Comprehensive analysis of key lncRNAs in HCV-positive hepatocellular carcinoma

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Abstract

Introduction: Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. Despite the therapeutic advances in HCC in the past few decades, the mortality rate of HCC is still high. Hepatitis C (HCV) infection is one of the major etiological risk factors of HCCs. However, the underlying mechanisms of HCV-induced hepatocarcinogenesis remain largely unclear.

Material and methods: Our study represented the comprehensive analysis of differentially expressed lncRNAs in HCV-positive HCC for the first time by analyzing the public dataset GSE17856. Co-expression network and gene ontology (GO) analysis revealed the functions of those differentially expressed lncRNAs.

Results: We identified 256 upregulated lncRNAs and 198 downregulated lncRNAs in HCV-positive HCC compared to the normal liver tissues. Co-expression network and GO analysis showed that these lncRNAs were involved in regulating metabolism, energy pathways, proliferation and the immune response. Seven lncRNAs (LOC341056, CCT6P1, PTTG3P, LOC643387, LOC100133920, C3P1 and C22orf45) were identified as key lncRNAs and co-expressed with more than 100 differentially expressed genes (DEGs) in HCV-related HCC. Kaplan-Meier analysis showed that higher expression levels of LOC643387, PTTG3P, LOC341056, CCT6P1 and lower expression levels of C3P1 and C22orf45 were associated with shorter survival time in the TCGA dataset.

Conclusions: We believe that this study can provide novel potential therapeutic and prognostic biomarkers for HCV-positive HCC.

Key words: long non-coding RNA, HCV, hepatocellular carcinoma, co-expression network, biomarkers.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and the second leading cause of cancer death worldwide [1]. Despite the therapeutic advances in HCC in the past few decades, the mortality rate of HCC is still high [2]. Chronic hepatitis B (HBV) and hepatitis C (HCV) infection were the major etiological risk factors of HCCs [3]. HCV is a sense-strand RNA virus and is unable to integrate into the host genome [4] and HCV infection represents the main risk factor of primary HCC [5]. Although a previous study showed that several proteins (such as COX-2)
were associated with decreased overall and disease-free survival [6], and HCV encoded proteins (such as E1, E2, P7, NS2 and NS3) could also promote HCC formation by activating oncogenic molecular pathways in vivo [7–9], the underlying mechanisms of HCV-induced hepatocarcinogenesis remain largely unclear.

LncRNAs are a class of non-coding RNAs longer than 200 nucleotides with little or no protein-coding potential [10]. Previous studies had reported that lncRNA expression was frequently dysregulated and could contribute to the progress of various human cancer types, including prostate [11], breast [12], colon [13] and liver cancers [14]. For example, Fan et al. revealed that more than 1000 lncRNA transcripts were significantly differentially expressed in HBV-related HCC patients [15]. Mechanically, lncRNAs could regulate protein-coding genes’ expression at epigenetic, transcriptional, and post-translational levels [16]. One of the most well-known mechanisms was acting as miRNAs sponges by interacting with miRNAs [17]. However, the potential roles of an enormous number of lncRNAs in HCC, especially in HCV-positive HCC, remains to be further elucidated.

In this study, we analyzed the public dataset GSE17856 to identify differentially expressed lncRNAs (DElncs) and mRNAs in HCV-related HCC patients. Next, a series of bioinformatics analyses (including GO, KEGG and co-expression analysis) were performed to explore potential roles of DElncs. The present study aimed to provide useful information to identify novel lncRNAs as biomarkers for HCV-related HCC.

Material and methods

Microarray data

Microarray data were obtained from the study by Tsuchiya et al. [18], which is referenced in the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) under accession number GSE17856. The platform is GPL6480 Agilent-014850 Whole Human Genome Microarray 4x44K G4112F Array. A total of 44 non-tumoral liver and 43 tumors sample were obtained from 47 subjects with HCV-associated HCC. LncRNAs having fold changes ≥ 2 and p-values < 0.05 were selected as of significantly differential expression.

