Basic Study

S100 calcium binding protein A6 and associated long noncoding ribonucleic acids as biomarkers in the diagnosis and staging of primary biliary cholangitis

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Biomarkers for the diagnosis and staging of PBC

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CONCLUSION
These four genes may potentially act as novel biomarkers for the diagnosis of PBC. Moreover, LINC00472 acts as a potential biomarker for staging in PBC.

Key Words: S100 calcium binding protein A6; Long noncoding ribonucleic acids; Primary biliary cholangitis; Biomarker; Diagnosis; Staging

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INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic and slowly progressing cholestatic disease, which causes damage to the small intrahepatic bile duct by immunoregulation, and may lead to cholestasis, liver fibrosis, cirrhosis and, eventually, liver failure. The injury mechanism of intrahepatic biliary epithelial cells (iBECs) is the key to investigate the pathogenesis of PBC, but the accurate relationship between cholestasis and liver fibrosis is still indistinct. Currently, liver injury caused by cholestasis is mainly studied using liver cell lines or liver cancer cell lines treated with hydrophobic bile acids[1], while iBECs, the main target cells of PBC, have rarely been studied.

S100 calcium binding protein A6 (S100A6), also known as calcyclin, is a Ca$^{2+}$ binding protein and is a member of the S100 family. Its distribution in the body is specific to cells and tissues, having a high expression in normal epithelial cells and fibroblasts, as well as in some tumor cells[2]. As an intracellular protein, S100A6 is involved in the regulation of various cellular functions, such as proliferation, apoptosis, cytoskeletal dynamics, and cell response to different stressors. It is believed that S100A6 may be involved in the ubiquitination of beta catenin and play an important role in controlling the cell cycle process[3]. S100A6 can interact with the calcyclin-binding protein/Siah-1-interacting protein, which is a component of the ubiquitin ligase complex[4].

Long non-coding ribonucleic acids (lncRNAs) are involved in the regulation of a variety of intracellular processes[5]. As a structural component, lncRNAs can form a nucleic acid protein complex with gene regulatory transcription factors[6]. LncRNAs can also bind to specific transcription factors and change their cellular localization, thus affecting the transcription of target genes. Abnormal expression of lncRNAs in plasma has been shown to accurately predict several human diseases[7,8].

As a general rule, PBC diagnosis depends on titers of antimitochondrial antibody (AMA), serum level of alkaline phosphatase (ALP) and liver biopsy[9-11]. However, it is difficult to achieve an early diagnosis in AMA-negative patients, or to differentiate from other autoimmune liver diseases; thus, an invasive liver biopsy is required to make a definitive diagnosis, and this not only increases the financial burden of patients, but also brings mental and physical trauma to patients, often delaying the best time for treatment. However, after definite diagnosis, some patients fail to respond to ursodeoxycholic acid treatment and often have a poor prognosis or even progress to liver failure. The majority of PBC cases are diagnosed mostly at an advanced stage, so diagnosis and staging biomarkers of PBC are urgently needed.

In this study, we explored the value of S100A6 and its associated lncRNAs as potential biomarkers for the diagnosis and staging of PBC.

MATERIALS AND METHODS

Study design

This study included three phases (Figure 1): (1) The discovery phase, in which candidate genes and lncRNAs were searched using bioinformatics methods, and were then verified by a mouse model and cell model; (2) The training phase, in which quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to evaluate the relative expression levels of target gene and lncRNAs in the plasma of PBC patients and healthy controls, as well as to estimate their diagnosis and staging value; and (3) the validation phase, in which the diagnosis and staging value of target genes and lncRNAs was verified in another independent PBC cohort.

Identification of differentially expressed genes from the gene expression omnibus dataset

The GSE29776 array dataset was analyzed on the gene expression omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/)[12]. The dataset contains 6 mouse liver tissue samples, including 3 bile duct ligation (BDL) mouse samples and 3 sham mouse samples. “GEO2R” in the webpage was used to analyze the array database.
LncRNAs selection

The PROMO usage database (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to predict the transcription factors of the S100A6 promoter[13]. There were multiple binding sites between the transcription factor estrogen receptor alpha (also known as ESR1) and the promoter of S100A6. Experiments with BDL mice and PBC patients suggested that the expression of estrogen receptor in bile duct epithelial cells was associated with cholestasis or bile duct epithelial cells in PBC[14]. We hypothesized that ESR1 could regulate the transcription of S100A6 as a transcription factor and thus play an important role in the injury of bile duct cells in PBC. The Gene-Cloud of Biotechnology Information database (https://www.gcbi.com.cn/gcanalyze/html/generadar/index) was used to screen lncRNAs associated with ESR1[15]. The binding force between IncRNAs and ESR1 was calculated by RNA-Protein Interaction Prediction (http://pridb.gdcb.iastate.edu/RPISeq/)[16]. As RF and SVM scores of LINC00312, LINC00472, and LINC01257 were all found to be close to 1.0, these three lncRNAs were selected as candidate lncRNAs in this study.

Animal studies, bile duct ligation model

Male C57BL/6j mice (aged 6-8 wk) were purchased from the Animal Experiment Department of China Medical University (Shenyang, Liaoning Province, China). All mice were weighed and randomly grouped with an average weight of 20-25 g into the BDL group and the sham group. To simulate cholestasis, 9 mice underwent BDL[17]. The animal protocol was designed to minimize pain or discomfort to the mice. The animals were acclimatized to laboratory conditions (24 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for 2 wk prior to experimentation. The BDL procedure was performed with the common bile duct doubly ligated under anesthesia via laparotomy[18]. The sham procedure was performed via a similar laparotomy without BDL. Animal experiments were approved by the Ethics Committee of the Animal Experiment Department of China Medical University.

