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Original Article

Comparison of LncRNA profile in mouse models of drug-induced liver injury and autoimmnue hepatitis

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Abstract: Aim: To analyze and compare the expression profile of long non-coding RNA (lncRNA) in the liver tissue of drug-induced liver injury (DILI) and autoimmune hepatitis (AIH). Methods: Mouse models of DILI and AIH were constructed, with non-treated mice as healthy controls. lncRNA microarray was used to detect the lncRNA expression profile in mice liver tissues. Defferentially expressed lncRNAs with the most meaningful fold changes were further validated by real-time PCR. Results: Compared with normal hepatic tissues, 8 lncRNAs were simultaneously up-regulated in both DILI and AIH, and 28 were simultaneously down-regulated in both diseases. Notably, among these simultaneously dysregulated lncRNAs, some had quite different fold changes in the two disease models. Besides, 32 dysregulated lncRNAs (13 up-regulated ones and 19 down-regulated ones) were observed only in DILI models, while 24 dysregulated lncRNAs (9 up-regulated ones and 15 down-regulated ones) were restrictedly expressed in AIH models. RT-PCR validation was consistent with the results of microarray analysis. Conclusion: Some of the dysregulated lncRNAs were differentially expressed between AIH and DILI, and the detection of lncRNA profile or of differentially expressed lncRNAs may help distinguish between the two diseases.

Keywords: lncRNAs, lncRNA microarray, differential diagnosis, drug-induced liver injury, autoimmune hepatitis

Introduction

As is known, mammalian genomes transcribe a broad spectrum of RNA molecules, including the classical long proteincoding mRNAs, some short regulatory noncoding RNAs (e.g., microRNAs, small interfering RNAs), and many more recently characterized long noncoding RNAs (lncRNAs) [1, 2]. Defined as non-coding RNA molecules greater than 200 nucleotides in length, lncRNAs have been shown to play significant regulatory roles in multiple major biological processes, including development [3], differentiation [4] and carcinogenesis [5]. Previous studies have also provided some concrete evidences regarding the correlation between the dysregulated lnRNA profile and the occurrence and development of many human diseases, from various cancers [6] to congenital defects such as brachydactyly syndromes [7]. In the research field of hepatic diseases, some recent studies have implicated lncRNAs involved in liver regeneration [8], hepatic ischemia/reperfusion injury [9], and hepatocellular carcinoma (HCC) [10]. However, few studies have focused on the potential value of lnRNAs and lnRNA profile in the differential diagnosis of different hepatic diseases.

Autoimmune hepatitis (AIH) is an unresolving progressive liver disease with unknown etiology characterized by hypergammaglobulinemia, autoantibodies detection and interface hepatitis [11]. While drug-induced liver injury (DILI) represents a diverse set of responses following exposure to any manufactured or naturally occurring chemical compound [12]. Timely diagnosis and proper management are critical in both conditions, for the control of AIH progression requires early immunosuppressive therapy while the first move in DILI intervention should be the prompt identification and discontinuation of offending drugs [13]. Failure to properly treat AIH and DILI could sometimes lead to devastating clinical outcomes such as acute liver failure (ALF) [14, 15]. However, in clinical practice, the diagnosis

and differential diagnosis of idiopathic AIH and DILI can be very challenging since both conditions have heterogeneous clinical manifestations as well as similar laboratory test results. Even the application of liver biopsy cannot guarantee a correct diagnosis due to the lack of individual histologic feature that is absolutely indicative of either DILI or AIH [13].

In this study, we compared the lncRNA expression profiles of AIH and DILI using the liver tissues in mouse models. The comparison analysis revealed that dysregulated LncRNAs may be useful for the diagnosis and differential diagnosis of AIH and DILI.

Materials and methods

Animal ethics statement

BALB/c mice were supplied by the Animal Experiment Center of Chinese Academy of Sciences (Shanghai, China). Animals were housed in pathogen free conditions under a 12-h light/dark cycle, with food and water freely available. All of the experimental procedures were approved by the Zhejiang Chinese Medical University Ethics Committee. All animal protocols complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China, and efforts were made to minimize numbers and suffering of animals used.

