

# Identification Of Novel lncRNAs For Detection Of HBV-Associated Hepatocellular Carcinoma

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**Purpose:** This study was conducted to investigate the differentially expressed profiles of long non-coding RNAs (lncRNAs) in HBV-associated HCC (HBV-HCC), which may serve as potential diagnostic biomarkers and therapeutic targets.

**Methods:** To examine the differentially expressed profiles of lncRNAs and mRNAs using microarray analysis, we collected 15 specimens: five HBV-associated HCC tissues, five paired adjacent peritumoral liver tissues (APLT), and five distant peritumoral liver tissues (DPLT). Then, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to predict the biological roles and potential signaling pathways of these dysregulated mRNAs. In addition, lncRNA-mRNA co-expression network and signal transduction pathway network (Signal-net) were employed to further explore the potential target genes and roles of dysregulated lncRNAs in HBV-HCC pathogenesis. Finally, quantitative real-time PCR (qRT-PCR) was used to confirm the expression of six selected dysregulated lncRNAs.

**Results:** A total number of 719 lncRNAs and 3438 mRNAs were significantly more dysregulated in HBV-HCC tissues than in APLT and DPLT (fold change > 2,  $P < 0.05$ , FDR < 0.05). Additionally, 337 GO terms and 53 KEGG pathways were established to be significantly enriched. These dysregulated mRNAs were mainly enriched in metabolism-related biological processes. Additionally, lncRNA-mRNA coexpression network analysis showed that NONHSAT053785 is at the core of the network. Furthermore, the Signal-net analysis showed that CYP3A4 was gene with the highest degree. Finally, the data of five of the six selected differentially expressed lncRNAs were in agreement with the microarray data obtained by qRT-PCR verification.

**Conclusion:** Our study revealed the differentially expressed profiles of lncRNAs and mRNAs for HBV-HCC, and five novel dysregulated lncRNAs were identified in HBV-HCC tissues. The aforementioned dysregulated lncRNAs may represent potential diagnostic biomarkers and therapeutic targets of HBV-HCC, which needs to be validated in future studies.

**Keywords:** long non-coding RNA, hepatocellular carcinoma, microarray, biomarker

## Introduction

Hepatocellular carcinoma (HCC), which accounts for almost 90% of primary liver cancer cases, is one of the most prevalent malignancies and leading causes of cancer-related death worldwide.<sup>1-4</sup> Globally, an estimated 841,080 (4.7%) new HCC cases and 781,631 (8.2%) cancer-related deaths occur annually.<sup>3</sup> Chronic hepatitis B virus (HBV) infection is the major risk factor in most high-risk HCC areas.<sup>5</sup> Over 90% of the cases of HCC globally have a viral etiology, and their majority has been caused by chronic hepatitis B infection.<sup>6</sup> Although HBV is a

known hepatocarcinogen, the molecular mechanisms that determine HBV-associated HCC (HBV-HCC) development remain elusive.<sup>7</sup> The lack of effective diagnostic and prognostic biomarkers leads limits the available options for HBV-HCC treatment. Thus, the further exploration of the underlying molecular pathogenesis of HBV-HCC and the subsequent development of new effective early diagnosis biomarkers and therapeutic targets for HBV-HCC are urgently needed.

The development of high-throughput RNA sequencing technology has enabled the discovery of thousands of non-coding RNA (ncRNA) genes. Generally, ncRNAs shorter than 200 nucleotides are usually described as small ncRNAs, whereas ncRNAs longer than 200 nucleotides are referred to as long non-coding RNAs (lncRNAs). The secrets of long noncoding RNAs are gradually elucidated.<sup>8</sup> lncRNAs were recently shown to have abundant functions in multiple biologic processes. The overexpression, deficiency, or mutation of lncRNA genes has been implicated in numerous human diseases.<sup>9</sup> Studies on cancer have revealed the prominent role of lncRNAs in various cancer forms by their promotion of growth and metastatic potential.<sup>10</sup> It is now clear that lncRNAs are also critically involved in HCC pathogenesis. Some lncRNAs, such as the HULC, MALAT1 and H19, are over-expressed and participate in the tumorigenesis of HCC.<sup>11–13</sup> In 2007, by the use of an HCC-specific cDNA microarray platform, HULC was identified as the first lncRNA with highly specific upregulation in HCC.<sup>11</sup> In another examination, Lin et al found that MALAT1 was closely related to HCC neoplasia and progression.<sup>13</sup> In addition, more recent research indicates that lncRNA H19 can serve as a ceRNA to sponge miR-326 and modulate TWIST1 levels in HCC pathogenesis and may serve as a novel HCC target.<sup>12</sup> The evidence of a correlation between the changes in the expression levels of lncRNAs and HCC development is increasing. However, currently, the research on lncRNAs in HBV-HCC is quite limited. Therefore, it is highly necessary and extremely urgent to

**Table 1** Information Of Clinical Specimens For Microarray And qRT-PCR

	Training Set (5 Groups)	Validation Set (20 Groups)
<b>Age</b> Mean ± SD	45.6±15.8	53.9±13.2
<b>Gender</b> Female Male	1 4	3 17
<b>TNM stage</b> I II III IV	3 1 1 1	8 6 4 2
<b>Tumor grade (differentiation)</b> G1 G2 G3	1 3 1	3 11 6

investigate the association between lncRNAs and HBV-HCC, which may play an important role in the tumorigenesis and pathogenesis, and may potentially be used as a diagnostic marker or therapeutic target.

In past decades, the mechanism of HCC development has been investigated, and different clinical therapies to overcome this disease have been examined. In particular, the application of high-throughput techniques in HCC research has generated a vast amount of data, which has provided important resources for further exploration of the molecular mechanisms and identification of HCC-related molecules. Doubtless, these data will help to improve the diagnostic, prognostic, and therapeutic classification. Therefore, in the present study, we applied microarray technology to analyze the expression profiles of lncRNAs and mRNAs in hepatocellular carcinoma with HBV infection. Additionally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were

**Table 2** Primer Sequences Of The qRT-PCR Used In This Study

LncRNA Name	F-Primer (5'–3')	R-Primer (5'–3')
NONHSAT138156	CCACAACATCCTTTATGACTTA	CACGAGAACAGTCAGCAGAT
ENST00000514608.1	TTGAGTGCCTGAAAGAACTGT	CGTATTAATTTCAAGAGGGTGA
ENST00000560295.1	ACTCGTTGCAGTCTTGTAACA	TGGAAGTCAAAGACCATGCTT
NONHSAT024276	TCTGTGGTAACTCAAGTGTTC	TTGTGGAATGTGTGTTTGTAT
NONHSAT124357	GCTCCAAAGCCATTTACTCAGT	TATCCGCTAAGGAAGTATGACAAT
NONHSAT053785	AGTTATAGATTTACGTCCACTTTAGA	CACCTGAAAAGTATGATACTA
GADPH	CCCTTCATTGACCTCAACTA	TGGAAGATGGTGTATGGGATT

