Brief Report

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Preliminary investigation of five novel long non-coding RNAs in hepatocellular carcinoma cell lines

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Summary **Hepatocellular carcinoma (HCC) is a highly prevalent cancer with a high mortality rate and HCC is always accompanied with a hepatitis B virus (HBV) infection, unlike many other types of cancers. Over the past few years, cancer-related long non-coding RNAs (lncRNAs) and virus-related lncRNAs have attracted the attention of many researchers, and a number of previous studies have examined the relationship between lncRNAs and various cancers and viruses. The current study used The Cancer Genome Atlas database to screen for lncRNAs up-regulated in HCC in order to identify cancer biomarkers. Results revealed five lncRNAs that were the most up-regulated. This result was then verified in 10 HCC cell lines and two normal liver cell lines. Quantitative real-time PCR revealed that the five lncRNAs were substantially up-regulated in HCC cell lines. Several of the five lncRNAs were expressed at higher levels in a few HCC cell lines that were infected with HBV or that were positive for its protein or DNA than in HCC cell lines that were not infected with HBV or that were negative for its protein or DNA. These findings suggest that the five lncRNAs might play a role in the progression of HCC and/or HBV infection, and these findings need to be studied in further detail.**

Keywords: lncRNA, hepatocellular carcinoma, cell line, hepatitis B virus

1. Introduction

The GENCODE project identified about 20,000 protein-coding genes in the human genome (*1*). The DNA in the human genome that is not genes and that does not produce proteins is referred to as "non-coding DNA" (*2*). Some parts of "non-coding DNAs" produce introns and the others are transcribed into functional non-coding RNAs (ncRNAs). NcRNAs are classified into small ncRNAs (less than 200 nts) and long ncRNAs (lncRNAs, more than 200 nts). Small ncRNAs, and particularly microRNAs (miRNAs), are widely considered to be post-transcriptional regulators of mRNAs and their roles in cancer progression are increasingly being studied (*3,4*). A great number of lncRNAs have been identified thus far, and there is a rapidly growing number of studies of the biological functions of lncRNAs in human cancers, such as hepatocellular carcinoma (HCC), gastric cancer, prostate cancer, bladder cancer, renal cancer, colorectal carcinoma, and glioma (*5-9*). Numerous studies have suggested that lncRNAs are related to the proliferation, invasion, and metastasis of cancers as well as their poor prognosis. The relationship between lncRNAs and cancers implies that lncRNAs might serve as biomarkers and therapeutic targets. As an example, TUG1 was found to increase in different types of cancers, including B-cell malignancies, esophageal

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squamous cell carcinoma, bladder cancer, HCC, and osteosarcoma (*10*). MALAT1 was identified as a prognostic marker for survival and metastasis in nonsmall cell lung cancer, cervical cancer, pancreatic cancer, and breast cancer (*11*). Another widely studied lncRNA, HOTAIR, was up-regulated and was used as an independent prognostic biomarker for breast cancer, cervical cancer, colon cancer, and gastric cancer (*12*). Unfortunately, most of the molecular mechanisms of lncRNAs have yet to be elucidated.

HCC is a type of highly malignant cancer with a poor prognosis according to many studies. Over the past few years, abnormal expression of a few lncRNAs has been found to be related to recurrence and metastasis of HCC as well as its poor prognosis (*13*). A special feature of HCC is the fact that some patients with the cancer are infected with a hepatitis virus. To the extent known, viruses play important roles in the progression of a few cancers, such as human papillomavirus in cervical cancer and the hepatitis B virus (HBV) and hepatitis C virus (HCV) in HCC (*14,15*). Numerous studies have suggested that some lncRNAs could affect virus infection and replication. For example, EGOT affects the antiviral response to HCV, lncRHOXF1 promotes replication of the Sendai virus, and NEAT1 is related to infection with the influenza virus (*16-18*). Thus, significant components of HCC treatment are steps to counter the tumor and viruses. The current study focused on five new lncRNAs from the Cancer Genome Atlas database. lncRNAs with the highest level of expression in HCC were identified and their level of expression was detected in HCC cell lines that were infected or not infected with HBV.

2. Materials and Methods

2.1. *Cell lines*

Ten human HCC cell lines, BEL-7402, HLF, HLE, HepG2, HepG2.2.15, Huh-1, Huh-7, PLC/PRF/5, Hep3B, and SK-Hep-1, and two normal human liver cell lines, hNHEPs and L02, were cultured in high glucose Dulbecco's Modified Eagle medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA) in a humidified chamber at 37°C in 5% CO_2.

2.2. *Identification of candidate lncRNAs from the TCGA database*

The Cancer Genome Atlas (TCGA) database was searched to identify candidate lncRNAs. The TCGA online database is the result of a project, begun in 2005, to catalogue genetic mutations responsible for cancer, using genome sequencing and bioinformatics. The TCGA database contains the results of high-throughput genome analysis of about 30 sets of paracancerous tissue and cancer tissue from patients with HCC. The current study arranged lncRNAs by level of expression from highest to lowest in order to identify lncRNAs that were up-regulated in cancer. The five lncRNAs that were up-regulated the most were selected to examine their expression in HCC.

2.3. *Reverse transcription PCR*

After cells were collected, total RNA was extracted from cells with the RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions. The mRNA was then reverse-transcribed to produce cDNA using a Reverse Transcription System (Promega, Madison, USA) and random primers in accordance with the manufacturer's instructions.