LncRNA classification pipeline

To evaluate the lncRNA expression pattern in microarray data, we applied a pipeline described by Zhang et al. [19]. Briefly, the probe set ID was mapped to the NetAffx Annotation Files (HG-U133 Plus 2.0, Annotations, CSV format, release 31, 08/23/10). The annotations included the probe set ID, gene symbol, gene title, ensemble gene ID, Ref-seq transcript ID and other informative items for the specific probe sets. The probe ID-centric gene expression data were joined with the annotation files on the probe set ID. Secondly, the probe sets which were assigned with a Refseq transcript ID and/or Ensemble gene ID in the, NetAffx annotations were extracted. For the probe sets with Refseq IDs, we only retained those labeled as “NR_” (NR indicates non-coding, RNA in the Refseq database). For the probe sets with Ensemble gene IDs, we only retained those annotated with “lncRNA”, “processed transcripts”, “non-coding” or “misc_RNA” in Ensemble annotations (accessible at the UCSC genome browser: http://www.genome.ucsc.edu/). Then, we filtered the probe sets obtained in step 2 by filtering out rRNAs, microRNAs and other short RNAs, including tRNAs, snRNAs and snoRNAs. Finally, 2448 annotated IncRNA transcripts with corresponding Affymetrix probe IDs were generated.

Identification of DElncs

A t-test [20] in the limma [21] package, in R [22] was used to identify genes which were differentially expressed between normal and HCV-positive HCC samples. The threshold for the DEGs was set as corrected p-value < 0.05 and | log2 fold change, (FC) | ≥ 1.

GO and KEGG pathway analysis

To identify functions of DEGs in HCC, we performed GO function enrichment analysis in three functional ontologies: biological process (BP), cellular component (CC) and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was also performed to identify pathways enriched in HCC using the DAVID system (https://david.ncifcrf.gov/). The p-value was calculated by hypergeometric distribution and a pathway with p < 0.05 was considered as significant.

Co-expression network construction and analysis

In this study, the Pearson correlation coefficient of DEG-lncRNA pairs was calculated according to the expression value of them. The co-expressed DEG-lncRNA pairs with the absolute value of Pearson correlation coefficient ≥ 0.5 were selected and the co-expression network was established using Cytoscape software.

Statistical analysis

The numerical data were presented as mean ± standard deviation (SD) of at least three determinations. Statistical comparisons between groups of normalized data were performed using the t-test or...
Mann-Whitney U-test according to the test condition. A value of $p < 0.05$ was considered to indicate statistical significance with a 95% confidence level.

**Results**

**Screening the DElncs in the HCV-positive hepatocellular carcinoma**

In the present study, the public dataset GSE17856 was analyzed to identify differentially expressed IncRNAs. A total of 44 non-tumoral liver and 43 tumor samples were obtained from 47 subjects with HCV-associated HCC. IncRNAs having fold changes $\geq 2$ and $p$-values $< 0.05$ were selected as of significantly differential expression. In total, 454 transcripts were observed to be expressed differentially compared to the normal liver tissues, including 256 upregulated transcripts and 198 downregulated transcripts. Hierarchical clustering showed systematic variations in the expression of IncRNAs in the HCV-positive HCC samples (Figure 1).

**Co-expression network analysis**

Here, we constructed up- and down-regulated IncRNA-mRNA co-expressed networks to reveal the potential roles of IncRNAs in HCV-positive HCC. The co-expressed IncRNA-mRNA pairs with the absolute value of the Pearson correlation coefficient $\geq 0.5$ were selected for network construction. As shown in Figure 2, a total of 125 IncRNAs and 1469 DEGs were included in the up-regulated IncRNA mediated co-expression network. In this network, we found that LOC341056, CCT6P1, PTTG3P, LOC643387, CXADRP2, LOC100128098, LOC100133920 played key roles and co-expressed with more than 100 DEGs. Meanwhile, we observed that the up-regulated IncRNA mediated co-expression network contained 67 IncRNAs and 558 DEGs (Figure 2 A). Twelve IncRNAs (FLJ35390, MGC45922, LOC92249, MTMR9LP, LOC440461, LOC92659, LOC100130557, LINC00304, RRP7B, FAM201A, PYY2, PIN1P1) co-expressed with more than 100 DEGs in the down-regulated IncRNA mediated network (Figure 2 B).