**Table 3** Number Of Significant Dysregulated Genes On Microarray In Groups

Gene	Expression	HCC vs APLT	DPLT vs HCC
lncRNA	Upregulated	525	528
	Downregulated	510	598
mRNA	Upregulated	2680	1647
	Downregulated	1633	2987

**Abbreviations:** HCC, hepatocellular carcinoma; APLT, adjacent peritumoral liver tissues; DPLT, distant peritumoral liver tissues.

performed to predict the biological roles and potential signaling pathways of these differentially expressed mRNAs. Moreover, the lncRNA-mRNA coexpression network and signal transduction pathway network (Signal-net) were conducted to further explore the potential roles of differentially expressed lncRNAs in HBV-HCC pathogenesis and analysis, followed by quantitative real-time PCR (qRT-PCR) verification.

## Materials And Methods

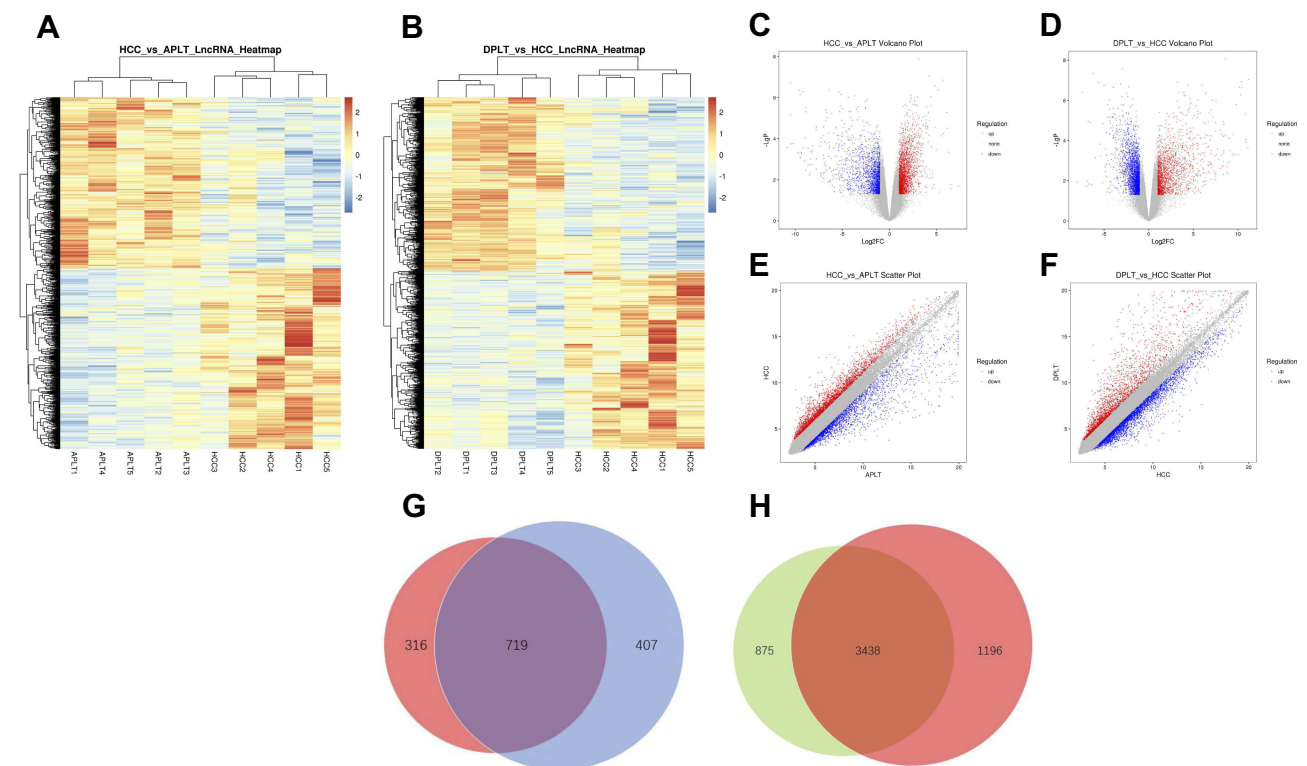
### Patient Samples

From July 2016 to December 2016, samples were randomly collected for transcriptome microarray profile analysis from

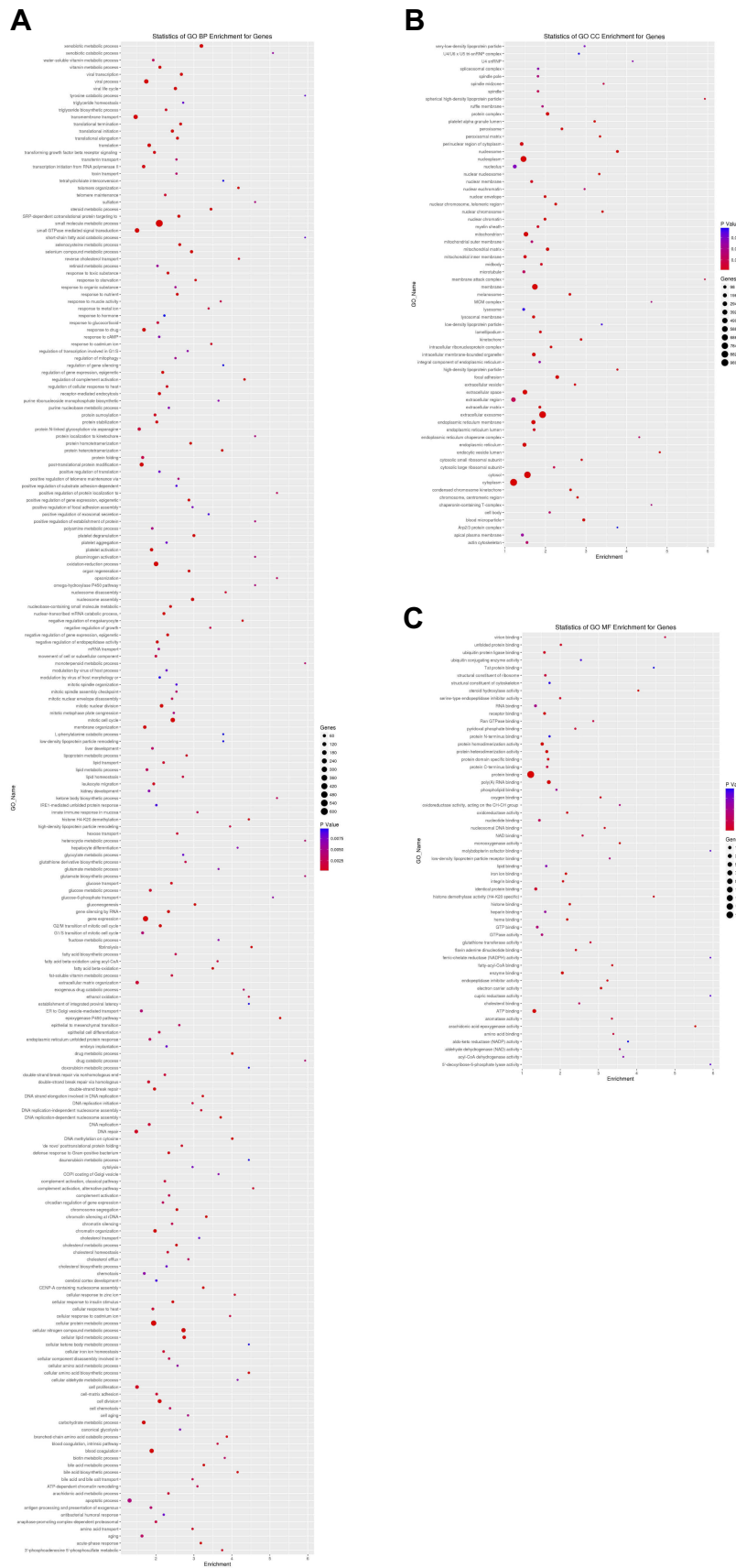
the Daping Hospital of the Army Medical University (Third Military Medical University), including five cases HBV-associated HCC tissues, paired adjacent peritumoral liver tissues (APLT, less than 2 cm away from the cancerous nest) and distant peritumoral liver tissues (DPLT, at least 5 cm away from the cancerous nest). Then, other 20 groups of specimens were collected from March 2016 to July 2017 for expressional validation (Table 1). Serological tests pathologically confirmed that all patients had HCC and were infected with HBV. The clinical specimens were frozen in liquid nitrogen immediately after collection. Written informed consent was obtained from the patients, and the study was approved by the Medical Ethics Committee of Daping Hospital.