Generation of AIH model and DILI model

Thirty male BALB/c mice five to six week old, weighing 19-22 g were acclimatized for 1 week before the study. Then these mice were randomly grouped into the AIH group, the DILI group and the control group, 10 mice in each group. The protocol for generation of murine AIH and DILI models had been reported by previous studies [16, 17]. Briefly, Concanavalin A (ConA) purchased from Sigma-Aldrich Corporation, US was fully dissolved in sterilized Saline to make ConA solution with final concentration of 1 g/L. Likewise, acetaminophen (APAP) from Tokyo Chemical Industry, Japan was prepared as 12.5 g/L solution. Mice in the AIH group received 0.02 mL/g ConA solution via tail vein, while mice in the DILI group received 0.04 mL/g APAP solution intraperitoneally (i.p.). Mice in the control group received no intervention.

Blood and tissue collection

Mice in the AIH group were euthanized 12 h after the injection, mice in the DILI group were euthanized 24 h after the injection, and mice that received no treatment were also euthanized as healthy controls. Bood and livers were collected.

Portions of mouse livers were fixed in formalin and snap frozen in liquid nitrogen for further analysis. The blood collected by cardiac puncture was centrifuged to obtain serum for further detection of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Detection of serum ALT, AST activities and histology of liver tissue

The detection of ALT and AST was performed using the Olympus AU400 Automatic Biochemistry Analyzer. Liver sections prepared from Formalin-fixed paraffin-embedded liver tissues were stained using Hematoxylin and Eosin (H&E) and observed under microscope.

RNA extraction

Total RNA was extracted from all the liver tissues using Trizol reagent (Invitrogen, US) according to the manufacturer's instruction. The quantity and quality of RNA was assessed by measuring OD260 nm, OD280 nm and OD260/OD280 with the NanoDrop ND-1000 spectrophotometer (NanoDrop, US) and RNA integrity was assessed using standard denaturing agarose gel electrophoresis.

LncRNA microarray and data analysis

The microarray (GeneChip Mouse Gene 2.0 Array, Affymetrix, US) used in this study covered more than thirty-five thousand lncRNAs coming from the most authoritative databases including RefSeq, Ensemble, FANTOM3, lncRNAdb, etc. Each transcript was represented by multiple probes to improve statistical confidence. And external positive controls (BioB, BioC, BioD and Cre) were also provided for quality control of the genechips.

For microarray hybridization, total RNA was reverse-transcribed to cDNA using the Double Strand cDNA Synthesis Kit (Invitrogen Corporation, US), and then labeled and hybridized

Table 1. Plasma activities of ALT and AST of mice in each group (U/L, mean ± SD)

Group	ALT	AST
AIH (n=10)	1302.90±143.24**	576.40±140.81**
DILI (n=10)	1477.60±202.18**	651.10±143.14**
Control (n=10)	36.10±7.23	125.32±14.38

Note: **stands for $P<0.01$ *vs* control.

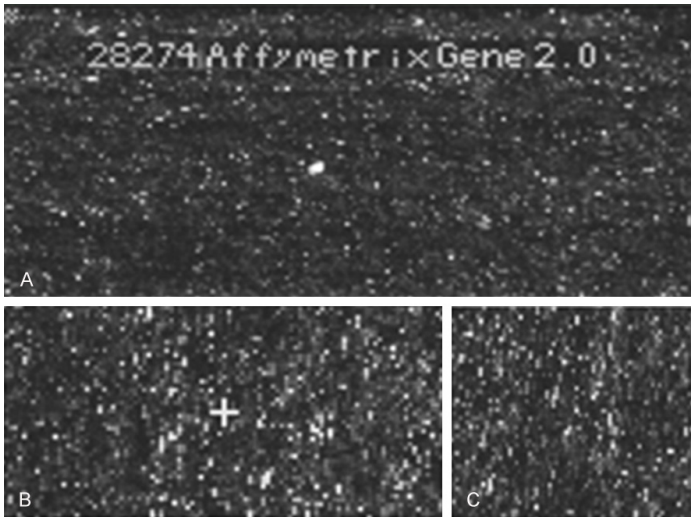

Figure 1. Profile of hybridized genechip of mouse liver tissues. A: A Corner of genechip and marker; B: Marker "+" for quanlity control of hybridization; C: A section of hybridized genechip profile.

using the GeneChip WT Terminal Labeling and Controls Kit from Affymetrix corporation.

For microarray analysis, fluorescent signals of the microarrays were scanned by GeneChip Scanner 3000 7G (Affymetrix, US), and scanned images (TIF format) were subsequently imported into Affymetrix GeneChip Operating Software for grid alignment and expression data analysis. Quantile normalization and subsequent data processing were performed using the Affymetrix Expression Console. Then differentially expressed LncRNAs were identified through fold change filtering: the threshold for up-regulation was fold change >1.5 and for down-regulation, fold change <0.7.

