Hepatocellular Carcinoma (HCC), a highly aggressive malignancy, is one of the most common cancers and represents the second leading cause of cancer-caused death worldwide [1]. In spite of the improvement in diagnostic technology, surgical therapy, chemotherapy, and molecular targeting therapy, the overall 5-year survival rate of HCC patients still remains unsatisfied due to the recurrence and metastasis [2, 3]. Although some important driver genes have been uncovered in HCC, the mechanism of HCC development, metastasis, and recurrence have not yet been well clarified [4, 5]. Therefore, a better understanding of the biological mechanisms involved in HCC pathogenesis and progression is critical for the developing of novel diagnostic biomarkers and therapeutic strategies.

In the past decade, advances in the high-throughput RNA sequencing techniques and bioinformatics methods have led to the identification that less than 2% of whole human genome sequence is protein coding gene, while...
the majority of the genome is transcribed into noncoding RNAs such as microRNAs, and long noncoding RNAs (lncRNAs) [6–8]. lncRNA is a novel member of ncRNA, which is longer than 200 nt in length and lacks protein coding capacity. Increasing evidence have revealed that lncRNAs are widely expressed in human tissues, and participate in many important cellular biological processes, such as cell fate decision, stem cells differentiation, X chromosome imprinting, cancer cells metastasis, and drug resistance [9–11]. Importantly, it has been reported that lots of lncRNAs dysregulation contributes to several diseases development [12]. In addition, analyses of several large-scale cancer tissue samples’ RNA sequencing and microarray datasets have uncovered that lots of lncRNAs showed altered expression pattern in multiple human cancers, and many of these lncRNAs could affect cancer cells phenotype via repression of tumor suppressors’ expression or activation of oncogenes transcription [13–15]. For example, overexpressed gastric cancer-associated lncRNA HOXA11-AS promotes cell proliferation and invasion through repressing KLF2 and PRS58 transcription through functioning as a scaffold of EZH2, DNMT1, and LSD1 [16].

Recently, the biological roles of lncRNAs have been frequently documented in HCC development and progression. For example, lncRNA FTX is down-regulated in HCC tissues, and it could inhibit HCC cells invasion and proliferation by repressing Wnt/β-catenin signaling activity and impeding DNA replication through competitively sponging miR-374a and binding to the DNA replication licensing factor MCM2 [17]. In addition, overexpressed lncRNA unigene56159 promotes HCC cells migration and invasion through binding to miR-140-5p and effectively acting as a ceRNA for miR-140-5p to derepress the SNAI2 expression [18]. Although functions and mechanisms of some lncRNAs have been characterized in HCC, the expression pattern and relevance of the majority lncRNAs in HCC remain unknown. To identify HCC-associated lncRNAs, we investigated lncRNAs profiles in HCC and adjacent nontumor samples by analyzing TCGA RNA sequencing data and GEO RNA sequencing and microarray datasets. This study reveals the aberrant lncRNAs expression in HCC tissues, which may provide new potential candidates for HCC diagnosis and treatment.

Methods and Materials

TCGA and public microarray datasets analyses

The RNA sequencing data and corresponding clinical data from TCGA liver hepatocellular carcinoma and paired normal tissues were downloaded from the GDC Data Portal (https://portal.gdc.cancer.gov). Another four HCC public datasets (GSE77509 [19], GSE64041 [20], GSE70880 [21], and GSE67260 [22]) were downloaded from Gene Expression Omnibus (GEO). Gene expression profiling of GEO microarray dataset GSE64041 was analyzed based on the Affymetrix Human Gene 1.0 ST Array platform, GSE77509 dataset was analyzed based on the Agilent-038314 CBC Homo sapiens lncRNA + mRNA microarray V2.0 platform, and GSE67260 dataset was analyzed based on the Agilent-052909 CBC lncRNA+mRNA_V3 platform. For TCGA differential gene expression analysis, Mann–Whitney U test was applied to the log2-transformed upper-quartile normalized FPKM values (from GDC Data Portal). For GEO Microarray datasets, the microarray probes were reannotated to lncRNAs, and the R package, limma, was used to calculate the differentially expressed lncRNAs.