A portion of the liver tissue was placed in a 4% p-formaldehyde solution and routinely processed for histological assessment, while the remaining tissue was snap frozen and stored at -80 °C.
Histological analysis
The mice were sacrificed by cervical dislocation and the liver was immediately removed by laparotomy. Part of the right lobe of the liver was fixed in 4% formaldehyde. The liver tissues were embedded in paraffin and sliced. Hematoxylin and eosin stained liver sections were observed under a light microscope at x 400 magnification to evaluate whether the cholestasis model was successfully established[19].

Double immunofluorescence
To identify whether the expression of S100A6 protein was up-regulated in bile duct epithelial cells in BDL mice, we performed double immunofluorescence[20] for S100A6 antibodies (Abcam, USA, Cat. No. ab181975) with cytokeratin 19 (CK19) antibodies (Abcam, USA, Cat. No. ab52625) which was specifically expressed in epithelial cells[21]. The primary antibody was replaced by rabbit or mouse IgG for negative controls. The working concentration of fluorescein isothiocyanate and tetraethyl rhodamine isothiocyanate was 1:50. Nuclei were counterstained with DAPI. The empirical procedure was performed according to the manufacturer’s instructions. The sections were counterstained with DAPI and evaluated under a conventional fluorescence microscope.

Cells culture and treatments
Human intrahepatic biliary epithelial cells (HiBECs) were purchased from Guangzhou Jennio Biotech Company Limited (Guangzhou, Guangdong Province, China). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (GEMINI, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin in incubators at 37 °C with 5% CO₂. HiBECs were treated with 1000 mmol/L glycochenodeoxycholate (GCDC)[22] for 24 h to mimic cholestasis in PBC patients.

Patients
A total of 80 untreated PBC patients and 60 healthy controls as the training set were enrolled in order to differentially evaluate S100A6 and IncRNAs. In addition, another cohort consisting of 65 PBC patients and 50 healthy controls was used as the validation set. PBC patients were diagnosed by the Department of Gastroenterology or Rheumatology of The First Affiliated Hospital of China Medical University between January 2017 and November 2020. The diagnosis of PBC needed to meet two of the following three criteria[9-11]: (1) AMA titer > 1:40; (2) ALP level 1.5-times higher than the normal upper limit for more than 24 wk; and (3) liver biopsy revealing non-suppurative cholangitis and interlobular bile duct damage. Written informed consent was obtained from all patients who participated in the study. This study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University and was carried out in accordance with the Declaration of Helsinki.

Percutaneous ultrasound-guided puncture biopsy of the right liver was performed in all PBC patients, followed by histopathological examination and pathological stage identification[23]. Four stages were defined based on intrahepatic bile duct injury[24]: Stage I: Cholangitis stage, chronic inflammation in the interlobular and septal bile duct. Lymphocytes and plasma cells around the damaged bile ducts infiltrate or form granuloma, but the inflammation in the portal area does not involve the liver parenchyma and there is no cholestasis; stage II: Periportal inflammation stage, with a continuous reduction in the number of interlobular bile ducts, reactive hyperplasia of bile ducts around the portal area, inflammation involving adjacent liver parenchyma and destruction of liver cells, and common focal necrosis, cholestasis also occurs; stage III: Progressive fibrosis stage, the portal area is continuously enlarged by inflammation and fibrosis progression, the fibrous septa formed gradually widens, and cholestasis is aggravated; stage IV: Liver cirrhosis stage, fibrous septa divides the liver parenchyma into patchy nodules, regenerating nodules, and forming pseudo lobules.

Extraction of total RNA from plasma samples and cells
The relative expression levels of S100A6 and IncRNAs in plasma were measured in PBC patients, as well as human intrahepatic biliary epithelial cell lines. Total RNA was extracted from plasma and HiBECs by an RNA extraction kit (Biotek, China), according to the manufacturer’s instructions.

Reverse transcription and quantitative PCR for S100A6 and IncRNAs
Total RNA was amplified by reverse transcription using a reverse transcription kit (PrimeScript™ RT Master Mix, TaKaRa, China)[25]. All reactions were completed in a
Themocycler (Mastercycler nexus, Eppendorf, Germany). Then, quantitative PCR was performed using SYBR® Premix Ex Taq™ II kit (Takara, China) on the LightCycler 480 (Roche, Germany). GAPDH was used as an internal reference, and served as an internal control for plasma RNA quality. S100A6 and lncRNAs expression were calculated by the $2^{-\Delta\Delta C_t}$ method [$26$]. The calculated result was the relative quantitative expression value of S100A6 and lncRNAs compared with the internal reference. Primers for reactions were designed by Primer Premier 6.0 (Canada) software ($27$).