Quantitative real-time PCR

The expression level of the most differently expressed lncRNAs was detected by real-time PCR using the Bio-Rad fluorescence quantitative PCR instrument following the manufacturer's instructions (All the primers are available on request). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. For quantitative results, the expression of each lncRNA was calculated according to fold change using $2^{-\Delta\Delta Ct}$ methods. All experiments were performed in triplicate.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed with the Student's t-test for comparisons of two groups and analysis of variance (ANOVA) for multiple comparisons. In both cases, differences with $P<0.05$ were considered statistically significant. The statistical significance of microarray results was analyzed in terms of fold change using the Student's t-test.

Results

Characterics of liver injury after ConA or APAP treatment

After ConA or APAP treatment, H.E. staining assay and markedly elevated plasma ALT and AST activities (shown in **Table 1**) revealed significant signs of liver injury of mice in the AIH group and DILI group. There were significant overlap of histologic findings in mouse models of AIH and DILI. Interface hepatitis, focal necrosis and portal inflammation were present in all cases of AIH and DILI. In the liver tissue of AIH mice, portal and intra-acinar plasma cells, rosette formation, prominent intraacinar eosinophils and emperiopolesis were more common; while in the DILI, portal neutrophils and hepatocellular cholestasis were more prevalent. These histologic features of AIH and DILI were in line with those reported in previous research [13].

However, mice in the control group did not show typical histological manifestations of liver injury, or elevated plasma aminotransferase level. All these pathophysiological changes indicated the success of AIH and DILI model in our study.

Quality control of hybridization

To investigate the differentially expressed lncRNAs in AIH and DILI, we analyzed the lncRNA expression profiles of these two diseas-

Table 2. The simultaneously dysregulated lncRNAs in DILI and AIH groups

lncRNA	Fold change		Regulation	Chromosome
	DILI	AIH		
A130040M12Rik	12.9456455***	4.5666081	up	chr11
Gm19933	2.8400553*	1.9066129	up	chr7
1300002E11Rik	2.6689791*	1.5022376	up	chr16
LOC100862007	2.5008994	2.8573564	up	chr3
Gm19899	1.7815303	1.5576564	up	chrX
Gm20024	1.5926099	1.6798786	up	chr15
Gm14379	1.5253074	1.5253074	up	chrX
4930529L06Rik	1.5153983	1.5677809	up	chr16
C730036E19Rik	0.1364777	0.1546990	down	chr1
Gm19894	0.1864612	0.1817279	down	chr10
1300015D01Rik	0.2124995***	0.5926219	down	chr2
9530091C08Rik	0.2790088*	0.4272190	down	chr9
2310001H17Rik	0.3028058	0.4091733	down	chr6
LOC100861972	0.3631790**	0.5806642	down	chr6
AW011738	0.4246084*	0.6245275	down	chr4
0610043K17Rik	0.4270408*	0.6334589	down	chr4
LOC100503186	0.4369330	0.4501266	down	chr5
B930025P03Rik	0.4557675	0.6470750	down	chr8
0610031O16Rik	0.5247095	0.4584111	down	chr3
LOC100505156	0.5761382	0.5863593	down	chr4
E230016M11Rik	0.5856229	0.5203282	down	chr6
Gm19522	0.5949408	0.5471896	down	chr16
Rpph1	0.6013471	0.5631456	down	chr14
4930573O16Rik	0.6017149	0.5925611	down	chr2
Gm19765	0.6066065	0.6066065	down	chr7
Gm19617	0.6124763	0.5742643	down	chr5
1110050K14Rik	0.6162736	0.6162736	down	chr8
Gm20199	0.6168796	0.4886400	down	chr9
Gm15545	0.6219458	0.6185143	down	chr7
1700025N23Rik	0.6259364	0.6259364	down	chr6
6720401G13Rik	0.6285127	0.6496893	down	chrX
Kcnq1ot1	0.6290383	0.5411610	down	chr7
4931406H21Rik	0.6326585	0.6326585	down	chr14
1700012D14Rik	0.6399397	0.6399397	down	chr7
B130024G19Rik	0.6401451	0.5514855	down	chr7
Gm20264	0.6662728	0.6071403	down	chrX

Note: Student t test; *for $P<0.05$ *vs* AIH group, **for $P<0.01$ *vs* AIH group, ***stands for $P<0.001$ *vs* AIH group.

es, as well as of healthy controls. The scanning results revealed that the array name marker and genechip arrays in the four corners could be clearly seen. The marker "+" in the middle of the genechip was clear too. In all the groups, signal values were stronger than background value, indicating normal background intensity. The signals of the external positive controls BioB, BioC, BioD and Cre were all clearly visible. All the above information (displayed in **Figure 1**) were evidences of good genechip and hybridization quality.