### RNA Isolation

Total RNA was extracted from each liver tissue sample using TRIzol (Invitrogen, Carlsbad, CA, USA) per the manufacturer's protocol. RNA quantity and quality were measured using a NanoDrop ND-1000 spectrophotometer (Thermo, Wilmington, DE, USA) and RNA integrity was evaluated by standard denaturing agarose gel electrophoresis.



**Figure 1** Differentially expressed lncRNAs (fold change  $>2$ ,  $P < 0.05$ ) in the microarray profiles. (A) Heatmap of five paired HCC and APLT. (B) Heatmap of five paired HCC and DPLT. (C) Volcano plot of five paired HCC and APLT. (D) Volcano plot of five paired HCC and DPLT. (E) Scatter plot of five paired HCC and APLT. (F) Scatter plot of five paired HCC and DPLT. (G) Venn diagram indicating that 719 lncRNAs are consistently in both microarray analyses. (H) Venn diagram indicating that 3438 mRNAs are consistently in both microarray analyses.



**Figure 2** GO terms for the dysregulated mRNAs ( $P < 0.01$ ). **(A)** GO terms for the dysregulated mRNAs in biological process. **(B)** GO terms for the dysregulated mRNAs in cellular component. **(C)** GO terms for the dysregulated mRNAs in molecular function.

## Microarray Profiling

Microarray analysis was performed using GeneChip<sup>®</sup> Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA, USA), which is designed for the global profiling of 245,349 protein coding transcripts and 40,914 non-protein coding transcripts from public transcriptome databases, including RefSeq, Ensembl, UCSC (known genes and lincRNA transcripts), as well as landmark publications and was carried out by Beijing Cnkingbio Biotechnology Co, Ltd (Beijing, China). Affymetrix<sup>®</sup> Transcriptome Analysis Console (TAC) Software was employed to analyze the results from Human Transcriptome Array 2.0.

## GO Enrichment Analysis

The Gene Ontology (GO) is a structured and controlled vocabulary of terms. The terms are subdivided in three non-overlapping ontologies, Molecular Function (MF), Biological Process (BP) and Cellular Component (CC), and now is used widely for annotating genes and gene products.<sup>14</sup> To detect the overrepresentation of Gene-Ontology annotations among the large list of genes from high-throughput experiments such as microarray that is effective in targeting

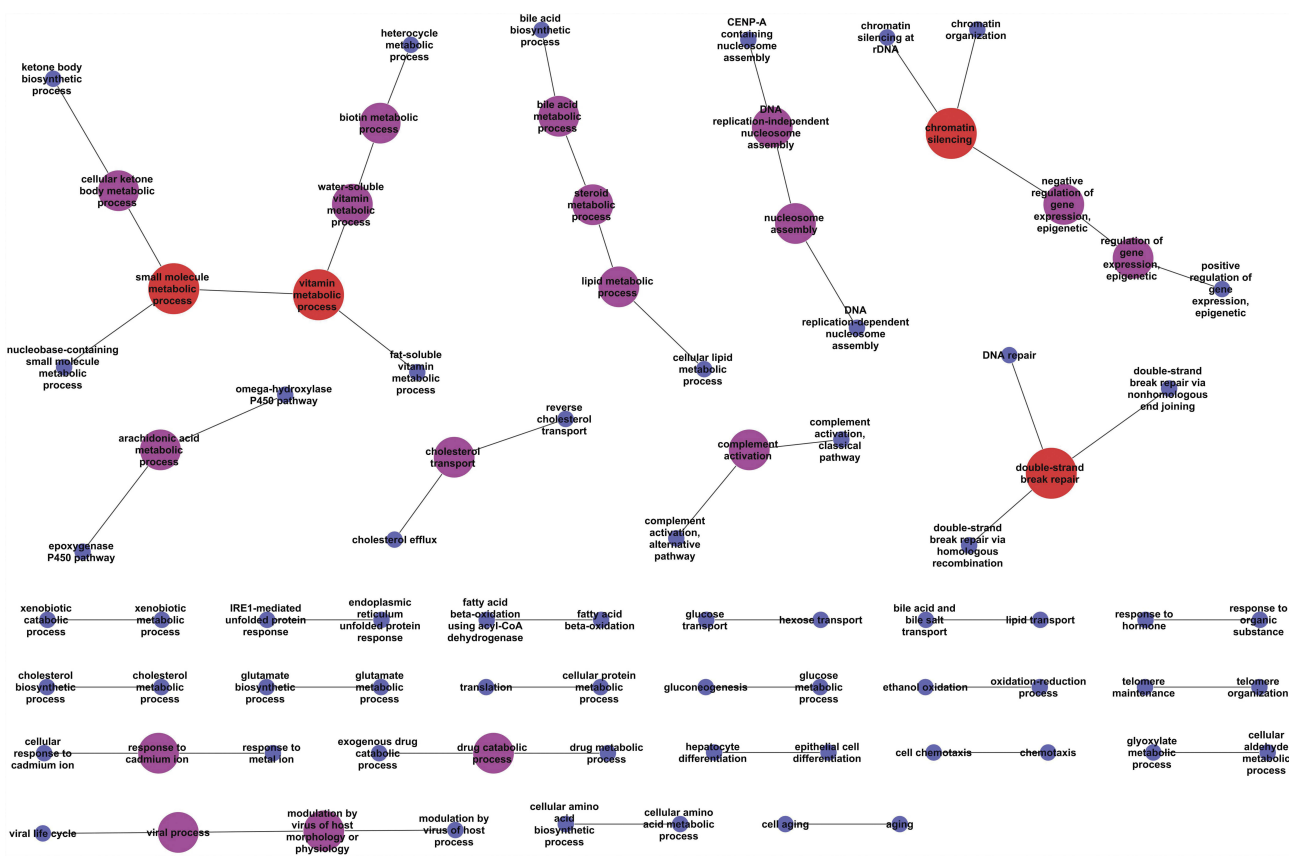
downstream events and molecules. Parent-Child-Intersection method was applied for enrichment analysis and Benjamini-Hochberg was utilized for multiple tests correction, and curated association (ie, all evidence codes except IEA, ND, and NR) was employed for enrichment analysis.<sup>15</sup>

