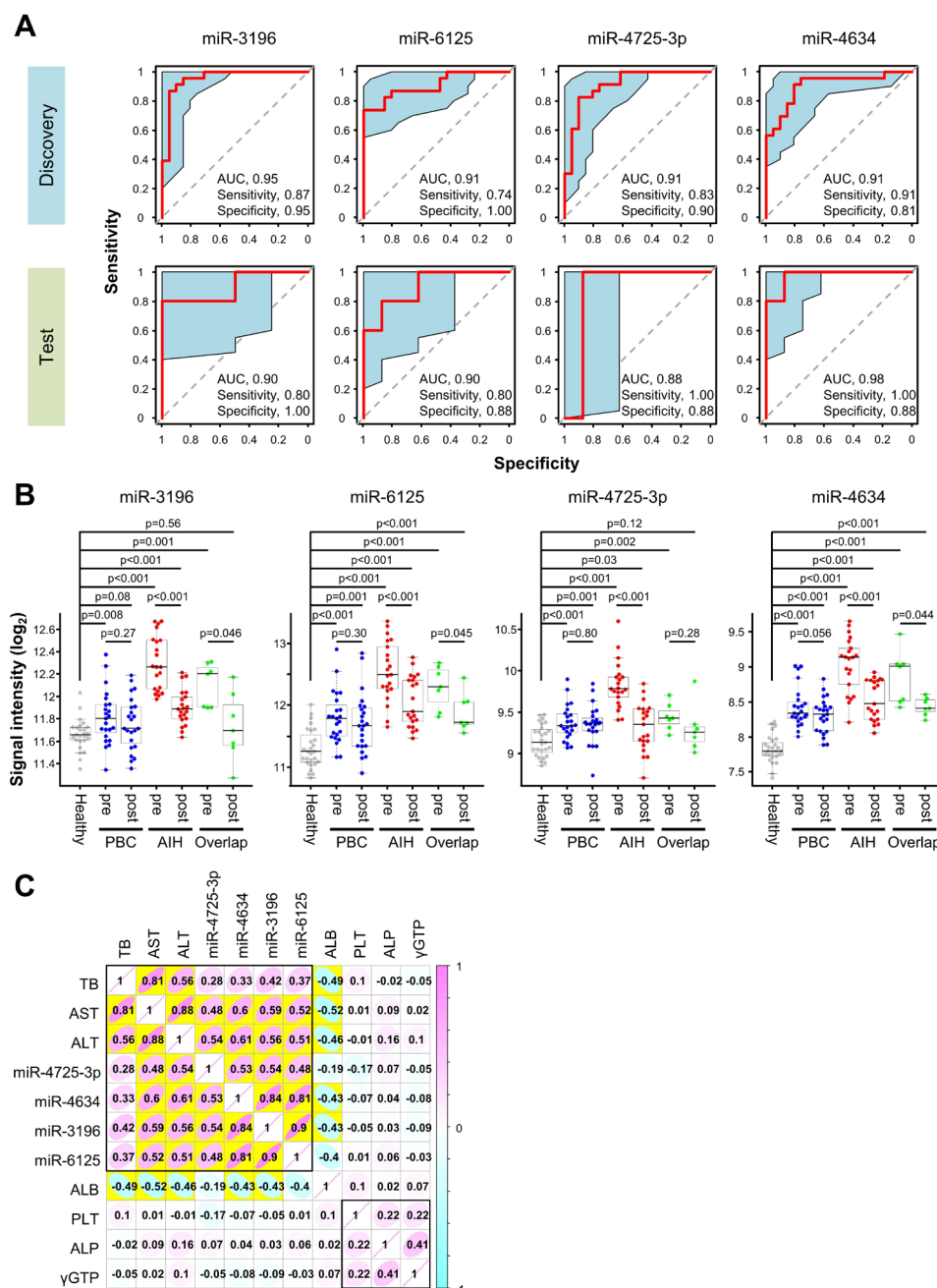
The correlation between the expression levels of the four miRNAs and liver function parameters was investigated (figure 2C). Their expression levels had significant positive correlations with the levels of aspartate aminotransferase ( $p<0.001$  for all;  $r=0.59, 0.52, 0.48$ , and  $0.60$  for miR-3196, miR-6125, miR-4725-3p and miR-4634, respectively) and ALT ( $p<0.001$  for all;  $r=0.56, 0.51, 0.54$ , and  $0.61$  for miR-3196, miR-6125, miR-4725-3p and miR-4634, respectively). The expression levels of miR-4634 and miR-3196 had significant negative correlations with albumin levels ( $p<0.001$  and  $r=-0.43$  for both).