Differently expressed lncRNAs in microarray

Results from the microarray showed that a total of 60 lncRNAs were differentially expressed (fold change >1.5 or <0.7, P<0.05) in the AIH group compared to the control group. Among these, 17 lncRNAs were upregulated while 43 lncRNA were downregulated. On the other hand, there were a total of 68 differentially expressed (fold change >1.5 or <0.7, P<0.05) lncRNAs in the DILI group compared to the control group, 21 upregulated ones and 47 downregulated ones. Of all these differentially expressed lncRNAs, 8 were found to be upregulated in both AIH group and DILI group, and 28 were downregulated in both of the liver injury groups. The results were displayed in detail in **Table 2**. Notably, among these simultaneously dysregulated lncRNAs, some had quite different fold changes in the two disease models, with statistically significant differences. For example, lncRNA A130040M12Rik in the DILI group and in the AIH group had fold changes of 12.95 and 4.57 respectively (P<0.001), as compared with the control group. Although in the same direction, these lnRNAs could be considered as differentially expressed between the AIH group and DILI group.

Table 3. The dysregulaed lncRNAs observed in the DILI group only

lncRNA	Fold change	Regulation	Chromosome
LOC100861856	4.7942794	up	chr12
E330011021Rik	2.2718381	up	chr16
Gm19270	2.1487759	up	chr17
4930515G01Rik	1.8495355	up	chr5
9330133014Rik	1.7647478	up	chr8
Snhg1	1.735603	up	chr19
3110070M22Rik	1.7333993	up	chr13
5033406009Rik	1.6993052	up	chr12
LOC100861804	1.683596	up	chr10
D930048N14Rik	1.6765587	up	chr11
3300005D01Rik	1.6705209	up	chr17
2610019E17Rik	1.5895022	up	chr17
Raver1-fdx1l	1.5214406	up	chr9
C730027H18Rik	0.3680753	down	chr10
1810008I18Rik	0.3681828	down	chr7
AU020206	0.4330108	down	chr7
D130020L05Rik	0.4367338	down	chr12
chr17:35266768-35267368	0.5400010	down	chr17
Rian	0.5650110	down	chr12
2900005J15Rik	0.5743830	down	chr5
2810013P06Rik	0.5848307	down	chr8
LOC628147	0.5865569	down	chr2
Gm19426	0.6084814	down	chr2
Terc	0.6085548	down	chr3
4930581F22Rik	0.6159747	down	chr9
Gm7854	0.6178631	down	chr5
4831440E17Rik	0.6313080	down	chr5
Scarna6	0.6321317	down	chr1
G630008E18	0.6465317	down	chr5
1700120K04Rik	0.6589272	down	chr7
Gm8883	0.6594065	down	chr1
Gm19263	0.6646091	down	chr19

Meanwhile, some of the dysregulated lncRNAs were limited only to AIH or observed only in DILI mice. In the current study, we found 13 upregulated lncRNAs (3 of them with fold change >2.0) and 19 downregulated lncRNAs (4 with fold change <0.5) in the DILI group that were expressed in normal levels (fold change <1.5, P>0.05 as compared with the control group) in the AIH group. On the other hand, in the AIH group, there were 9 upregulated lncRNAs (2 with fold change >2.0) and 15 downregulated lncRNAs (1 with fold change <0.5) that were in normal expression in the DILI group. The detailed information of these dysregulated lncRNAs in the liver was provided in **Tables 3** and **4**.

Selection and verification of differently expressed lncRNAs

To further validate the accuracy of lncRNA profile determined by microarray technology, some of the dysregulated lncRNAs with the most meaningful change in fold (lncRNA A130040M12Rik, lncRNA 1300015D01Rik, lncRNA LOC100861856 and lncRNA Gm19585) were analyzed by real time PCR assays (**Figure 2**). Overall, these findings were consistent with those of the microarray analysis, in that all 4 lncRNAs were differentially expressed among the groups with the same trend (up- or down-regulated) and reached statistical significance (P<0.05).