## KEGG Pathway Analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a knowledge base utilized for systematic analysis of gene functions, linking genomic information with higher order functional information, which is now widely used for pathway-related analysis.<sup>16</sup> Hypergeometric distribution was used to calculate the pathway enrichment, and FDR (false discovery rate) was implemented to adjust the P-values in the multiple comparisons.

## Co-Expression Analysis

The co-expression network between lincRNA and genes was constructed by combining the expression profile data of differentially expressed lincRNA components with the expression profile data of differentially expressed genes. Coexpression network can search for the core regulated



**Figure 3** GO network. Significantly changed GO functions were connected in a GO network to show the relationship between these functions.

genes in the network and locate the functions and signal transduction pathways in which lncRNA may participate through genes it regulates. The co-expression network clearly reflects the relationship between lncRNA and genes as well as the overall interaction, enabling the discovery of the regulatory relationship between lncRNA and genes and the exploration of its possible functional effects.

## Global Signal Transduction Network

After parsing the whole KEGG database, all study genes involved pathways were extracted, and the study pathway network was generated with the help of the pathway topology in the KEGG database. The specific gene network of one pathway was generated based on the pathway topology analysis, and the study gene network was generated after mapping to the generated reference KEGG gene network (ie a collection of gene networks from each pathway).

## Quantitative Real-Time PCR (qRT-PCR)

The expression levels of selected lncRNAs were validated by qRT-PCR. The specimens of 20 groups were used for further

validation. cDNA was synthesized by EvoScript Universal cDNA Master RT reagent Kit (Roche, Germany) under the manufacturer's document. The lncRNA expression level was quantified using TaqMan® Fast Advanced Master Mix (ABI, USA) on the CFX96™ Real-Time system (BIO-RAD, USA). All experiments were performed in triplicate (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control). Finally, the  $2^{-\Delta\Delta Ct}$  method was used to calculate the relative expression. The primers, whose sequences are listed in Table 2, were obtained from Sangon Biotech (Shanghai) Co, Ltd (Shanghai, China).

## Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 (San Diego, CA, USA). The qRT-PCR data were statistically analyzed by Student's *t*-test with SPSS 21.0; differences were considered statistically significant at  $P < 0.05$ .

## Results

### lncRNA And mRNA Expression Profiles In HBV-HCC

Fifteen specimens (five HCC, five APLT, and five DPLT) were subjected to microarray analysis of lncRNA-mRNA. We found that 1035 lncRNAs and 4313 mRNAs were differentially expressed between HCC and APLT, whereas 1126 lncRNAs and 4634 mRNAs were differentially expressed between HCC and DPLT (fold change  $> 2$ ,  $P < 0.05$ , FDR  $< 0.05$ ; Table 3). The Venn diagram (Figure 1G–H) showed that 719 of the differentially expressed lncRNAs and 3438 of the differentially expressed mRNAs were identified in both sets. They were considered to be candidate tumor-derived RNAs and subjected to subsequent analysis. The differential lncRNA expression was jointly evaluated by heatmap, volcano plot, and scatter plot, and categorized in the HCC, APLT, and DPLT (Figure 1A–F).

### GO Enrichment And KEGG Pathway Analysis

To predict the functions of the differentially expressed genes, we conducted functional enrichment analysis of the 3438 dysregulated mRNAs, and 337 GO terms were identified as significantly enriched. As can be seen in Figure 2, the GO analysis revealed that the most enriched GO terms were small-molecule metabolic process (ontology: biological process, GO: 0044281), extracellular exosome (ontology: cellular



**Figure 4** KEGG pathways analysis ( $P < 0.05$ ). Major pathways of the dysregulated mRNAs.

component, GO: 0070062) and poly(A) RNA binding (ontology: molecular function, GO:0044822). GO network analysis was performed to generate the interaction network among these enriched GO terms, of which the vitamin metabolic process (degree = 3) had the highest degrees (Figure 3). Furthermore, the KEGG pathway analysis indicated 53 KEGG pathways were significantly enriched and the dysregulated mRNAs were mainly involved in metabolism-related biological processes, such as the regulation of systemic lupus erythematosus, complement and coagulation cascades, valine, leucine, and isoleucine degradation, alcoholism, and carbon metabolism (Figure 4). We performed pathway network analysis to generate an interaction network covering significantly changed pathways (Figure 5). The alanine, aspartate and glutamate metabolism (degree = 19) showed the highest degrees, suggesting that it may plays a core role.