Copy number variation analysis

The HCC copy number variation data were downloaded from Broad GDC FireBrowser website (https://portal.gdc.cancer.gov). GISTIC 2.0 [23] was used to determine significantly recurrent lncRNAs genomic regions of somatic copy number alterations (SCNA). The amplification and deletion peaks with \( q \)-values < 0.25 were considered as significant. The lncRNAs regions were mapped to the GISTIC peaks.

lncRNAs Survival analysis

The univariable Cox regression analysis was used to determine the relationship between each lncRNA expression and HCC patients’ overall survival (OS) and recurrence-free survival (RFS). Only the lncRNAs with the \( P \) < 0.05 were considered statistically significant. Then, the HCC patients were divided into high- and low-expression groups according to the median lncRNAs expression level. Log-rank test was applied to compare the survival distribution between two groups. The Python package, lifelines, was used for the survival analysis.

Cell culture and transfection

HCC cell line HuH1 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HuH1 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing 100 U/mL penicillin, and 1 \( \mu \)g/mL streptomycin (Invitrogen), 10% fetal bovine serum (Invitrogen, Shanghai, China) at 37°C with 5% CO\(_2\). The SNHG7, PVT1, and negative control siRNAs (Invitrogen, Carlsbad, CA) were transfected into HuH1 cells using RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours post transfection, the cells were harvested for RNA extraction. The SNHG7 siRNA sequences are siRNA 1, 2.
GGAGAAGGTCATTCTCAATGTA, siRNA 2#, CCAGTCGHTCTGTGTTCCATTTGA. The PVT1 siRNA sequences are siRNA 1#, GCUGAGAGGGGUUGAGAUCUCUGUUU, siRNA 2#, UGGACAGUCUGGGCUGGGUGGGAA.

**RNA extraction and qPCR**

RNA was extracted from HuH1 cells using TRIZOL reagent, according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed into cDNA using PrimeScript RT Reagent Kit (Takara, Dalian, China). To examine SNHG7 and PVT1 expression levels, SYBR Premix Ex Taq (Takara) was used. The housekeeping gene GAPDH was used as an internal control. The primer sequence of SNHG7 is, forward 5′-GTGTGTCCCTTGGTGGAGAG-3′, reverse 5′-TCCCAGATACAGCGAGGA-3′. The primer sequences of PVT1 are: forward 5′-TGAGAACTGTCCTTACGTGACC-3′; reverse 5′-AGAGCACCAAGACTGGCTCT-3′. The primer sequences of GAPDH are: forward 5′-AGAAGGCTGGGGCTCATTTG-3′; reverse 5′-AGGGGCCATCCACAGTCTTC-3′. QPCR analyses were performed on ABI7500, and comparative cycle threshold (CT) (2^-ΔΔCT) method was used to analyze the data.

**Cell migration and invasion assays**

Transwell assays (Corning, Tewksbury, MA, USA, 8.0-μm pores) were performed to measure HuH1 cell invasive ability after transfection with SNHG7 or negative control siRNAs. Cells (1 × 10^5) in 300 μL medium containing 1% FBS were placed in the upper chamber of an insert (coated with Matrigel for invasion assay, Sigma-Aldrich); 700 μL medium with 10% FBS was added to the lower chamber. Twenty-four hours after incubation, the HuH1 cells invaded through the membrane were fixed with methanol, then stained with 0.1% crystal violet, and imaged using an IX71 inverted microscope (Olympus, Tokyo, Japan).

**Statistical analysis**

The one-way ANOVA and Student’s t test (two-tailed) was used to analyze the in vitro data using R. A P < 0.05 was considered statistically significant.

**Results**

**Identification of lncRNAs alterations in HCC tissues**

To investigate the lncRNA expression profiles in HCC tissues, we used the RNA sequencing data and microarray gene profiling data (GSE77509, GSE64041, GSE70880) from TCGA and GEO in HCC and nontumor tissue samples. The TCGA project includes 374 HCC and 50 normal tissue samples, while the GSE77509, GSE64041, and GSE70880 datasets contains 20, 60, and 16 paired HCC and adjacent normal samples, respectively. Analyses of these datasets revealed that 1939 lncRNAs expression were dysregulated in the TCGA dataset (1295 up-regulated and 644 down-regulated); 657 lncRNAs was differentially expressed in the GSE77509 dataset (249 up-regulated and 408 down-regulated); 103 lncRNAs were dysregulated in the GSE64041 dataset (31 up-regulated and 72 down-regulated); and 335 lncRNAs were differentially expressed in the GSE70880 dataset (176 up-regulated and 159 down-regulated) (Fig. 1A–D, and Table S1). Further intersection analysis revealed that 347 lncRNAs were consistently up-regulated or down-regulated in at least two datasets (Fig. 1E and F). These findings indicate that a large number of lncRNAs have dysregulated expression levels in HCC, and some of these lncRNAs may be potential biomarkers for HCC diagnosis.