**Statistical analysis**

Statistical Package for Social Science 23.0 software (IBM Solutions Statistical Package for the Social Sciences Incorporated, USA) and GraphPad Prism 8 (GraphPad Software, Incorporated, San Diego, CA, USA) were used for all statistical analyses. The normal distribution data were recorded (mean ± SD), and comparisons between the two groups were performed using the unpaired $t$-test. The paired $t$-test was used to compare the expression levels before and after treatment. Non-normal distribution data were analyzed using the non-parametric Mann-Whitney $U$ test [$28$]. Categorical data were analyzed using the $\chi^2$ test. The correlation between the plasma level of S100A6 mRNA and lncRNAs was analyzed using Pearson or Spearman correlation analysis. Receiver operating characteristic (ROC) curves were constructed and the areas under the curves (AUC) were used to evaluate the value of plasma S100A6 mRNA and lncRNAs as biomarkers for the diagnosis and staging of PBC [$29$]. $P < 0.05$ was considered statistically significant.

## RESULTS

**Identification of the target gene**

“GEO2R” was used to analyze the differentially expressed genes in liver tissues of BDL and sham mice of GSE29776. The top 10 up- and down-regulated genes of GSE29776 in the BDL and sham group are listed in Table 2. To identify potential biomarkers for PBC diagnosis and staging, we used qRT-PCR to validate the analysis of bioinformatics up-regulated genes in plasma of 30 PBC patients and 30 healthy controls. It was found that S100A6 showed the greatest change in the plasma of PBC patients ($t = 20.28$, $P < 0.0001$) ($26$). Therefore, S100A6 was selected as the target gene in this study.

**Expression of S100A6 protein in the BDL mouse model**

HE staining revealed histological changes in liver tissues, with the BDL group showing liver cell swelling, vacuolar degeneration, and coagulative necrosis. Inflammatory cell infiltration was observed in the portal area and around the bile duct, and fibrosis around the bile duct ($26$). In the sham group, there was no or minimal inflammatory cell infiltration around the portal area and bile duct ($26$).

Double immunofluorescence staining was used to label CK19 and S100A6 proteins. A fluorescence microscope was used for observation and Image J software was used for graph analysis. The results showed that S100A6 labeled with fluorescein isothiocyanate showed emerald green fluorescence and CK19 labeled with tetraethyl rhodamine isothiocyanate showed red fluorescence. Red and green fluorescent overlapping images showed that CK19 and S100A6 proteins were positively expressed in the iBECs of BDL mice ($26$), while these two proteins were weakly expressed in the iBECs of mice in the sham group ($26$).

**The expression of S100A6 and IncRNAs analyzed in HiBECs**

To investigate the mechanism of S100A6 and lncRNAs, the expression of S100A6 and lncRNAs was studied in HiBECs. Normal and HiBECs treated with GCDC were detected by qRT-PCR. The relative expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated and LINC00312 was down-regulated in HiBECs treated with GCDC compared with controls ($26$). The expression of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated and LINC00312 was down-regulated in HiBECs treated with GCDC compared with controls ($26$). The expression of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated and LINC00312 was down-regulated in HiBECs treated with GCDC compared with controls ($26$).

**Demographics and clinical features of PBC patients compared with healthy controls**

There were no differences in age and gender between the training set and validation
**Table 1 Primer sequences used in this study**

| Target gene | Forward sequence (5'→3') | Reverse sequence (5'→3') |
|-------------|--------------------------|-------------------------|
| S100A6      | AATGTGCGTTGTGTAAGC       | CGGTCCAAGTCCTCCATC      |
| LINC00312   | GGAAGGAATACCAACAGAAGT    | TGAAGAACAGGACATTGACA    |
| LINC00472   | AGAGTTGCTGTAGAAGAAGG     | AGGAGGAGAGTAGAAGAGAC    |
| LINC01257   | TGCTGCGAATGATGACTT       | AGGACTTGAATCTGCTACTG    |
| HMGB2       | TTACGTTCCTCCCAAAAGGTG    | TCTTTGGCTGACTGCTCAGA    |
| RC3H2       | TTGCAAAGAAATGCGTTGAG     | GATTGGCAGACAACTGCTGA    |
| ADAMTS1     | CCTCTCTGCTCCTCCAACAC     | GTCGACCGAGCTCTGGCTGA    |
| SERPINE1    | CTCCTCTGCTGCTCAACAC      | GTGGAGAGGCTCTTGGTCTG    |
| PALD1       | GCCGAAGGGTGTCTCCATTTA    | GCTGGACAGGCTCTGCTGA     |
| GSTA4       | TCCCTGAGATGGGTCTTTAGC    | GTCCAAGAGATGGTCTTG      |
| ACTA2       | TTCAATGTCCCAGCCATGTA     | GAAGGAATAGCCACGCACCAG   |
| GAPDH       | GCACCGTCAAGGCTGAGAAC     | TGCTGAGACGCCAGTGGGA     |

ACTA2: Actin alpha 2, smooth muscle; ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GSTA4: Glutathione S-transferase alpha 4; HMGB2: High mobility group box 2; PALD1: Phosphatase domain containing paladin 1; RC3H2: Ring finger and CCCH-type domains 2; S100A6: S100 calcium binding protein A6; SERPINE1: Serpin family E member 1.

Moreover, there were no differences in age and gender between PBC patients and healthy controls (P = 0.58 and P = 1.0, respectively). Clinical serological data including alanine aminotransferase, aspartate aminotransferase, ALP, gamma-glutamyl transpeptidase, total bilirubin, direct bilirubin, total bile acid, hyaluronic acid, laminin, collagen type IV (C-IV) and procollagen III were all significantly higher in PBC patients than in healthy controls (P < 0.0001).