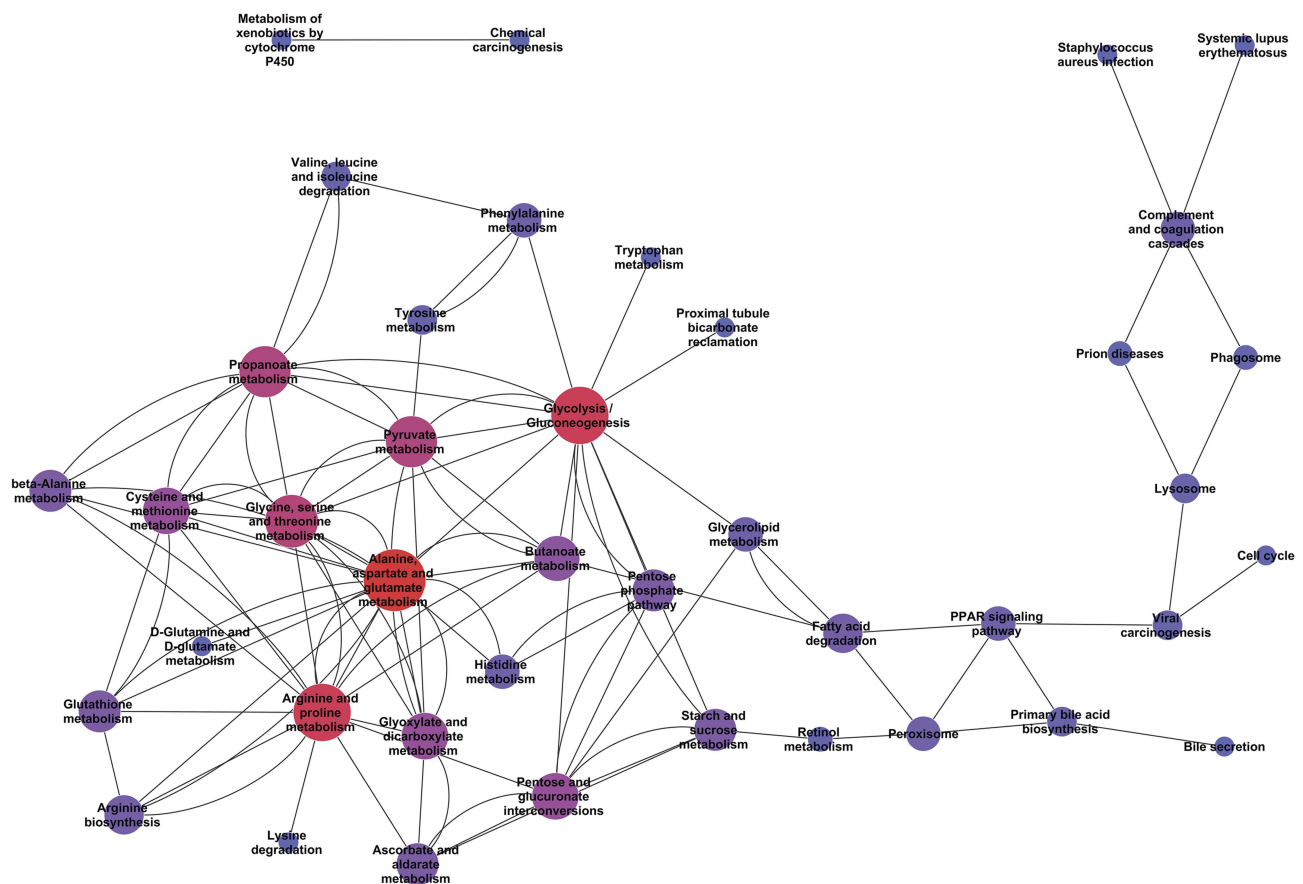
## lncRNA-mRNA Coexpression Network Analysis

To investigate the potential roles of these dysregulated lncRNAs in HCC, we selected dysregulated mRNAs with

both significantly changed GOs and pathways through GO and KEGG analysis, and constructed a coexpression network of these mRNAs with dysregulated lncRNAs. As can be observed in Figure 6, the whole coexpression network profile consisted of 336 network nodes and 416 connections among 127 differentially expressed lncRNAs and 209 differentially expressed mRNAs. There were 350 positive and 66 negative interactions within the network. Moreover, our data showed that one lncRNA may correlate with 1–28 mRNAs. Among them, NONHSAT053785, RP1-81D8.6, UGT2B25P, HIST1H2APS4, and RP11-371E8.2 were at the core of the co-expression network and were related to at least 20 mRNAs. The top five mRNAs related to NONHSAT053785, RP1-81D8.6, UGT2B25P, HIST1H2APS4, and RP11-371E8.2 ranked by their correlation value, are listed in Table 4.

## Global Signal Transduction Network

The signal transduction network analysis was used to define the core key regulatory mRNA genes which had a strong capacity to modulate adjacent mRNA genes. Based



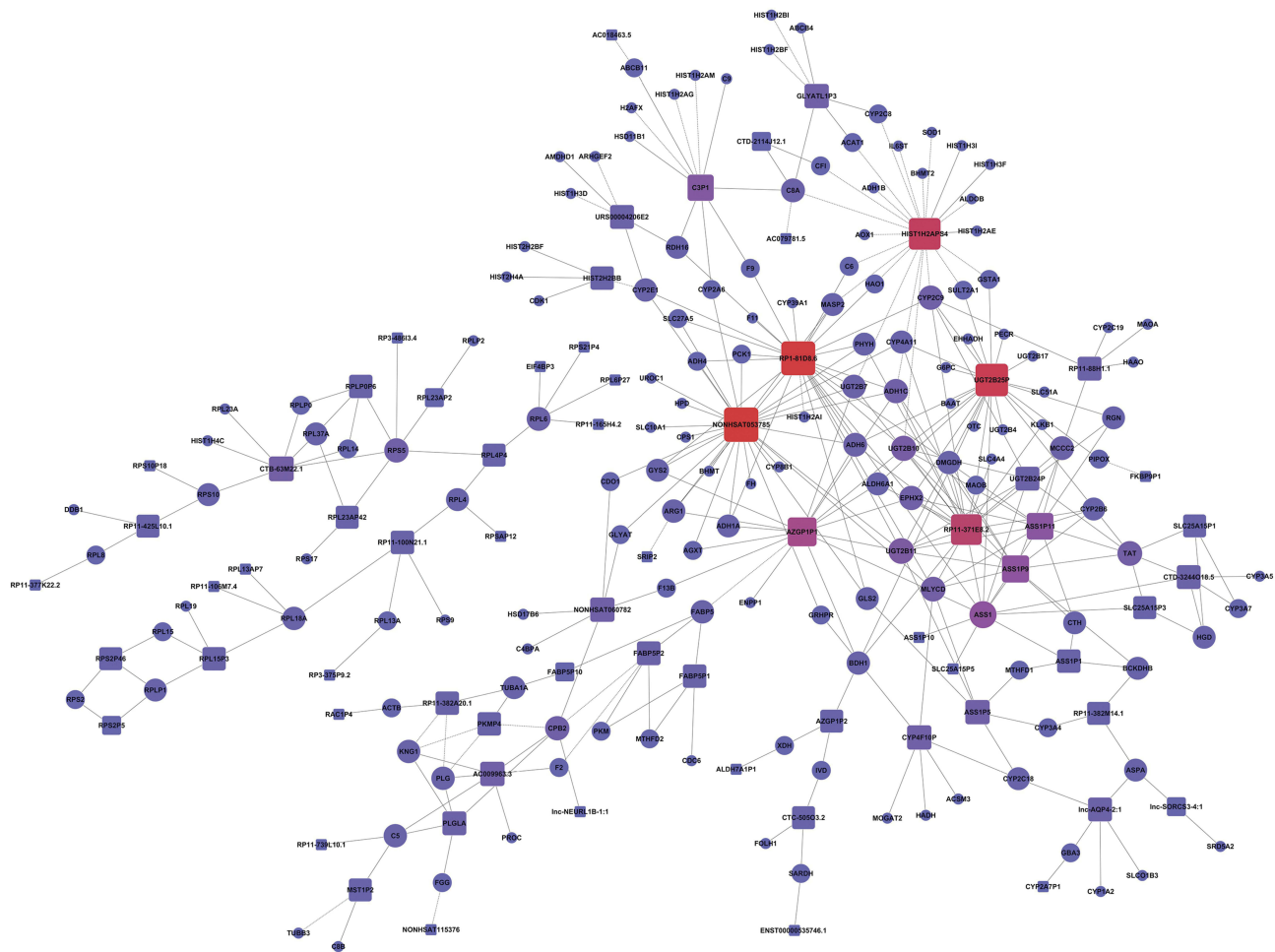
**Figure 5** Pathway network. Significantly changed pathways were connected in a pathway network to show the relationship between these pathways.