### Localisation of the identified miRNAs in liver tissues

In situ hybridisation was performed to determine the localisation of the four miRNAs in liver tissues. Diffused expressions of the four miRNAs were observed in the cytoplasm of hepatocytes in patients with AIH (figure 3B). miR-3196 and miR-6125 were very strongly stained, while miR-4725-3p and miR-4634 were moderately stained. In patients with PBC and OS, these miRNAs were also expressed in the cytoplasm of hepatocytes, but their expression levels were considerably lower than those in patients with AIH (figure 3A,C). miR-3196 was strongly stained, miR-6125 was moderately stained, while miR-4725-3p and miR-4634 were very weakly stained. In contrast, the expression of these miRNAs was almost undetectable in control liver tissues (figure 3D).

### Relationship between circulating miRNA expression levels and histological findings

The correlation between the expression levels of the four miRNAs and hepatic necroinflammation/fibrosis



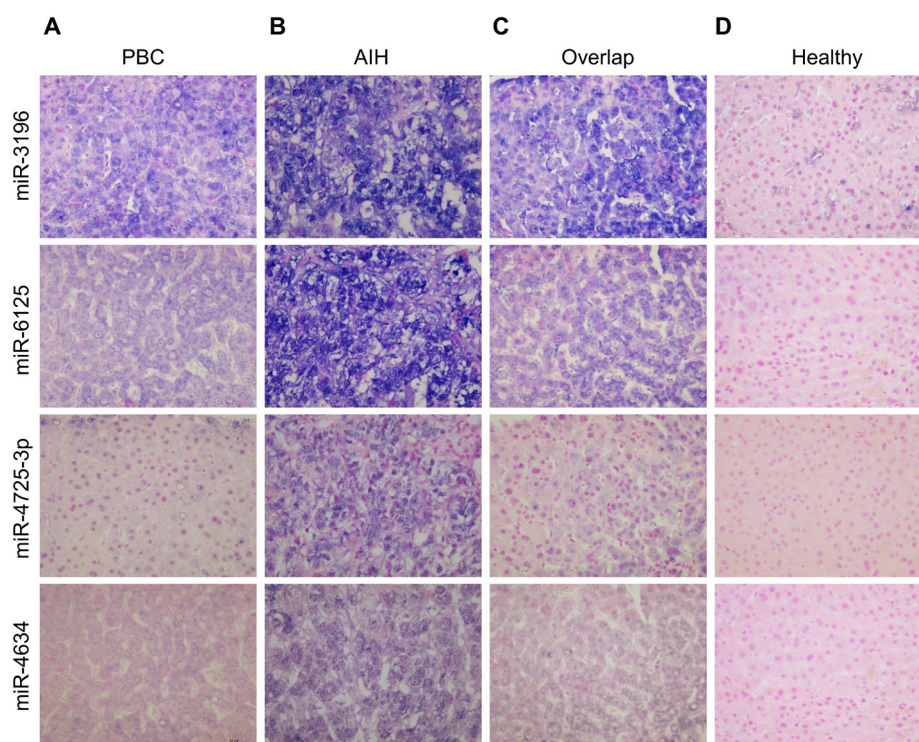
**Figure 2** (A) Receiver operating characteristic (ROC) curve analysis of miR-3196, miR-6125, miR-4725-3p and miR-4634 in the discovery and test sets. The area under the curve (AUC), sensitivity and specificity values are shown for each curve. Sky-blue areas indicate 95% CIs for ROC curves. (B) The expression of the four microRNAs (miRNAs) in patients with autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and overlap syndrome prior to and after treatment, and in healthy individuals. Box-and-whisker plots show the full range of variation, IQR and median values. Outliers are points that fell more than 1.5 times out of the IQR above the third quartile or below the first quartile. P values versus healthy individuals were calculated by unpaired t-test. P values between prior to and after treatment were calculated by paired t-test. (C) Correlation between the expression levels of the four identified miRNAs and liver function parameters in patients prior to treatment (n=64). Numbers indicate Pearson's correlation coefficients. Yellow boxes represent statistical significance ( $p < 0.000455$ ), considering the Bonferroni correction for multiple testing. ALB, albumin, ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, gamma-glutamyltransferase; PLT, platelet; TB, total bilirubin.