**Differential expression of S100A6 and IncRNAs in PBC patients compared with HCs**

To compare the mean expression levels of S100A6 mRNA, LINC00312, and LINC01257 in PBC patients and healthy controls, the Kolmogorov–Smirnov test was used to check normality. The results showed that these variables had a normal distribution (P > 0.05), and the t-test was used for analysis, as the relative expression of LINC00472 showed a skewed distribution, and was normally distributed after logarithmic conversion based on 10. The results showed that the expression levels of S100A6 mRNA, log10 LINC00472 and LINC1257 in PBC patients were significantly up-regulated compared to the healthy controls (3.01 ± 1.04 vs 2.09 ± 0.87; 2.46 ± 1.03 vs 1.77 ± 0.84; 3.49 ± 1.64 vs 2.37 ± 0.96, P values were all less than 0.0001, Figure 6A, C and D).

The mean expression level of LINC00312 was significantly lower in PBC plasma samples compared with HCs (1.70 ± 0.33 vs 2.07 ± 0.53, P < 0.0001, Figure 6B).

**Distribution of S100A6 and IncRNAs expression levels in different stages of PBC**

The unpaired t-test analysis of variance was performed to evaluate differences in the expression of S100A6 and IncRNAs among different PBC stages and healthy controls (Figure 7). The results showed that the mean expression of S100A6 in the advanced stage (III and IV) of PBC was up-regulated compared to that in HCs and the early stage (II) (3.38 ± 0.71 vs 2.09 ± 0.87, P < 0.0001; 3.38 ± 0.71 vs 2.57 ± 1.21, P = 0.0003, respectively); and in the early stage (II), it was higher than that in HCs (2.57 ± 1.21 vs 2.09 ± 0.87, P = 0.03) (Figure 7A). The mean expression of LINC00312 in the advanced stage was lower than that in the early stage and HCs (1.39 ± 0.29 vs 1.56 ± 0.33, P = 0.01; 1.39 ± 0.29 vs 2.07 ± 0.53, P < 0.0001, respectively) (Figure 7B); in addition, the mean expression of LINC0312 in the early stage was lower than that in HCs (1.56 ± 0.33 vs 2.07 ± 0.53, P < 0.0001) (Figure 7B). The mean expression of log10 LINC00472 in the advanced stage was higher than that in the early stage and HCs (2.99 ± 0.87 vs 1.81 ± 0.83, P < 0.0001; 2.99 ± 0.87 vs 1.77 ± 0.84, P < 0.0001, respectively) (Figure 7C). The mean expression of LINC01257 in both the early stage and advanced stage were up-regulated compared with HCs (3.88 ± 1.55 vs 2.37 ± 0.96, P < 0.0001; 3.57 ± 1.79 vs 2.37 ± 0.96, P < 0.0001, respectively) (Figure 7D).
Table 2 Top 10 dysregulated genes in bile duct ligation and sham mice

| Gene name | Transcript | Lg fold change |
|-----------|------------|---------------|
| **Up-regulated** | | |
| Hmgb2     | ENSMUSG000000054717 | 3.53 |
| Rc3h2     | ENSMUSG000000075376 | 3.33 |
| Adamts1   | ENSMUSG00000022893 | 3.15 |
| Serpine1  | ENSMUSG00000037411 | 3.08 |
| S100a6    | ENSMUSG00000001025 | 2.98 |
| Pald1     | ENSMUSG0000002092  | 2.67 |
| Gsta4     | ENSMUSG00000032348 | 2.50 |
| D17H6S56E-5 | NM_033075    | 2.46 |
| Acta2     | ENSMUSG00000035783 | 2.39 |
| Ifi204    | ENSMUSG00000073489 | 2.33 |
| **Down-regulated** | | |
| Mcm10     | ENSMUSG00000026669 | 3.23 |
| Upp2      | ENSMUSG00000026839 | 2.85 |
| 2810043O03Rik | AK012901.1 | 2.59 |
| Dnaaf5    | ENSMUSG00000025857 | 2.41 |
| Sex       | ENSMUSG00000023289 | 2.40 |
| Naca      | ENSMUSG00000061315 | 2.35 |
| Dhps      | ENSMUSG00000060038 | 2.33 |
| Ch15      | ENSMUSG00000031962 | 2.26 |
| Gzmm      | ENSMUSG00000054206 | 2.20 |
| Alox12    | ENSMUSG00000003220 | 2.15 |

2810043O03Rik: RIKEN complementary deoxyribonucleic acid 2810043O03 gene; Acta2: Actin alpha 2, smooth muscle; Adamts1: A disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 1; Alox12: Arachidonate 12-lipoxygenase; Cd15: Cadherin 15; D17H6S56E-5: Deoxyribonucleic acid segment, Chr 17, human D6S56E 5; Dhps: Deoxyhypusine synthase; Dnaaf5: Dynein, axonemal assembly factor 5; Gsta4: Glutathione S-transferase alpha 4; Gzmm: Granzyme M (lymphocyte met-ase 1); Hmgb2: High mobility group box 2; Ifi204: Interferon activated gene 204; Mcm10: Minichromosome maintenance 10 replication initiation factor; Naca: Nascent polypeptide-associated complex alpha polypeptide; Pald1: Phosphatase domain containing paladin 1; Rc3h2: Ring finger and CCCH-type domains 2; S100A6: S100 calcium binding protein A6; Serpine1: Serpin family E member 1; Sva: Seminal vesicle antigen; Upp2: Uridine phosphorylase 2.