Discussion

In the current study, we constructed murine models of DILI and AIH and compared their lncRNA profiles using microarray analysis. Dysregulated lncRNAs were found in both disease models as compared with healthy controls. Moreover, some of these dysregulated lncRNAs were differentially expressed between AIH and DILI, indicating that detection of lncRNA profiles or of differentially expressed lncRNAs may help distinguish AIH from DILI.

From our results, we proposed that lncRNAs with obvious up- or down-regulation (fold change >2.0 or <0.5) in only one of the diseases, i.e. in AIH or DILI, are of the most value in the diffeeantial diagonosis between the two diseases. These differentially expressed lncRNAs may have participated in some specific mechanisms of AIH or DILI. For instance, there are lncRNA Gm19585 (fold change 3.09), Gm19705 (fold change 2.04) and Neat1 (fold change 0.461) in AIH. On the other hand, there are a bunch of dysregulated lncRNAs which appear only in DILI. These include LOC100861856 (fold change 4.79), E330011021Rik (fold change 2.27), Gm19270 (fold change

Table 4. The dysregulaed lncRNAs observed in the AIH group only

lncRNA	Fold change	Regulation	Chromosome
Gm19585	3.0886813	up	chr17
Gm19705	2.0408639	up	chr1
Gm11827	1.8000675	up	chr4
Gm19528	1.7730141	up	chrX
AI662270	1.7106362	up	chr11
Gm19763	1.6342356	up	chrX
2810408B13Rik	1.6067595	up	chr13
Gm3279	1.5657067	up	chr6
LOC100862033	1.5336306	up	chr2
Neat1	0.4609017	down	chr19
1110028F11Rik	0.5062300	down	chr11
Gm20342	0.5473012	down	chr1
Gm19664	0.5529868	down	chr15
Gm16157	0.5599613	down	chr7
LOC100861653	0.5626891	down	chr8
A530065N20	0.5639679	down	chr13
Gm17066	0.5849815	down	chr14
Malat1	0.5944058	down	chr19
9530051G07Rik	0.6098837	down	chrX
5730420D15Rik	0.6109266	down	chr10
1700091H14Rik	0.6186391	down	chr14
LOC100862607	0.6315536	down	chr6
4930568G15Rik	0.6487709	down	chr1
LOC100503496	0.6603478	down	chr11

2.15), C730027H18Rik (fold change 0.368), 1810008I18Rik (fold change 0.368), AU020-206 (fold change 0.433) and D130020L05Rik (fold change 0.434). According to previous studies, lncRNA Neat1 is required for corpus luteum formation and the establishment of pregnancy [18], as well as mammary gland development [19]. Other studies have reported that lncRNA Neat1 promotes laryngeal squamous cell cancer [20] and glioma pathogenesis [21], and that inhibition of Neat1 impairs myeloid differentiation in acute promyelocytic leukemia cells [22]. In our study, the specific down-regulation of lncRNA Neat1 in AIH model may have further suggested its correlation with immune regulation. Yet other dysregulated lncRNAs observed in the present study have seldom been reported before. And their significance in the occurrence and development of liver diseases need to be further studied.

In the current study, we also found some simultaneously dysregulated lncRNAs (8 upregulated ones and 28 down-regulated ones) in both AIH and DILI models. These simultaneously dysregulated lncRNAs may be involved in the common mechanisms of both AIH and DILI, like the destruction and (or) regeneration of liver cells, etc. Further studies are required for the clarification of the role that lncRNAs play in these mechanisms. Nevertheless, some of the simultaneously dysregulated lncRNAs detected in our study have quite different fold changes in the liver samples of AIH and DILI. The most prominent one among the simultaneously upregulated lncRNAs is A130040M12Rik, with fold change 12.95 in DILI and 4.57 in AIH; while the most distinguished simultaneously downregulated lncRNA is 13000-15D01Rik, with fold change 0.212 in DILI, 0.593 in AIH. These two lncRNAs are also considered to be differentially expressed between AIH and DILI, and may also help in their differential diagnosis. What's notable is that lncRNA A130040M12Rik (with alias of VL30, CA782090 and H3053C11) has been identified as a major tumor promotor in mice [23], because the expression of VL30-1 RNA is 5 to 8 fold higher in mouse melanoma cell lines than in normal mouse fibroblast or myoblast lines, and that increasing the expression of VL30-1 RNA promotes cell proliferation in culture and tumorigenesis in mice. Likewise, the simultaneous up-regulation of A130040M12Rik (or VL30) observed in our study in both DILI and AIH may be reliable indicator of cell proliferation after liver injury, and that the extremely high expression of A130040M12Rik in DILI models may suggest either effective tissue repair or the initiation of early oncogenesis. Of course, the detailed mechanisms remain to be investigated by future studies.