in patients with AIH and OS was assessed (figure 4A). The median expression levels of miR-3196, miR-6125 and miR-4634 in the necroinflammatory activity grade A3 group were distinctly higher than those in the grade

A0–A2 groups. In contrast, no correlation was found between miRNA expression levels and fibrosis stages.

It was also evaluated whether the expression levels of the four miRNAs were correlated with histological





**Figure 3** In situ hybridisation detection of miR-3196, miR-6125, miR-4725-3p and miR-4634 in liver tissues from patients with autoimmune liver disease and healthy subjects. The blue-violet colour represents positive staining. miRNAs expression in patients with (A) primary biliary cholangitis (PBC), (B) autoimmune hepatitis (AIH), (C) overlap syndrome and (D) in healthy control (magnification,  $\times 400$ ).

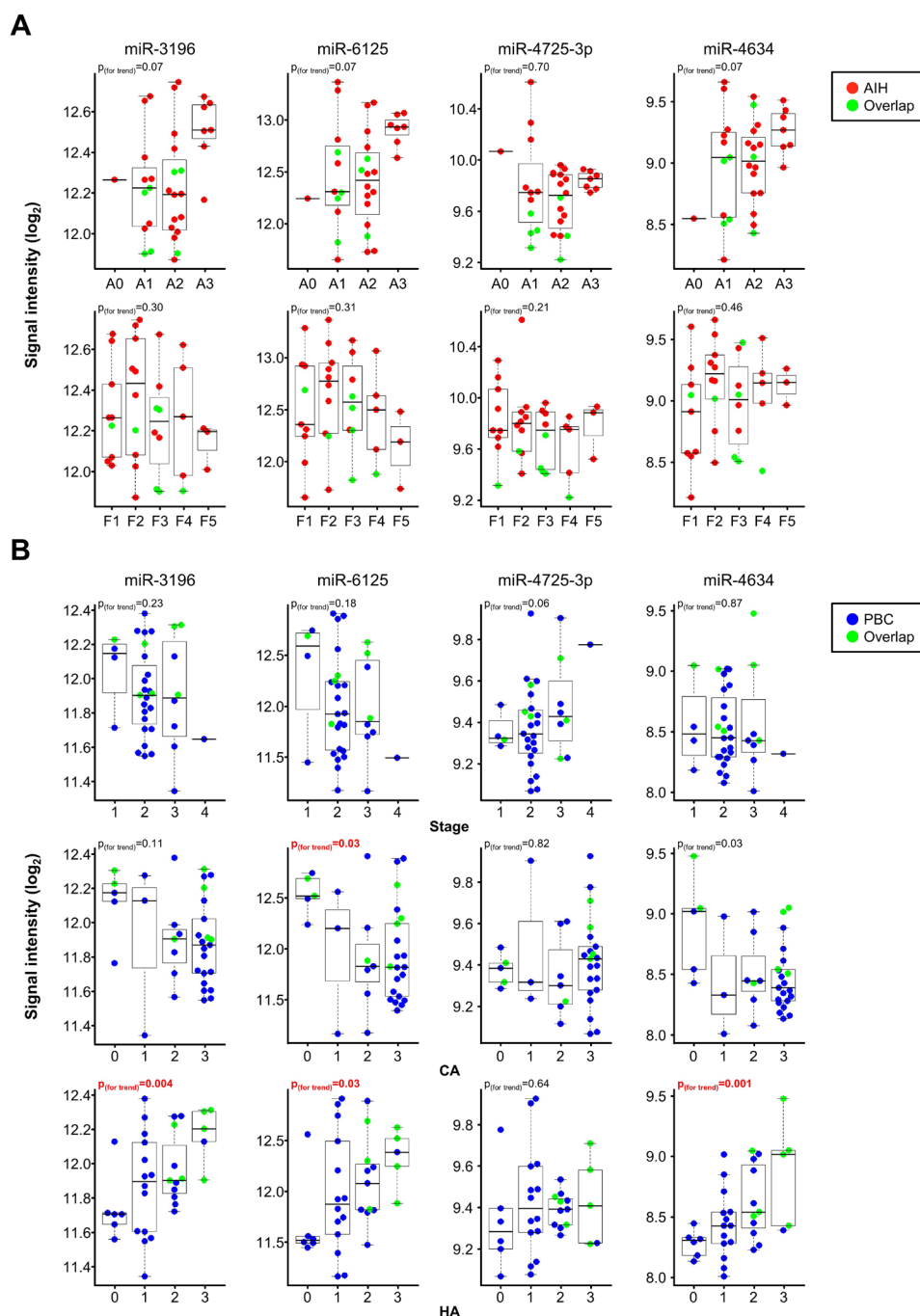
severity scores in patients with PBC and OS (figure 4B). The median expression levels of miR-3196, miR-6125, and miR-4634 significantly increased in a stepwise manner with an increase in hepatitis activity scores. In contrast, the median expression levels of miR-6125 significantly decreased in a stepwise manner with an increase in cholangitis activity. Taken together, these findings suggest that the expression levels of miR-3196, miR-6125, and miR-4634 were positively associated with hepatocellular inflammatory activity.