**Somatic copy number alterations of lncRNAs in HCC**

To determine whether genomic alterations contribute to lncRNA dysregulation in HCC, we analyzed the copy number alterations of these differentially expressed lncRNAs (in TCGA data) using TCGA data. For HCC, the gene-containing loci SCNAs frequencies of each lncRNAs were calculated. An alteration that occurs in all HCC samples with q value less than 0.25 was defined as significant alteration. The results showed 31 and 41 lncRNAs loci had recurrent DNA gain and loss, respectively (Fig. 2A and B, and Table S2). These findings suggest that genome copy number variations are involved in some lncRNAs dysregulation in HCC.

**Identification of survival-related lncRNAs in HCC**

To evaluate whether the lncRNAs alterations are related with HCC patients’ survival time, we performed univariable Cox regression analysis. The results revealed that 22 up-regulated lncRNAs and 18 down-regulated lncRNAs are significantly associated with HCC patients OS (P < 0.05), while 11 up-regulated lncRNAs and 10 lncRNAs down-regulation are significantly associated with HCC patients RFS (Fig. 3A, Table S2). For example, HCC patients with higher RP1-228H13.5 and TMCC1-AS1 expression levels had shorter OS time, while HCC patients with higher RP11-307C12.11 and LINC00205 expression levels had shorter RFS time (Fig. 3B and C). These
findings indicate that these HCC survival-associated dysregulated lncRNAs could be used as biomarkers for HCC patients’ prognostic prediction.

**Identification of lncRNAs alteration in HCC tissues with metastasis**

Cancer cells invasion, metastasis, and tumor recurrence are few of the main reasons of HCC-caused death. To identify lncRNAs that may be involved in HCC recurrence and metastasis, we downloaded the GSE67260 dataset from GEO, which consists of five tissue samples with early recurrence (less than 1 year, with invasion and metastasis out of liver) and five samples with late recurrence (longer than 2 years, without invasion and metastasis out of liver). Analysis of the data revealed that 156 lncRNAs expression were up-regulated and 36 lncRNAs expression were down-regulated in HCC samples with early recurrence when compared with the HCC tissues with late recurrence (Fig. 4A). Further intersection analysis showed that five up-regulated lncRNAs in early recurrence samples are also overexpressed in HCC tissues compared with normal tissues, while two down-regulated lncRNAs in early recurrence samples are also decreased in HCC tissues compared with normal tissues (Fig. 4B). These findings indicated that lncRNAs not only contribute to HCC development,
but may also be involved in cancer cells metastasis and HCC recurrence.

Knockdown of PVT1 and SNHG7 impaired HCC cells invasion

To determine whether these lncRNAs could affect HCC cells invasion, we chose up-regulated lncRNAs PVT1 and SNHG7 for further study. Recent studies reveal that PVT1 is overexpressed in multiple cancers and correlated with cancer progression, including colorectal cancer [24], gastric cancer [25], and cervical cancer [26]. In addition, a recent study showed that SNHG7 overexpression promotes cell migration and invasion in lung cancer by enhancing the FAIM2 expression [27]. Here, we firstly analyzed PVT1 and SNHG7 expression levels in HCC cell lines using CCLE data [28], and the results showed that HuH1 cell has highest PVT1 and SNHG7 expression levels (Fig. 5A). Then, we designed siRNAs for PVT1 and SNHG7, and transfected them into HuH1 cells to knockdown PVT1 and SNHG7 expression. The qPCR results showed that PVT1 and SNHG7 expression was significantly reduced in siRNA-transfected cells compared with controls (Fig. 5B). Next, transwell assays revealed that knockdown of PVT1 and SNHG7 could impair HuH1 cells invasive ability compared with control cells (Fig. 5C–F). These results suggest that PVT1 and SNHG7 might play important roles in HCC invasion and progression.