**Diagnosis and staging value of plasma S100A6 and IncRNAs for PBC patients**

ROC curves were used to evaluate the potential diagnostic value of each biomarker for PBC. The AUC for S100A6, LINC00312, log10 LINC00472 and LINC01257 in PBC diagnosis were 0.759, 0.7292, 0.6942 and 0.7158, respectively (Figure 8A-D). Furthermore, AUC for these four genes in PBC staging were 0.666, 0.661, 0.839 and 0.5549, respectively (Figure 8E-F).

Pearson or Spearman correlation analysis was performed to evaluate the correlation between relative expression of S100A6 mRNA and IncRNAs, as well as relative expression of S100A6 mRNA or IncRNAs and clinical serological data in PBC patients. Relative expression of S100A6 mRNA was positively correlated with log10 LINC00472 (r = 0.683, P < 0.0001); serum level of C-IV was positively correlated with relative expression of log10 LINC00472 (r = 0.482, P < 0.0001); relative expression of S100A6 mRNA was positively correlated with serum level of C-IV (r = 0.732, P < 0.0001) (Figure 9).

**Comparison of expression levels of biomarkers before and after treatment**

A total of 58 PBC patients were followed up after their treatment for one year. Paired t-test analysis was used to compare the expression levels of these four genes before and after treatment. The relative expression of S100A6 mRNA, log10 LINC00472, and
Table 3 Demographics and clinical characteristics in the training and validation datasets

| Characteristics | Training | Validation | P value |
|-----------------|----------|------------|---------|
| No.             | 140      | 115        | -       |
| Age, mean ± SD, yr | 56.0 ± 13.9 | 57.2 ± 13.2 | 0.504   |
| Gender, n (%)   |          |            |         |
| Male            | 17 (12.1)| 13 (11.3)  |         |
| Female          | 123 (87.9)| 102 (88.7)| 1.0     |
| Pathological stage |        |            |         |
| I and II        | 36 (45.0)| 26 (40.0)  |         |
| III and IV      | 44 (55.0)| 39 (60.0)  | 0.614   |

Normally distributed data are expressed as mean ± SD. Categorical variable values are described as n (%). SD: Standard deviation.

Table 4 Demographics and clinical characteristics of primary biliary cholangitis patients and healthy controls

| Characteristics | PBC (n = 145) | HCs (n = 110) | P value |
|-----------------|---------------|---------------|---------|
| Age, mean ± SD, yr | 56.1 ± 13.4  | 55.3 ± 11.8  | 0.38    |
| Gender, n (%)   |               |               |         |
| Male            | 17 (12.5)     | 13 (16.7)    |         |
| Female          | 128 (87.5)    | 97 (83.3)    | 1.00    |
| ALT, U/L        | 78.6 ± 35.7   | 18.4 ± 6.5   | <0.001  |
| AST, U/L        | 104.8 ± 43.5  | 20.2 ± 4.3   | <0.001  |
| ALP, U/L        | 257.4 ± 79.9  | 64.7 ± 14.5  | <0.001  |
| γGT, U/L        | 416.7 ± 209.2 | 26.3 ± 10.4  | <0.001  |
| TBIL, μmol/L    | 66.8 ± 10.6   | 11.8 ± 4.0   | <0.001  |
| DBIL, μmol/L    | 51.9 ± 11.4   | 6.4 ± 0.5    | <0.001  |
| TBA, μmol/L     | 71.3 ± 11.6   | 2.8 ± 0.4    | <0.001  |
| HA, ng/mL       | 146.9 (104.6-190.1) | 67.0 (53.9-79.7) | <0.001 |
| LN, ng/mL       | 148.9 (76.7-182.8) | 70.4 (58.7-82.9) | <0.001 |
| C-IV, ng/mL     | 154.8 (121.1-192.0) | 60.1 (55.2-66.7) | <0.001 |
| PC-III, ng/mL   | 161.0 (135.1-184.5) | 57.3 (49.9-65.3) | <0.001 |
| Pathological stage |            |               |         |
| I and II        | 62 (42.8)     | -             |         |
| III and IV      | 83 (57.2)     | -             |         |

†Normally distributed data are expressed as means ± SD, variables with a skewed distribution are presented as median (interquartile range). Categorical variable values are described as n (%). γGT: Gamma-glutamyl transpeptidase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; C-IV: Collagen type IV; DBIL: Direct bilirubin; HA: Hyaluronic acid; LN, Laminin; PC-III: Procollagen III; TBA: Total bile acid; TBIL: Total bilirubin; SD: Standard deviation; PBC: Primary biliary cholangitis; HCs: Healthy controls.

LINC01257 were significantly decreased after treatment (2.35 ± 1.02 vs 3.06 ± 1.04, P = 0.0018; 1.99 ± 0.83 vs 2.33 ± 0.96, P = 0.036; 2.84 ± 0.92 vs 3.69 ± 1.54, P = 0.0006, respectively); in addition, the relative expression of LINC00312 increased significantly after treatment compared with before treatment (1.95 ± 0.35 vs 1.73 ± 0.32, P = 0.0007) (Figure 10).