## DISCUSSION

miRNAs regulate the activities of immune cells, such as B, T and dendritic cells. Dysregulated miRNAs are involved in the pathogenesis of autoimmune diseases.<sup>35</sup> Circulating miRNAs could serve as biomarkers for the diagnosis and staging/grading of various diseases, including autoimmune disease.<sup>31–36</sup> However, an extensive analysis of circulating miRNAs in AIH has not sufficiently proceeded because AIH is relatively rare, so samples are difficult to obtain. In this study, the miRNA expression profiles between untreated patients with AIH and PBC were compared using a highly sensitive miRNA microarray technology; the four circulating miRNAs (miR-3196, miR-6125, miR-4725-3p and miR-4634) were identified as highly expressed in untreated patients with AIH, and these could discriminate AIH from PBC with high sensitivity and specificity. Of note, their circulating levels in patients with AIH drastically decreased after

prednisolone treatment. Furthermore, in situ hybridisation analysis revealed that the four miRNAs were very strongly or moderately expressed in the cytoplasm of hepatocytes in untreated patients with AIH. Given that it is generally challenging to determine cells that release circulating miRNAs, this new finding that the four miRNAs are released by hepatocytes will be meaningful when they are identified as other disease biomarkers. These results suggest that the four miRNAs are closely associated with the pathogenesis of AIH and may be potential biomarkers for disease activity.

A previous study reported that 11 circulating miRNAs were up-regulated in untreated patients with AIH compared with healthy controls. Among the 11 miRNAs, miR-21-5p and miR-122-5p levels decreased in the remission phase after treatment.<sup>22</sup> However, another study reported that these two miRNAs were also up-regulated in patients with PBC, suggesting that both might not be specific for AIH.<sup>23</sup> In this study, we failed to find the up-regulated expression of the two miRNAs. Conversely, none of the 10 miRNAs up-regulated in our discovery set were increased in the previous study.<sup>22</sup> There may be several possible explanations for these differences. First, the previous study only included a small number of patients with acute-onset AIH alone ( $n=5$ ), and AIH-specific miRNAs were identified by comparing patients with AIH to healthy controls (not including patients with PBC). Second, our study included 28 patients with AIH (23 discovery and five test) with various disease stages and



