

Dear editors and reviewers,

The authors are very grateful to the editors and technical reviewers for their careful reviews of the manuscript entitled **“S100A6 and Associated lncRNAs as Biomarkers in the Diagnosis and Staging of Primary Biliary Cholangitis”** (Manuscript NO.: **60827, Basic Study**) and offering their insightful comments and suggestions to improve the quality of the article. We revised the manuscript according to your decision letter, and all the detailed correction were shown in our cover letter text material.

The following responses have been prepared to address the Editor's and the reviewers' comments. The responses to the comments are marked as **blue text**. And the tracking version of manuscript is uploaded again. Thank you!

Comment 1: In order to consider S100A6 and associated lncRNAs as PBC-specific “diagnosis” biomarkers, it is necessary to show that their expression levels are different from those of other diseases. However, only PBC and healthy controls were compared in the present study. On the other hand, S100A6 and associated lncRNAs will be able to the biomarkers for “staging”. The entire manuscript, including the title, should be revised.

**Response:** Your suggestions are very helpful to our article. In this study, we ignored the expression of S100A6 and lncRNAs in other diseases. So, we looked up references and related websites and added relevant content in the discussion section. “Staging” is added where diagnosis biomarkers are found throughout the manuscript according to the comment.

The corrected information as below:

We have added a discussion on lncRNAs in paragraph 5 of the discussion section:

**“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 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 were all related to tumors. This study is the first time to explore the relationship between these three genes and autoimmune diseases. While, our study investigated the relationship between the expression of LINC01257 and diseases for the first time."

The second paragraph of "In this study, S100A6 mRNA was overexpressed in plasma of PBC patients compared with healthy controls." was moved to the beginning of the paragraph.

Comment 2: If there were changes in expression levels of S100A6 and associated lncRNAs before and after treatment, the data should be shown.

Response: Your comment is very appropriate. We also tried to compare the expression levels of S100A6 and lncRNAs before and after treatment. Paired *t*-test was used to analyze the difference of S100A6 mRNA and lncRNAs expression in plasma of 58 patients with PBC before and after one year treatment.

The corrected information as below:

- (1) Added in the Statistical Methods section: "Paired *t*-test was used to compare the expression levels before and after treatment."
- (2) Results of comparison of before and after treatment were added in the Results section:

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

Also add Figure 10:

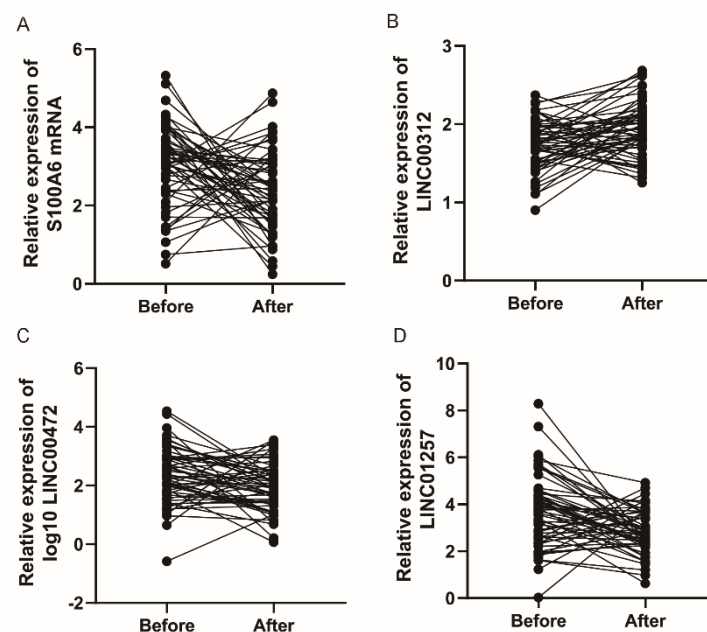


Figure 10 Comparison and analysis of S100A6 mRNA, LINC00312, log10 LINC00472, LINC01257 expression levels in PBC patients before and after treatment using paired *t*-test. A: S100A6 mRNA; B: LINC00312; C: log10 LINC00472; D: LINC01257.

(3) Relevant content has also been added in paragraph 5 of the discussion section:

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

Comment 3: Was S100a6 the gene with the greatest change in expression level between disease model mice and control? That data should be shown in a Figure or Table.

Response: Your suggestion is very thoughtful. Although we screened the differential genes and gave the results directly, we did not specify the screening process. Therefore, we added relevant content in the results section according to your comment.

The corrected information as below:

(1) We added Table 2 to list the top 10 up- and down-regulated genes in the BDL and sham group in GSE29776.

Table 2 Top 10 dysregulated genes in BDL and sham mice

Gene name	Transcript	Lg fold change
Up-regulated		
Hmgb2	ENSMUSG00000054717	3.53
Rc3h2	ENSMUSG00000075376	3.33
Adamts1	ENSMUSG00000022893	3.15
Serpine1	ENSMUSG00000037411	3.08
S100a6	ENSMUSG00000001025	2.98
Pald1	ENSMUSG00000020092	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
Sva	ENSMUSG00000023289	2.40
Naca	ENSMUSG00000061315	2.35
Dhps	ENSMUSG00000060038	2.33
Cdh15	ENSMUSG00000031962	2.26
Gzmm	ENSMUSG00000054206	2.20
Alox12	ENSMUSG00000000320	2.15

2810043O03Rik: RIKEN cDNA 2810043O03 gene; Acta2: Actin alpha 2, smooth muscle; Adamts1: A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1; Alox12: Arachidonate 12-lipoxygenase; Cdh15:

Cadherin 15; D17H6S56E-5: DNA 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.