on the significantly regulated GOs and pathways, we performed Signal-net analysis to screen the important candidate genes involved in HCC associated with hepatitis B virus infection. As illustrated in Figure 7, 995 genes were obtained in the Signal-net. The resulting network indicated that genes with high degrees were CYP3A4, PRKX, CYP1A1, CYP2C9, CYP1A2, CYP2A6, MAPK1, MAPK3, PIK3R1, and ITGA2 (Table 5).

### The Validation Of Novel lncRNAs That May Become Potential Biomarkers

Based on the high normalized intensity in the raw data, the results of bioinformatics analysis, high fold change (fold change > 5), significant P-value (P < 0.01) and sorted by adjustment of the P-value, we selected six lncRNAs for qRT-PCR verification to confirm the reliability of the microarray data. Sorted by the above parameters, the six lncRNAs were

all ranked in the front position. Among these six dysregulated lncRNAs, three (ENST00000514608.1, NONHSAT138156, and ENST00000560295.1) were upregulated, whereas the other three (NONHSAT024276, NONHSAT124357.2, and NONHSAT053785) were downregulated. Then, we analyzed their expression levels by qRT-PCR in another 60 specimens (20 HCC, 20 APLT, and 20 DPLT samples). The result of qRT-PCR indicated that five lncRNAs were consistent with the microarray analysis and showed the same trends of up- or downregulation for each lncRNA with statistical significance. ENST00000514608.1, NONHSAT138156 were significantly upregulated and NONHSAT024276, NONHSAT124357, NONHSAT053785 were significantly downregulated in HCC cases (all P < 0.05 and detection rate = 100%). No significant differences in ENST00000560295.1 were found among the three groups. The  $\Delta$ CT of each lncRNA is presented in Figure 8.



**Figure 6** lncRNA-mRNA coexpression network. Square nodes represent dysregulated lncRNAs, round nodes represent dysregulated mRNAs. The dashed lines between lncRNAs and mRNAs indicate a negative correlation, while the solid lines indicate a positive correlation.



**Table 4** Top 5 mRNAs Ranked By Correlation After Coexpression Network Analysis

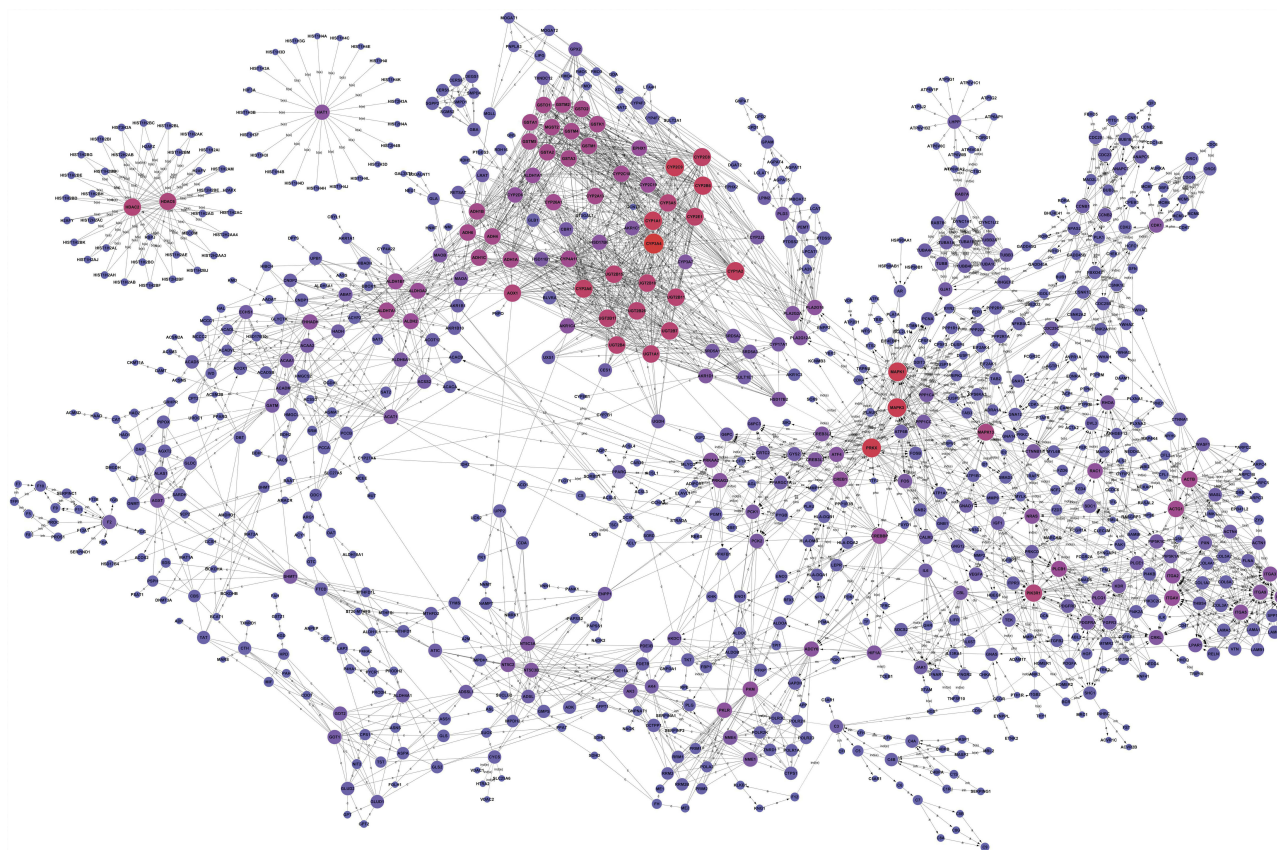
LncRNA	mRNA	Correlation	Regulation
NONHSAT053785	ADH1A	0.9760	Positive
	ARG1	0.9742	Positive
	ADH4	0.9736	Positive
	AGXT	0.9723	Positive
	SLC27A5	0.9715	Positive
RPI1-81D8.6	F11	0.9765	Positive
	ADH1C	0.9724	Positive
	ADH4	0.9695	Positive
	MLYCD	0.9690	Positive
	ADH1A	0.9682	Positive
UGT2B25P	UGT2B11	0.9787	Positive
	MCCC2	0.9704	Positive
	TAT	0.9681	Positive
	UGT2B10	0.9681	Positive
	ADH6	0.9676	Positive
HIST1H2APS4	HIST1H2AE	0.9514	Positive
	ACAT1	-0.9500	Negative
	ALDOB	-0.9506	Negative
	GSTA1	-0.9517	Negative
	CFI	-0.9521	Negative
RPI1-371E8.2	DMGDH	0.9660	Positive
	MCCC2	0.9650	Positive
	MAOB	0.9640	Positive
	ASS1	0.9636	Positive
	ADH6	0.9612	Positive