**Figure 4** (A) Correlation between the expression levels of the four microRNAs (miRNAs) and histological findings in patients with autoimmune hepatitis (AIH) and overlap syndrome (OS). The scores of A0–A3 represent the necroinflammatory activity grades, and the scores of F1–F5 represent the fibrosis stages. (B) Correlation between the expression levels of the four miRNAs and histological findings in patients with primary biliary cholangitis (PBC) and OS. P values for trend were calculated using the Pearson's correlation test. CA, cholangitis activity; HA, hepatitis activity.

activities and compared them to patients with PBC and OS, as well as healthy controls. Third, despite the same microarray system, we normalized the miRNA signals using three unique internal controls (miR-149–3p, miR-2861 and miR-4463), which are stably expressed in any samples. Lastly, we excluded miRNAs with signal values  $<2^6$  because candidate miRNAs should have a certain expression level as a disease-specific biomarker.

We also focused on the expression patterns of the four miRNAs in patients with OS with both AIH and PBC features. Their median levels were highest in untreated patients with AIH, followed by those in untreated patients with OS. Additionally, their expression levels drastically decreased after prednisolone treatment in patients with AIH and moderately declined in patients with OS after the initial or additional treatment or increased dose



of prednisolone. In contrast, their expression levels in untreated patients with PBC did not significantly change despite the treatment. These findings suggest that the expression levels of the four miRNAs are indicative of AIH-related and treatment-responsive disease activity.

This study demonstrated that the median levels of the four miRNAs were highest in untreated patients with AIH, especially in those with necroinflammatory activity grade A3, and these increased in a stepwise manner with an increase in hepatitis activity in untreated patients with PBC and OS. Their expression levels significantly decreased after prednisolone treatment; however, the reduced levels were still higher compared with those in healthy controls, suggesting that hepatic necroinflammation may still continue even in the remission phase of AIH. Previous studies reported that approximately half of AIH patients with biochemical remission after immunosuppressive treatment showed persistent histological activity on second liver biopsy specimens, which was associated with fibrosis progression and poor prognosis.<sup>7,8</sup> Therefore, they suggested that follow-up liver biopsy should be considered even in AIH patients with clinical remission.<sup>8</sup> However, liver biopsy is invasive and a burden to patients, making it difficult to obtain a consent for repeat liver biopsy, especially in patients with clinical remission. The circulating miRNAs may serve as non-invasive surrogate biomarkers to estimate the persistence or disappearance of histological activity and to formulate therapeutic strategies, such as dose reduction or withdrawal of immunosuppressive agents.

This study had several limitations. First, little is known about the biological and physiological properties of the four miRNAs identified in this study. Most studies on these miRNAs have focused on their role and behaviour exclusively in malignant tumours,<sup>37–39</sup> and no studies have investigated their involvement in the pathogenesis and clinical significance in autoimmune liver diseases. Accordingly, the four miRNAs may represent novel biomarkers of hepatic inflammatory activity in AIH. Second, this study included a small number of patients (especially in the test set) and no patients with other liver diseases, such as viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease. Therefore, it is unclear whether the four miRNAs reflect AIH-specific or non-specific inflammatory activity. Third, although the in situ hybridisation revealed that the four miRNAs were highly expressed in the cytoplasm of hepatocytes in patients with AIH, it is unclear whether the upregulation of these miRNAs is involved in the development of AIH or is a result of hepatic necroinflammation. A large-scale validation study that includes other liver diseases is required to determine whether the four miRNAs are specific and useful biomarkers to estimate the disease activity of AIH. In addition, functional analyses, including the identification of target genes and mechanisms underlying autoimmune liver disease, are required to clarify the biological and physiological properties of these miRNAs.

In conclusion, we identified the four circulating miRNAs that could serve as non-invasive biomarkers associated with hepatic necroinflammatory activity in patients with AIH, using a comprehensive and highly sensitive miRNA microarray technology. These findings may be helpful in elucidating the mechanisms involved in the pathogenesis of AIH and in developing therapeutic strategies for it.

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