**Functional annotation of differentially expressed IncRNAs**

Based on co-expression networks, we performed GO and KEGG analysis for differentially expressed IncRNAs using the set of co-expressed
mRNAs. According to the GO analysis, we found that up-regulated IncRNAs were mainly involved in regulating metabolism, energy pathways, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism, regulation of the cell cycle, amino acid and derivative metabolism, cell cycle, DNA repair, and DNA replication (Figure 3 A). Meanwhile, we observed that the downregulated IncRNAs were enriched in regulating metabolism, energy pathways, immune response, regulation of biological process, and cell-cell adhesion (Figure 3 B).

Identification of key IncRNAs in regulating HCV-positive HCC development

For further explore the key IncRNAs in regulating key biological processes in HCV-positive HCC development, we constructed IncRNA-mRNA-biological processes. We observed that LOC341056, CCT6P1, PTTG3P, LOC643387, LOC100133920 were mainly involved in regulating metabolism and regulation of nucleobase and cell proliferation processes were most significant in the upregulated network (Figure 4 A). In the down-regulated network, C3P1 and C22orf45 played crucial roles in regulating immune response and energy pathways (Figure 4 B).

Key IncRNAs were associated with hepatocellular carcinoma prognosis

Furthermore, we evaluated the possible prognostic value of LOC341056, CCT6P1, PTTG3P, LOC643387, LOC100133920, C3P1 and C22orf45 in HCC using TCGA RNA-seq data. As shown in Figure 5 A–D, according to Kaplan-Meier analysis, we found that higher expression levels of LOC643387, PTTG3P, LOC341056, and CCT6P1
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Figure 2. Cont. Co-expression network of downregulated lncRNAs with DEGs (B). Triangle nodes, lncRNAs; square nodes, lncRNAs co-expressed genes

were associated with shorter survival time in the TCGA dataset. However, we found that C3P1-high (Figure 5 F) and C22orf45-high (Figure 5 E) patients showed higher survival rates compared to C3P1-and C22orf45-low patients.

Discussion

Hepatocellular carcinoma is one of the most common cancers worldwide [1]. Hepatitis C infection was one of the major etiological risk factors of HCCs [8]. However, the underlying mechanisms of HCV-induced hepatocarcinogenesis remain largely unclear. LncRNAs were a class of non-coding RNAs of more than 200 nucleotides in length [10]. A previous study demonstrated the key roles of lncRNAs in various human cancer types, including liver cancers [14]. In this study, we analyzed the public dataset GSE17856 and found that 454 lncRNAs (including 256 upregulated lncRNAs and 198 downregulated lncRNAs) were differentially expressed compared to the normal liver tissues.

Recently, emerging studies have shown that lncRNAs were also associated with the progression of HCC. For example, authors [23–25] identified differently expressed lncRNAs in HCV-related HCC using RNA sequencing and expression profile analysis. Zhu et al. reported that LINC00052 upregulated EPB41L3 to inhibit migration and invasion of hepatocellular carcinoma by binding miR-452-5p [26]. LncRNA TSLNC8 was reported as a tumor suppressor by inactivating the IL-6/STAT3 signaling pathway [27]. However, very few reports have focused on the molecular function of lncRNAs in HCV-positive HCC. In this study, we performed bioinformatics analysis to explore the potential roles of lncRNAs in regulating HCV-pos-
Figure 3. GO analysis of up-regulated lncRNAs in biological process (A) and down-regulated lncRNAs in biological process (B)
Figure 4. Identification of key IncRNAs in regulating HCV-positive HCC development. We constructed upregulated IncRNA-mRNA-biological processes. LOC341056, CCT6P1, PTTG3P, LOC643387, and LOC100133920 were involved in regulating metabolism, regulation of nucleobase and cell proliferation processes (A). Down-regulated IncRNAs (C3P1 and C22orf45) were involved in regulating immune response and energy pathways (B). Triangle nodes, IncRNAs; square nodes, IncRNAs co-expressed genes; circle, biological processes.
A
TCGA HCC survival

Overall survival (%)

LOC643387 – high (n = 186)
LOC643387 – low (n = 186)

Time [months]

0 10 20 30 40

p = 0.00007

B
TCGA HCC survival

Overall survival (%)

PTTG3P – high (n = 186)
PTTG3P – low (n = 186)

Time [months]