## Discussion

In recent years, numerous studies have shown that HBV is involved in the carcinogenesis, invasion, and metastasis of liver cells, playing a key role in the occurrence and development of liver cancer.<sup>17–19</sup> LncRNA was reported to be involved in the development of various hepatocellular carcinomas.<sup>20,21</sup> LncRNA was also found to participate in HBV-HCC pathogenesis; HULC promoted hepatoma cell proliferation via downregulating p18, thereby exacerbating the malignancy of HCC.<sup>22</sup> More studies on lncRNAs in HBV-related HCC are necessary to understand the process of HCC pathogenesis. Currently, high-throughput gene chip technology enabled the exploration of the occurrence and development of diseases at the whole-genome or transcriptome level, and has been widely used in some analysis of disease gene expression profile, gene cloning, and the search for disease-specific molecular markers.<sup>23–25</sup> Thus, in our study, we utilized microarray and bioinformatics to seek crucial lncRNAs and reveal their potential

functions in association with HBV-HCC. We identified six crucial lncRNAs and used qRT-PCR to validate their expression levels.

In our present research, using microarray analysis, we found aberrant expression in 719 lncRNAs and 3438 mRNAs. Using bioinformatics analyses, including GO, KEGG pathway, lncRNA-mRNA network, and signal-net analyses, we studied the potential role of these identified genes and their relationship with the development of HCC. GO analysis identified the significant biological functions of the aberrantly expressed protein-coding genes. As a result, 337 GO terms were significantly enriched, which were related to the biological processes, cellular component, and molecular functions. The most significant GO terms were the small-molecule metabolic process (GO:0044281), extracellular exosome (GO: 0070062), and poly(A) RNA binding (GO:0044822), respectively, in these three aspects, which were all important pathophysiological and morphological changes in HCC. HCC patients with high PKM2 expression suffered from more severe metabolic damage, transcriptional regulation imbalance, and poor prognosis.<sup>26</sup> The pathway analysis revealed 53 enriched pathways, including the systemic lupus erythematosus signaling pathway, complement and coagulation cascades signaling pathway, and valine, leucine, and isoleucine degradation signaling pathway. To further investigate the complicated regulatory network between lncRNAs and mRNAs, we conducted the lncRNA-mRNA coexpression network analysis based on the aberrantly expressed lncRNAs and mRNAs. A total number of 127 lncRNAs and 209 mRNAs were included in this network, involving 336 network nodes and 416 connections. These numerous connections, which were lncRNA-mRNA coexpressed pairs (positive or negative correlation), suggested that the aberrantly expressed lncRNAs interacted with aberrantly expressed mRNAs. NONHSAT053785 was at the core of the co-expression network and was associated with 28 mRNAs, which had not been reported up to that time. In the future, we intend to study its role in HCC cell proliferation and recurrence. To study the regulatory relationships among these mRNAs, we used the intersecting genes between GO analysis and pathway analysis to perform the signal-net analysis. Our results showed that genes with high degrees were CYP3A4, PRKX, CYP1A1, CYP2C9, CYP1A2, CYP2A6, MAPK1, MAPK3, PIK3R1, and ITGA2. We selected lncRNAs in the genomic interzone, and finally determined six lncRNAs based on the calibrated *P*-value, which were validated by qRT-PCR analysis. The result of qRT-PCR indicated that the data of five lncRNAs



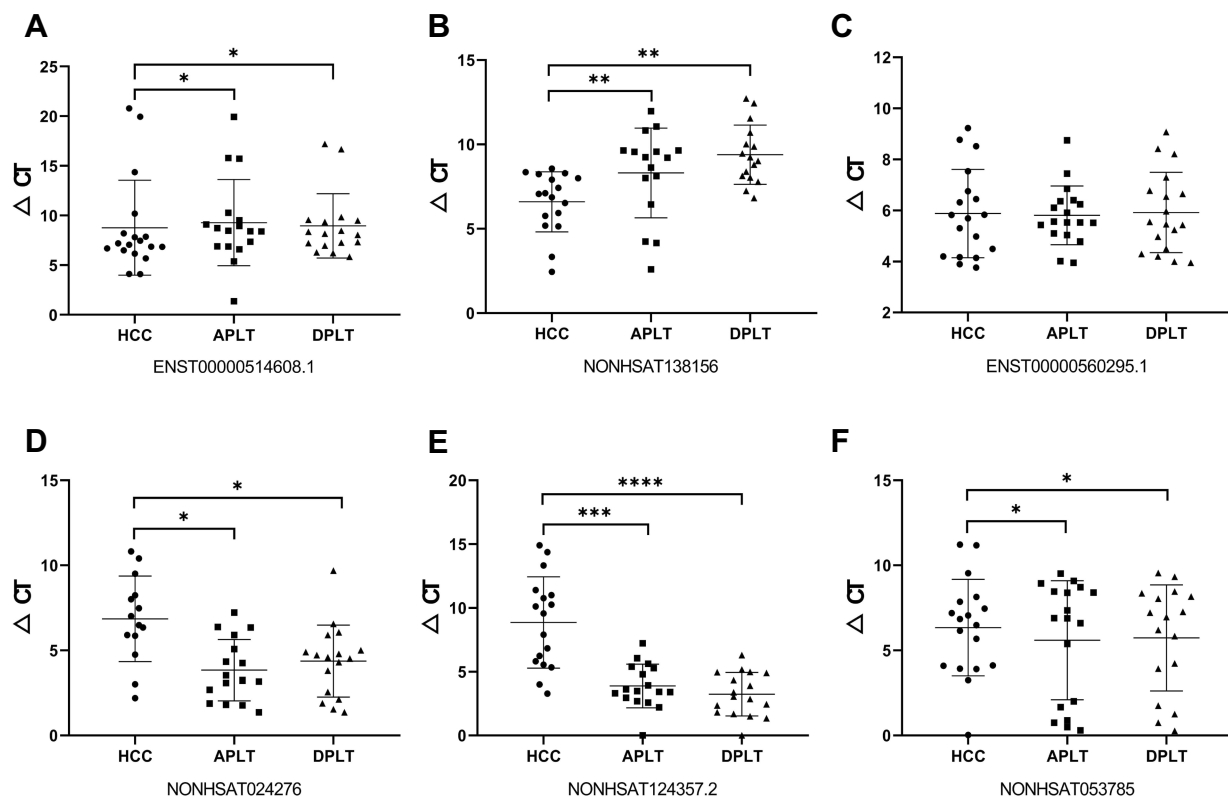
**Figure 7** Global signal transduction network. The area of the circle represents the degree. The larger area indicates a higher degree. Interaction between the genes is denoted as follows: a: activation, b(a): binding/association, c: compound; dep: dephosphorylation, dis: dissociation, ex: expression, ind(e): indirect effect, inh: inhibition, mis(i): missing interaction, pho: phosphorylation, rep: repression, s(c): state change, and ubi: ubiquitination.