Differences between PBC patients with high and low levels of LINC00472
According to ROC curves analysis, the AUC of log10 LINC00472 was 0.839 (P < 0.0001)
and the Youden index was 1.551. Accordingly, the patients in the PBC group were divided into L1 (log10 LINC00472 < 2.33) and L2 (log10 LINC00472 ≥ 2.33) subgroups. The baseline characteristics of PBC patients classified by the relative expression of the log10 LINC00472 cutoff value (2.33) is shown in Table 5. The relative expression of S100A6 mRNA and serum level of C-IV were lower in the L1 subgroup (P < 0.0001, Table 5); in addition, the relative expression of LINC01257 was higher in the L1 subgroup compared to the L2 subgroup (P = 0.005, Table 5).

**Validation of diagnosis and staging value**

The parameters estimated from the training data set were used to predict the probability of being diagnosed with PBC and staging of PBC for the independent validation data set. ROC curves were also constructed to predict the probability of diagnosis and staging. The AUC of S100A6 mRNA, LINC00312, log10 LINC00472 and LINC01257 in PBC diagnosis were 0.769, 0.772, 0.755 and 0.695, respectively (Figure 11A-D). Moreover, the AUC for log10 LINC00472 in PBC staging was 0.835 (Figure 11E).
Table 5 Characteristics of primary biliary cholangitis patients based on the expression of log10 LINC00472 cutoff value

| Relative Expression of log10 LINC00472 | L1 (< 2.33) (n = 38) | L2 (≥ 2.33) (n = 42) | P value |
|-----------------------------------------|----------------------|----------------------|---------|
| Age, mean ± SD, years                   | 60.3 ± 14.9          | 55.1 ± 14.0          | 0.109   |
| Gender, n (%)                           |                      |                      |         |
| Male                                    | 5 (13.2)             | 5 (11.9)             |         |
| Female                                  | 33 (86.8)            | 37 (88.1)            | 0.886   |
| ALT, U/L                                | 73.2 (52.7-100.1)    | 73.2 (46.2-100.7)    | 0.985   |
| AST, U/L                                | 109.2 ± 45.9         | 103.0 ± 44.6         | 0.543   |
| ALP, U/L                                | 264.0 ± 89.4         | 252.2 ± 78.3         | 0.532   |
| γGT, U/L                                | 420.2 ± 197.9        | 413.2 ± 237.1        | 0.887   |
| TBA, μmol/L                             | 73.2 ± 12.4          | 70.9 ± 13.3          | 0.438   |
| TBil, μmol/L                            | 68.0 (63.0-73.0)     | 63.0 (58.0-73.0)     | 0.166   |
| DBil, μmol/L                            | 50.6 ± 9.8           | 52.2 ± 11.4          | 0.505   |
| LINC00312                               | 1.51 ± 0.32          | 1.43 ± 0.31          | 0.261   |
| S100A6                                   | 2.40 ± 1.05          | 3.57 ± 0.66          | < 0.0001|
| LINC01257                               | 4.25 ± 1.39          | 3.22 ± 1.78          | 0.005   |
| HA, ng/mL                               | 144.8 (101.6-208.8)  | 135.5 (95.4-195.4)   | 0.537   |
| LN, ng/mL                               | 126.1 (48.4-178.4)   | 156.1 (57.6-175.8)   | 0.78    |
| C-IV, ng/mL                              | 127.2 (100.9-170.4)  | 176.0 (154.7-232.0)  | < 0.0001|
| PC-III, ng/mL                            | 156.6 (125.8-190.1)  | 161.6 (128.0-184.5)  | 0.916   |

SD: Standard deviation; γGT: Gamma-glutamyl transpeptidase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; C-IV: Collagen type IV; DBil: Direct bilirubin; HA: Hyaluronic acid; LN: Laminin; PC-III: Procollagen III; S100A6: S100 calcium binding protein A6; TBA: Total bile acid; TBil: Total bilirubin.

DISCUSSION

PBC is a type of cholestatic liver disease which is a pathophysiological process caused by the obstruction of bile secretion and excretion. After analyzing the expression levels of the top 10 up-regulated genes of GSE29776 in the plasma of PBC patients, it was found that the difference in S100A6 mRNA expression levels between PBC patients and healthy controls was greatest (t = 20.28, P < 0.0001). Therefore, S100A6 was selected as the target gene. BDL is a common procedure for biliary obstruction widely used in rodent models of cholestasis and liver damage.[30] Immunofluorescence double labeling analysis was performed to identify the overexpression of S100A6 protein in the intrahepatic bile duct epithelial cells of BDL mice compared with sham mice, which verified the results predicted by bioinformatics analysis. In this study, the bile duct cells proliferated greatly in the liver tissue 10 d after the operation in the BDL group,[31,32] and S100A6 protein was expressed in large quantities during the corresponding period. However, the number of bile duct cells in the sham group was relatively low, and the expression of S100A6 protein was also relatively low. Therefore, it can be seen that proliferation of bile duct cells was specifically enhanced when cholestatic liver injury occurred; thus, there was a difference in S100A6 expression between the two groups. S100A6 is expressed as a 89-amino acid protein in mice and rats, a 90-amino acid protein in humans and rabbits, and subtypes A (92 amino acids) and B (91 amino acids) in chickens, which may be produced by mRNA selective splicing[33]. In this study, the S100A6 antibodies used were universal in humans and mice, so the results of the BDL mouse model could indirectly reflect the up-regulation of S100A6 expression in human intrahepatic cholestasis.