However, this study also has certain limitations: First, we only used mouse liver tissues in lncRNA profiling, while non-or-mininal-invasive samples like blood or feces may be better choices considering the potential clinical value of this technique [9]; Second, the models in this study, although constructed with classic and reliable methods, can hardly mimic the various subtypes and complex clinical manifestations of AIH and DILI. All the above limitations need to be addressed by future research.

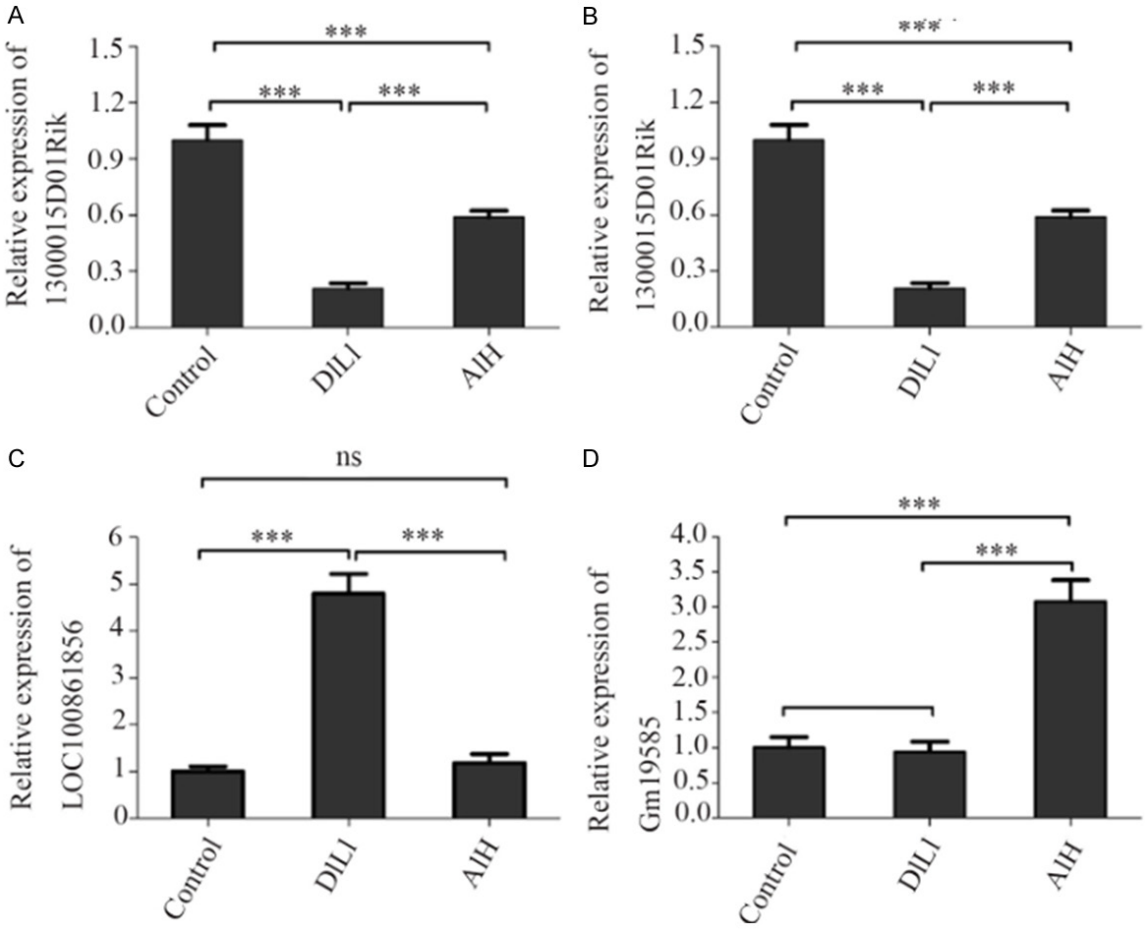

Figure 2. Differentially expressed lncRNAs identified by qRT-PCR (One way ANOVA; * for $P<0.05$, ** for $P<0.01$, *** for $P<0.001$).

In summary, the detection of lncRNA profiles or differentially expressed lncRNAs may have potential clinical value in the differential diagnosis between AIH and DILI.

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Disclosure of conflict of interest

None.

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