Discussion

In the past decade, increasing evidence have highlighted the critical roles of noncoding elements of human genomic in cellular process and their alteration in human diseases, such as cancer development and progression [29, 30]. Recently, lots of studies have revealed the involvement of lncRNAs in tumorigenesis and cancer progression by functioning as oncogenes or tumor suppressors dependent on the circumstance. Moreover, high throughput RNA sequencing in diverse cancers and normal tissues has uncovered that some lncRNAs expressions pattern is tissue-specific, and few cancer-specific lncRNAs have been characterized in different cancers [8]. These findings suggest that lncRNAs may be better biomarker candidates for human cancers diagnostic and prognostic prediction. However, the HCC-associated lncRNAs and their function remain not well known.
In this study, we analyzed lncRNAs expression profiles in HCC tissues compared with parental normal tissues or nontumor tissues using TCGA RNA sequencing data, as well as RNA sequencing and microarray gene profiling datasets from GEO. Interestingly, our findings revealed that thousands of lncRNAs were differentially expressed in HCC tissues compared with normal tissues. Moreover, genomic alterations analyses showed that somatic copy number variations contribute to some of these lncRNAs dysregulation in HCC tissues. Importantly, survival analysis indicated that lots of lncRNAs overexpression or down-regulation is significantly associated with HCC patients’ poorer OS or RFS, which suggests that these lncRNAs could be valuable prediction factors for HCC patients’ survival time. Cancer cells invasion, metastasis, and tumor recurrence are the main reason of HCC- caused death. Here, we identified several lncRNAs that may be involved in HCC metastasis by comparing lncRNA expression profiles.

Figure 3. Hepatocellular carcinoma (HCC) Patients’ overall survival (OS) and recurrence-free survival (RFS)-associated lncRNAs. (A) A heatmap was drawn to show the OS and RFS-associated lncRNAs (p value, and Hazard ratio value were shown) in HCC. (B) The Kaplan–Meier curve for OS of HCC patients with high- RP1-228H13.5 or TMCC1-AS1 expression and low- RP1-228H13.5 or TMCC1-AS1 expression using the TCGA data. The significant differences were examined using the two-sided log-rank test. (C) The Kaplan–Meier curve for RFS of HCC patients with high- LINC00205 or RP11-307C12.11 expression and low- LINC00205 or RP11-307C12.11 expression using the TCGA data. The significant differences were examined using the two-sided log-rank test.
pattern in HCC tissues with or without invasion and metastasis, such as PVT1 and SNHG7.

lncRNA PVT1 has been found to be up-regulated in several cancers, and overexpressed PVT1 exerts oncogenic function by promoting cancer cells proliferation, invasion, and metastasis. For example, PVT1 is significantly up-regulated in gastric cancer tissues, and increased PVT1 predicted poor prognosis. PVT1 promotes gastric cancer cell proliferation and invasion through directly binding with FOXM1 protein and increasing FOXM1 in a post-translation regulation manner [25]. In addition, Wan et al. reported that PVT1 is highly expressed in non-small-cell lung cancer tissues and cells, and enhances cells proliferation and inhibits apoptosis by interacting with EZH2 and repressing tumor suppressor LATS2 transcription [31]. Besides, PVT1 could promote HCC cells proliferation and stem cell-like property of HCC cells by stabilizing NOP2 [32]. Higher PVT1 expression level is associated with tumor progression and predicts recurrence in HCC patients [33]. Tian reported that PVT1 promotes the tumorigenesis and metastasis of hepatocellular carcinoma by acting as a competing endogenous RNA for miR-186-5p [34], which is consistent with our analyses results. Moreover, our finding also reveals that knockdown of PVT1 impedes HCC cells invasion. Similarly, down-regulation of another candidate SNHG7 also impairs cells invasion. SNHG7 overexpression has been found in lung cancer, and SNHG7 could promote lung cancer cells proliferation, invasion,
and inhibit apoptosis by enhancing the FAIM2 expression [27]. These findings suggest that our analyses results could provide valuable lncRNAs candidates for further investigation of lncRNAs function in HCC.

In summary, our findings uncover thousands of Dysregulated lncRNAs in HCC tissues compared with their parental normal tissues. Some of these lncRNAs are significantly associated with HCC patients’ prognosis, and might play critical roles in HCC development, progression, and recurrence through regulation of cell invasion ability. Our study highlights the critical roles of lncRNAs in HCC and may provide valuable candidates as diagnostic markers and potential targets for HCC patients. This study also has a few limitations; for example, only a few lncRNA candidates were validated in this study. This will need to be further investigated by other researchers.

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**Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

1. Siegel, R., D. Naishadham, and A. Jemal. 2013. Cancer statistics, 2013. CA Cancer J. Clin. 63:11–30.
2. Siegel, R., J. Ma, Z. Zou, and A. Jemal. 2014. Cancer statistics, 2014. CA Cancer J. Clin. 64:9–29.
3. Chen, W., R. Zheng, P. D. Baade, S. Zhang, H. Zeng, F. Bray, et al. 2016. Cancer statistics in China, 2015. CA Cancer J. Clin. 66:115–132.