In this study, S100A6 mRNA was overexpressed in the plasma of PBC patients compared with healthy controls. S100A6 expression is up-regulated in breast cancer, thyroid cancer, colorectal cancer, various types of skin tumors, acute myelogenous leukemia, epithelial tissues and other highly proliferating cell lines[34]. Apoptosis in
PBC is considered to be the cell effector injury mediated by T cells. Changes in apoptosis and apoptosis-related molecular expression of bile duct cells have been reported in bile duct lesions, but immune-mediated injury of bile duct epithelial cells has not been fully elucidated[35]. Joo et al[36] found that S100A6 may be involved in the process of apoptosis by regulating the transcriptional regulation of caspase-3. Therefore, it seems that S100A6 may play an important role in the pathogenesis of PBC.
The expression of lncRNAs is not only closely related to the occurrence and development of tumors[37], but also associated with autoimmune diseases[38]. In this study, the expression of lncRNAs selected by bioinformatics analysis was differentially expressed in the plasma of PBC patients compared with healthy controls. The levels of plasma LINC00312 was significantly down-regulated in PBC patients, while LINC00472 and LINC01257 were up-regulated in PBC patients, indicating that these lncRNAs might be valuable for PBC diagnosis. ROC curves were used to evaluate the diagnostic value of each marker. The differential expression in plasma between PBC patients and healthy controls indicated that S100A6 mRNA (AUC = 0.76, \( P < 0.0001 \)), LINC00312 (AUC = 0.73, \( P < 0.0001 \)), log10 LINC00472 (AUC = 0.69, \( P < 0.0001 \)) and LINC01257 (AUC = 0.72, \( P < 0.0001 \)) may be potential biomarkers for the diagnosis of PBC.

Furthermore, the ROC curves analysis also showed that plasma S100A6 mRNA (AUC = 0.67, \( P = 0.01 \)), LINC00312 (AUC = 0.66, \( P = 0.01 \)) and log10 LINC00472 (AUC = 0.84, \( P < 0.0001 \)) could also be used to predict disease progression in PBC. In particular, LINC00472 had high diagnostic value for PBC staging (sensitivity was 77.27%, specificity was 77.78%). According to the cutoff value (2.33) of log10 LINC00472, the relative expression of S100A6 mRNA and serum level of C-IV in the high-level group were higher than those in the low-level group.

LINC00312, also known as NAG7, was found to inhibit proliferation and induce apoptosis in nasopharyngeal carcinoma (NPC) cells but also stimulate NPC cell invasion. LINC00312 was significantly down-regulated in NPC tissues compared with non-cancerous nasopharyngeal epithelium tissues. Positive expression of LINC00312 was negatively correlated with tumor size but positively correlated with lymph node metastasis[39]. High expression of LINC00472 was associated with less aggressive breast tumors and better prognosis. Patients with high expression of LINC00472 had a significantly reduced risk of recurrence and death compared to those with low expression. Patients with high expression of LINC00472 also responded better to adjuvant chemotherapy or hormone therapy than those with low expression[40]. Therefore, studies on S100A6, LINC00312 and LINC00472 have all been related to tumors. This study is the first to explore the relationship between these three genes and autoimmune diseases. In addition, we investigated the relationship between the expression of LINC01257 and diseases for the first time.
Figure 7 Scatter plot and distribution of expression levels of S100 calcium binding protein A6 protein messenger ribonucleic acid and long non-coding ribonucleic acids in different stages of primary biliary cholangitis compared with healthy controls. The unpaired t-test analysis of variance was performed to examine differences in S100 calcium binding protein A6 protein messenger ribonucleic acid and long non-coding ribonucleic acids expression levels between various groups. A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: log10 LINC00472; D: LINC01257. *P < 0.05, **P < 0.0001. HCs: Healthy controls.

The expression levels of plasma S100A6, LINC00312, LINC00472 and LINC01257 in PBC patients before and after treatment were analyzed by the paired t-test. It was found that the elevated biomarkers decreased after treatment, while the reduced biomarker increased. This provides further evidence that these four genes are biomarkers for PBC diagnosis.

The correlation analysis showed that relative expression of S100A6 mRNA was positively correlated with log10 LINC00472 ($r = 0.683, P < 0.0001$) and the serum level of C-IV ($r = 0.732, P < 0.0001$). C-IV serves as a histochemical marker of perisinusoidal basement membrane formation in liver disease[41]. It was further illustrated that S100A6 may be associated with PBC liver injury. The relative expression of log10 LINC00472 was positively correlated with the serum level of C-IV ($r = 0.482, P < 0.0001$), indicating that it was related to the disease severity of PBC. It was suggested that LINC00472 can be used as a marker of PBC staging. However, in our study, the four biomarkers did not correlate with the cholestasis indicator ALP, and we think this may be due to the following reasons: (1) The S100A6 protein was expressed in large quantities during the early period of cholestasis. This process may precede the increase in serum ALP level; (2) Proliferation of bile duct cells is characterized by irregular proliferation of intrahepatic bile ducts not only confined to portal areas, but also sprouting into periportal and parenchymal regions. This implies that the newly formed bile ducts are functionally ineffective[42,43]; and (3) In the late stage of liver fibrosis, considerable hepatocyte necrosis occurs.