(2) At the same time, we also modified Table 1 and added primers used in the pre-experiment.

Table 1 Primer sequences used in this study

Target gene	Forward sequence (5'→3')	Reverse sequence (5'→3')
S100A6	AATGTGCGTTGTGTAAGC	CGGTCCAAGTCTTCCATC
LINC00312	GGAAGGAATACCACAGAAGT	TGAAGAACAGGACATTGACA
LINC00472	AGAGTTGCTGTAGAAGAAGG	AGGAGGAGAGTAGAAGAGAC
LINC01257	TGCTGCGAATGATGACTT	AGGACTTGAATCTGCTACTG
HMGB2	TTACGTTCCCTCCCAAAGGTG	TCTTTGGCTGACTGCTCAGA
RC3H2	TTGCAAAGAAATGCGTTGAG	GATTGGCAGACAACCTGCTGA
ADAMTS1	CCTCTGTCTGTGTGCAAGGA	GTGGCTCCAGTTGGAATTGT
SERPINE1	CTCTCTCTGCCCTCACCAAC	GTGGAGAGGCTCTTGGTCTG
PALD1	GCCGAAGTTGTTCCCATTTA	GCTGAAAGTCAGAGCCAACC
GSTA4	TCCGTGAGATGGGTTTTAGC	TGCCAAAGAGATTGTGCTTG
ACTA2	TTCAATGTCCCAGCCATGTA	GAAGGAATAGCCACGCTCAG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

ACTA2: Actin alpha 2, smooth muscle; ADAMTS1: ADAM metalloproteinase 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.

(3) We used qRT-PCR to analyze and compare the differences of the top 10 up-regulated genes between 30 PBC patients and 30 healthy controls. We listed the expression differences of these genes in Figure 2.

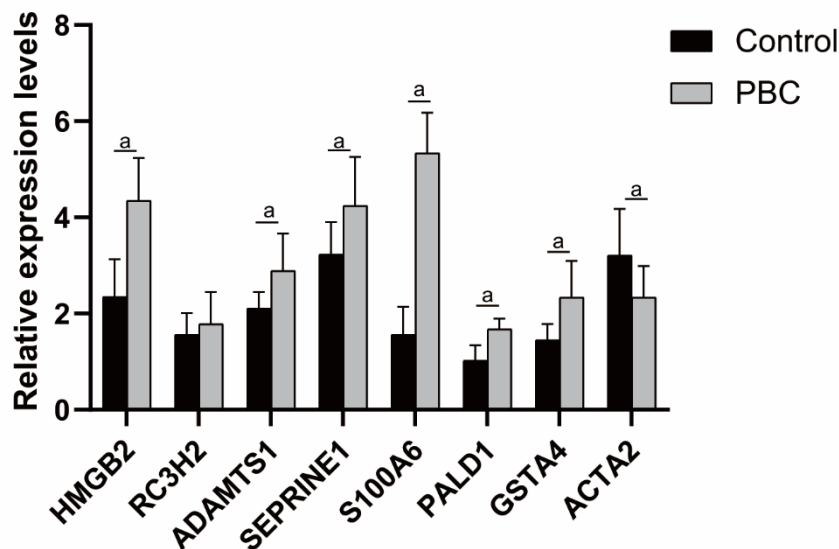


Figure 2 Validation of top10 up-regulated genes in plasma of PBC patients and healthy controls by qRT-PCR. <sup>a</sup> $P < 0.0001$ .

(4) At the same time, we give a detailed description in the results.

*Identification of target gene*

“GEO2R” was used to analyze the differentially expressed genes (DEGs) in liver tissues of BDL and sham mice of GSE29776. The top 10 up- and down-regulated genes of GSE29776 in the BDL and the sham group were 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 was the greatest change in plasma of PBC patients ( $t = 20.28$ ,  $P < 0.0001$ ) (Figure 2). Therefore, S100A6 was selected as the target gene in this study.

(5) We have also added relevant content in paragraph 1 of the discussion section:

“After analyzing the expression levels of the top 10 up-regulated genes of GSE29776 in plasma of PBC patients, it was found that the difference of S100A6 mRNA expression levels between PBC patients and healthy controls was the greatest ( $t = 20.28$ ,  $P < 0.0001$ ).”

According to the editor's comments, we also made some modifications to the manuscript to meet the requirements of your journal as much as possible. These changes will be reflected in the manuscript. We appreciate the Editors/Reviewers so much and hope that the correction will meet with approval.

Thank you again for your time and consideration!

Your sincerely,

Xihua Dong, Hui Kang

1. Zhang W, Huang C, Gong Z, Zhao Y, Tang K, Li X, Fan S, Shi L, Li X, Zhang P, Zhou Y, Huang D, Liang F, Zhang X, Wu M, Cao L, Wang J, Li Y, Xiong W, Zeng Z, Li G. Expression of LINC00312, a long intergenic non-coding RNA, is negatively correlated with tumor size but positively correlated with lymph node metastasis in nasopharyngeal carcinoma. *J Mol Histo* 2013;**44**:545-54
2. Shen Y, Katsaros D, Loo LW, Hernandez BY, Chong C, Canuto EM, Biglia N, Lu L, Risch H, Chu WM, Yu H. Prognostic and predictive values of long non-coding RNA LINC00472 in breast cancer. *Oncotarget* 2015;**6**:8579-92