were consistent with those of the microarray analysis. ENST00000514608.1 and NONHSAT138156 were significantly upregulated, whereas NONHSAT024276, NONHSAT124357, and NONHSAT053785 were significantly downregulated in HCC cases. On the other hand, ENST00000560295.1 (also called CASC7) showed no significant difference among the three groups. However, lncRNA CASC7 was reported to inhibit the proliferation

and migration of colon cancer cells via microRNA-21 suppression.<sup>27</sup> Apart from CASC7, all other (five) lncRNAs were novel and not yet reported. LncRNA SNHG20 expression was more upregulated in tumor tissues of HBV-positive HCC patients than in tumor tissues of HBV-negative HCC patients.<sup>28</sup> Nonetheless, more experiments are required to establish whether the differences in the screened lncRNAs are indeed associated with HBV. The current study on long

**Table 5** Top Ten Genes Ranked By Degree After Analysis Of Signal-Net

Gene Symbol	Description	Degree	Outdegree	Indegree
<i>CYP3A4</i>	Cytochrome P450 family 3 subfamily A member 4	84	55	29
<i>PRKX</i>		65	62	3
<i>CYP1A1</i>	Cytochrome P450 family 1 subfamily A member 1	64	53	11
<i>CYP2C9</i>	Cytochrome P450 family 2 subfamily C member 9	64	56	8
<i>CYP1A2</i>	Cytochrome P450 family 1 subfamily A member 2	61	58	3
<i>CYP2A6</i>	Cytochrome P450 family 2 subfamily A member 6	61	48	13
<i>MAPK1</i>	Mitogen-activated protein kinase 1	60	38	22
<i>MAPK3</i>	Mitogen-activated protein kinase 3	60	38	22
<i>PIK3R1</i>	Phosphoinositide-3-kinase regulatory subunit 1	58	18	40
<i>ITGA2</i>	Integrin subunit alpha 2	57	5	52



**Figure 8** The  $\Delta$ CT of the Screening lncRNAs in HCC, APLT, and DPLT by qRT-PCR. **(A)**  $\Delta$ CT of lncRNA ENST00000514608.1. **(B)**  $\Delta$ CT of lncRNA NONHSAT138156. **(C)**  $\Delta$ CT of lncRNA ENST00000560295.1. **(D)**  $\Delta$ CT of lncRNA NONHSAT024276. **(E)**  $\Delta$ CT of lncRNA NONHSAT124357.2. **(F)**  $\Delta$ CT of lncRNA NONHSAT053785. HCC group, n = 20; APLT group, n = 20; DPLT, n = 20. \* $P$  < 0.05, \*\* $P$  < 0.005, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001.

non-coding RNA provided novel insights into the expression profile in liver cancer and predicted the potential roles of long non-coding RNAs in hepatocellular carcinogenesis.

Despite the interesting profiling of lncRNA expression in HBV-HCC, several limitations in our study should be acknowledged. First of all, the small numbers of the samples in the microarray assay limited its reliability and biases might have occurred, affecting the results of the further analyses. Moreover, our study predicted mainly the functions of differentially expressed lncRNAs, but we were unable to exactly reveal the regulatory roles of these lncRNAs. In the future, we aim to further investigate that subject, focusing on lncRNA molecular mechanisms in HCC. At the same time, we will also focus on the differential expression of HBV-HCC and non HBV-HCC.

In summary, in this study, we comprehensively analyzed the profiles of key lncRNA in the progression of HBV-HCC. We discovered new lncRNAs that may play an important role in the progression of HBV-HCC, such as NONHSAT053785. Nevertheless, further studies on the role and mechanism of the above key lncRNA in

the occurrence and development of HBV-HCC are expected to provide new molecular and potential new targets for the diagnosis, treatment and prognosis of HBV-HCC.

## Conclusion

Our study identified five novel dysregulated lncRNAs in HCC with HBV infection. Among them, NONHSAT053785 was at the core of the lncRNA-mRNA co-expression network, which may play an important role in the tumorigenesis of HBV-HCC. Therefore, our study provides an important foundation for further exploration of the molecular mechanisms of HCC and identification of the related molecules.

## Abbreviations

lncRNA, long non-coding RNA; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBV-HCC, HBV-associated HCC; HULC, highly upregulated in liver carcinoma; MALAT1, metastasis associated lung adenocarcinoma transcript 1; ncRNA, non-coding RNA; APLT, adjacent peritumoral liver tissues; DPLT, distant peritumoral liver tissues; GO, Gene Ontology; KEGG, Kyoto

Encyclopedia of Genes and Genomes; MF, molecular function; BP, biological process; CC, cellular component; Signal-net, signal transduction pathway network; FC, fold change; FDR, false discovery rate; qRT-PCR, quantitative real-time PCR.

## Ethical Approval And Informed Consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants, and the study was approved by the Medical Ethics Committee of Daping Hospital.

## Availability Of Data And Material

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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## Author Contributions

All authors contributed to data analysis, drafting, and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors report no conflicts of interest in this work.

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