Hepatocytes exposed to bile acids have been used in many studies on PBC. The most commonly used bile acid is GCDC, which is a type of toxic hydrophobic bile acid and can induce apoptosis of iBECs, form apoptotic bodies, and can lead to the pyruvate dehydrogenase complex E2 subunit as an autoimmune antigen to be exposed. A series of immune responses are then activated[44]. Hisamoto et al[45] studied the effects of hydrophobic bile acid on human BECs and autologous spleen mononuclear cells, especially the effects of GCDC on anion exchange protein...
Figure 8 Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis and staging in the training set. A-D: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis in the training set; E-H: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis staging in the training set. AUC: Area under the curve; CI: Confidence interval.
Figure 9 Correlation analysis of biomarkers and clinical serological indices. A: The positive correlation between relative expression of s100 calcium binding protein A6 protein messenger ribonucleic acid and log10 LINC00472, \( r = 0.683, P < 0.0001 \); B: The positive correlation between relative expression of log10 LINC00472 and serum level of collagen type IV, \( r = 0.482, P < 0.0001 \); C: The positive correlation between serum level of collagen type IV and relative expression of s100 calcium binding protein A6 protein messenger ribonucleic acid, \( r = 0.732, P < 0.0001 \).

Figure 10 Comparison and analysis of s100 calcium binding protein A6 protein messenger ribonucleic acid, LINC00312, log10 LINC00472, LINC01257 expression levels in primary biliary cholangitis patients before and after treatment using the paired t-test. A: S100 calcium binding protein A6 protein messenger ribonucleic acid; B: LINC00312; C: log10 LINC00472; D: LINC01257.

expression of BECs and on the phenotype of BECs and local inflammatory response. It was proved that GCDC reduced the expression of anion exchange in BECs and accelerated the aging of BECs by inducing reactive oxygen species. Therefore, this study used GCDC to treat HiBECs to simulate a cholestatic environment and assess its damage to HiBECs. In this study, the expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated while LINC00312 was down-regulated in GCDC-treated HiBECs compared with controls, consistent with the expression in plasma of PBC patients. It was further proved that these four indicators are related to PBC diagnosis and staging.
Figure 11 Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis and staging in the validation set. A-D: Receiver operating characteristic curves of s100 calcium binding protein A6 protein, LINC00312, LINC00472 and LINC01257 for primary biliary cholangitis diagnosis in the validation set; E: Receiver operating characteristic curves of LINC00472 for primary biliary cholangitis staging in the validation set. AUC: Area under the curve; CI: Confidence interval.

The value of the above four biomarkers should be validated in an additional cohort of PBC patients and their specificity needs to be examined in other patient populations[46]. We chose another PBC cohort as the validation set. The AUC of the four genes were close to those in the training set. Therefore, the value of these four biomarkers in the diagnosis and staging of PBC was validated. However, in China, the vast majority are Han Chinese; therefore, it is difficult to verify these findings in other ethnic groups.

CONCLUSION

In conclusion, the expression of S100A6 protein in BDL mice was up-regulated, the expression of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated, while LINC00312 was down-regulated both in the plasma of PBC patients and HiBECs treated with GCDC compared with controls. Although our study was confined to the expression analysis of S100A6 mRNA, LINC00312, LINC00472 and LINC01257, warranting further studies to investigate the mechanisms underlying the functional role of these four markers, nevertheless their potential as biomarkers for diagnosis and staging of PBC was elucidated by multiple evaluations in this study.

ARTICLE HIGHLIGHTS

Research background

Primary biliary cholangitis (PBC) is an autoimmune liver disease that mostly affects women. Fatigue and persistent pruritus are the most obvious symptoms. PBC may lead to cholestasis, liver fibrosis, cirrhosis and, eventually, liver failure. The injury mechanism of intrahepatic biliary epithelial cells is the key to investigating the pathogenesis of PBC, but the accurate relationship between cholestasis and liver...
fibrosis is still indistinct.

**Research motivation**
To explore the target genes of intrahepatic biliary epithelial cell injury in PBC. To search for plasma biomarkers for early diagnosis and staging of PBC. To lay a foundation for further study on the pathogenesis of PBC.

**Research objectives**
To explore the potential diagnosis and staging value of plasma S100 calcium binding protein A6 (S100A6) messenger ribonucleic acid (mRNA), LINC00312, LINC00472, and LINC01257 in primary biliary cholangitis.

**Research methods**
The up-regulation of S100A6 was identified by double immunofluorescence in a bile duct ligation mouse model. We used quantitative reverse transcription-polymerase chain reaction to analyze the relative expression levels of S100A6 mRNA, long noncoding ribonucleic acids (lncRNAs) LINC00312, LINC00472 and LINC01257 both in patients with PBC and in human intrahepatic biliary epithelial cells treated with glycochenodeoxycholate.

**Research results**
The relative expression levels of S100A6 mRNA, LINC00472 and LINC01257 were up-regulated while LINC00312 was down-regulated in both the plasma of patients with PBC and in human intrahepatic biliary epithelial cells treated with glycochenodeoxycholate.

**Research conclusions**
These four genes may potentially act as novel biomarkers for the diagnosis of PBC. Moreover, LINC00472 acts as a biomarker for staging in PBC.

**Research perspectives**
Although we have demonstrated that S100A6 and related lncRNAs may be biomarkers for the diagnosis and staging of PBC, their detailed value needs to be analyzed in a large sample. The specific mechanisms of S100A6 and lncRNAs require further investigation.

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