0 10 20 30 40

p = 0.0023

C
TCGA HCC survival

Overall survival (%)

LOC341056 – high (n = 186)
LOC341056 – low (n = 186)

Time [months]

0 10 20 30 40

p = 0.0006

D
TCGA HCC survival

Overall survival (%)

CCT6P1 – high (n = 186)
CCT6P1 – low (n = 186)

Time [months]

0 10 20 30 40

p = 0.0987

E
TCGA HCC survival

Overall survival (%)

C22orf45 – high (n = 186)
C22orf45 – low (n = 186)

Time [months]

0 10 20 30 40

p = 0.0027

F
TCGA HCC survival

Overall survival (%)

C3P1 – high (n = 186)
C3P1 – low (n = 186)

Time [months]

0 10 20 30 40

p = 0.0014

Figure 5. Key lncRNAs were associated with hepatocellular carcinoma prognosis. According to Kaplan-Meier analysis, we found that higher expression levels of LOC643387 (A), PTTG3P (B), LOC341056 (C), and CCT6P1 (D) were associated with shorter survival time in TCGA dataset. We found that C22orf45- (E) and C3P1- (F) high patients showed higher survival rates compared to C3P1- and C22orf45-low patients. Significance was defined as p < 0.05 (*p < 0.05; **p < 0.01; ***p < 0.001).
itive HCC development. We first constructed dysregulated IncRNA-mRNA co-expressed networks. We found 125 up-regulated IncRNAs co-expressed with 1469 DEGs and 67 down-regulated IncRNAs co-expressed with 558 DEGs. Bioinformatics analysis showed that dysregulated IncRNAs in HCV-positive HCC were mainly involved in regulating metabolism (such as energy pathways, nucleic acid metabolism and amino acid and derivative metabolism) and cell proliferation related biological processes (including cell cycle, DNA repair, and DNA replication). Of note, emerging studies have indicated the important roles in HCC progression [28, 29]. However, very few reports have focused on exploring the roles of IncRNAs in HCC metabolism regulation. Our study for the first time provides useful information for exploring the effect of IncRNA on HCC metabolism.

The roles of most IncRNAs in HCC have remained unclear. In the present study, we identified IncRNAs (LOC341056, CCT6P1, PTTG3P, LOC643387, LOC100133920, C3P1 and C22orf45) as key IncRNAs. Furthermore, we constructed key IncRNA mediated mRNA-bp networks to reveal the potential roles of these key IncRNAs. Of these IncRNAs, PTTG3P has been reported to promote gastric tumor cell proliferation and invasion. However, the other IncRNAs were never reported in human diseases. According to bioinformatics analysis, we found that LOC341056, CCT6P1, PTTG3P, LOC643387, LOC100133920 were mainly involved in regulating metabolism and cell proliferation related processes, and C3P1 and C22orf45 were associated with immune response and energy pathways. Moreover, we performed Kaplan-Meier analysis to evaluate the prognostic value of these key IncRNAs. Higher expression levels of LOC643387, PTTG3P, LOC341056, C3P1 and C22orf45 were associated with shorter survival time in the TCGA dataset. These results suggested that these IncRNAs in HCV-positive HCC development could serve as biomarkers for hepatocellular carcinoma prognosis.

In conclusion, our study represents a comprehensive analysis of differentially expressed IncRNAs in HCV-positive HCC for the first time by analyzing the public dataset GSE17856. We identified 256 upregulated IncRNAs and 198 downregulated IncRNAs in HCV-positive HCC compared to the normal liver tissues. Co-expression network and GO analysis showed that these IncRNAs were involved in regulating metabolism, energy pathways, proliferation and immune response. Seven IncRNAs (LOC341056, CCT6P1, PTTG3P, LOC643387, LOC100133920, C3P1 and C22orf45) were identified as key IncRNAs and co-expressed with more than 100 DEGs in HCV-positive HCC. Kaplan-Meier analysis showed that higher expression levels of LOC643387, PTTG3P, LOC341056, CCT6P1 and lower expression levels of C3P1 and C22orf45 were associated with shorter survival time in the TCGA dataset. We believe that this study can provide novel potential therapeutic and prognostic targets for HCV-positive HCC.

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Conflict of interest

The authors declare no conflict of interest.

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