4. Dutta, R., and R. I. Mahato. 2017. Recent advances in hepatocellular carcinoma therapy. Pharmacol. Ther. 173:106–117.

5. Kudo, M., F. Trevisani, G. K. Abou-Alfa, and L. Rimassa. 2016. Hepatocellular Carcinoma: therapeutic Guidelines and Medical Treatment. Liver Cancer 6:16–26.

6. Griffiths-Jones, S. 2007. Annotating noncoding RNA genes. Annu. Rev. Genomics Hum. Genet. 8:279–298.

7. ENCODE Project Consortium. 2012. An integrated encyclopedia of DNA elements in the human genome. Nature 489:57–74.

8. Derrien, T., R. Johnson, G. Bussotti, A. Tanzer, S. Djebali, H. Tilgner, et al. 2012. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. 22:1775–1789.

9. Mercer, T. R., M. E. Dinger, and J. S. Mattick. 2009. Long non-coding RNAs: insights into functions. Nat. Rev. Genet. 10:155–159.

10. Ponting, C. P., P. L. Oliver, and W. Reik. 2009. Evolution and functions of long noncoding RNAs. Cell 136:629–641.

11. Ulitsky, I., and D. P. Bartel. 2013. lincRNAs: genomics, evolution, and mechanisms. Cell 154:26–46.

12. Shi, X., M. Sun, H. Liu, Y. Yao, and Y. Song. 2013. Long non-coding RNAs: a new frontier in the study of human diseases. Cancer Lett. 339:159–166.

13. Cabanski, C. R., N. M. White, H. X. Dang, J. M. Silva-Fisher, C. E. Rauck, D. Cicka, et al. 2015. Pan-cancer transcriptome analysis reveals long noncoding RNAs with conserved function. RNA Biol. 12:628–642.

14. Chen, J., Z. Fu, C. Ji, P. Gu, P. Xu, N. Yu, et al. 2015. Systematic gene microarray analysis of the IncRNA expression profiles in human uterine cervix carcinoma. Biomed. Pharmacother. 72:83–90.

15. Yan, X., Z. Hu, Y. Feng, X. Hu, J. Yuan, S. D. Zhao, et al. 2015. Comprehensive genomic characterization of long non-coding RNAs across human cancers. Cancer Cell 28:529–540.

16. Sun, M., F. Nie, Y. Wang, Z. Zhang, J. Hou, D. He, et al. 2016. LncRNA HOXA11-AS promotes proliferation and invasion of gastric cancer by scaffolding the chromatin modification factors PRC2, LSD1, and DNMT1. Cancer Res. 76:6299–6310.

17. Liu, F., J. H. Yuan, J. F. Huang, F. Yang, T. T. Wang, J. Z. Ma, et al. 2016. Long noncoding RNA FTX inhibits hepatocellular carcinoma proliferation and metastasis by binding MCM2 and miR-374a. Oncogene 35:5422–5434.
30. Fatica, A., and I. Bozzoni. 2014. Long non-coding RNAs: new players in cell differentiation and development. Nat. Rev. Genet. 15:7–21.
31. Wan, L., M. Sun, G. J. Liu, C. C. Wei, E. B. Zhang, R. Kong, et al. 2016. Long noncoding RNA PVT1 promotes non-small cell lung cancer cell proliferation through epigenetically regulating LATS2 expression. Mol. Cancer Ther. 15:1082–1094.
32. Wang, F., J. H. Yuan, S. B. Wang, F. Yang, S. X. Yuan, C. Ye, et al. 2014. Oncofetal long noncoding RNA PVT1 promotes proliferation and stem cell-like property of hepatocellular carcinoma cells by stabilizing NOP2. Hepatology 60:1278–1290.
33. Ding, C., Z. Yang, Z. Lv, C. Du, H. Xiao, C. Peng, et al. 2015. Long non-coding RNA PVT1 is associated with tumor progression and predicts recurrence in hepatocellular carcinoma patients. Oncol. Lett. 9:955–963.
34. Lan, T., X. Yan, Z. Li, X. Xu, Q. Mao, W. Ma, et al. 2017. Long non-coding RNA PVT1 serves as a competing endogenous RNA for miR-186-5p to promote the tumorigenesis and metastasis of hepatocellular carcinoma. Tumour Biol. 39:1010428317705338.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. The lncRNAs profiling in TCGA and GEO datasets.

Table S2. The lncRNAs CNV, and survival associated lncRNAs in HCC.

Table S3. The lncRNAs profile in HCC tissues with or without metastasis.