2.4. *Real-time PCR*

cDNA was quantified using the StepOneTM Real-Time PCR System (Applied Biosystems, USA). A polymerase chain reaction (PCR) was performed using the primers (designed with Primer 3Plus online software and synthesized by Invitrogen) shown in Table 1. GAPDH served as a positive control. FastStart Universal SYBR Green Master (Rox) (Roche) was used to amplify and detect DNA during the reaction. Thermal cycling parameters for the target genes and GAPDH consisted of a hot start for 2 min at 94°C, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s, and then 72°C for 30 s. The specificity of the PCR products was verified using melting curve analysis.

2.5. *Statistical analysis*

All experiments were performed in triplicate and the results were analyzed with one-way analysis of variance (ANOVA) in GraphPad Prism 4, followed by Student's *t* test in Microsoft Office Excel. $p < 0.05$ was considered to indicate a significant difference.

3. Results and Discussion

3.1. *Identification of candidate lncRNAs from the TCGA database*

The TCGA database was searched to identify candidate lncRNAs. As a result, several lncRNAs with abnormal levels of expression were identified. Five of these lncRNAs, PRC1-AS1 (Chromosome 15: 90966369-90988624), CRNDE (Chromosome 16: 54918863-54929189), RP11-334E6.12 (Chromosome 11: 119417951-119419114), LINC00665 (Chromosome 19: 36313061-36331718), and AC092171.4 (Chromosome 7: 5475804-5479811), were significantly up-regulated, so these lncRNAs were selected for further research (Figure 1).

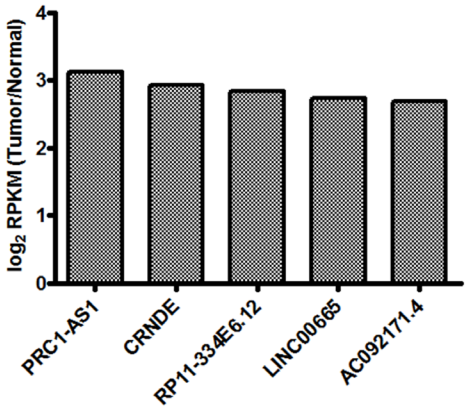

Figure 1. Five up-regulated lncRNAs identified from the TCGA database.

Table 1. Primers for quantifying lncRNAs

Primer	Sequence (5' to 3')	Product size (bp)
PRC1-AS1_F	CTCAGAGCTTTCGGTGGTTC	108 bp
PRC1-AS1_R	GGATTTCGTGGCTGGAGATA	
CRNDE_F	TGCCACTGGAAATGTTGAAA	189 bp
CRNDE_R	CTTCTGCGTGACAACTGAGG	
RP11-334E6.12_F	GACAGACCATGTCCGTGCTA	135 bp
RP11-334E6.12_R	ATGTGAGGGGTAGTGGGATG	
LINC00665_F	CCATCCACCTTTCTTGTGGT	153 bp
LINC00665_R	CAGCTGGCCTCTTTTTCACT	
AC092171.4_F	GGTTGCAGGGGACACTAAAA	160 bp
AC092171.4_R	CCTGGGTGTCCTGTTCTCAT	
GAPDH_F	AGGTGAAGGTCGGAGTCAAC	117 bp
GAPDH_R	AGTTGAGGTCAATGAAGGGG	

3.2. *Five lncRNAs were substantially up-regulated in 10 HCC cell lines*

The level of expression of the five candidate lncRNAs was measured in 10 HCC cell lines (BEL-7402, HLF, HLE, HepG2, HepG2.2.15, Huh-1, Huh-7, PLC/PRF/5, Hep3B, and SK-Hep-1) and two normal liver cell lines (hNHEPs and L02). Reverse transcription PCR and quantitative real-time PCR were used to detect the expression of these five lncRNAs in the 12 liver cell lines (Table 1). Results indicated that the five lncRNAs was significantly up-regulated in all of the 10 cancer cell lines in comparison to two normal liver cell lines (Figure 2 and Table 2).

All five lncRNAs were highly up-regulated in all of the cancer cell lines, but they tended to be expressed at a much higher level in certain cell lines. PRC1-AS1, which was the lncRNA with the highest level of expression in TCGA database, was markedly up-regulated in BEL7402, HepG2.2.15, Huh-1, and Hep3B; that level of expression was more than four-fold that in normal liver cell lines (Figure 2A). The lncRNA that was expressed at the next highest level was CRNDE, which was up-regulated in all HCC cell lines. CRNDE was expressed at higher levels in HLF, HLE, HepG2.2.15, and Huh-1 (Figure 2B). RP11-334E6.12 was expressed at a higher level in HLF, HepG2, HepG2.2.15, and Huh-1 (Figure 2C). LINC00665 was markedly expressed in HepG2, HepG2.2.15, Huh-1, and Hep3B (Figure 2D). AC092171.4 was expressed at a higher level in HLE, HepG2, Huh-1, and Hep3B (Figure 2E). These results indicate that all five of the lncRNAs were significantly up-regulated in HCC cell lines, but each lncRNA was expressed at higher levels in certain cell lines. This finding suggests that some cell lines are a better choice for differential expression of lncRNAs.

The level of lncRNA expression in normal liver cell lines and HCC cell lines was analyzed. Figure 2F shows that all five of the lncRNAs were expressed at a significantly higher level in cancer cell lines than in normal liver cell lines. This finding coincides with the results from the TCGA database. Although each lncRNA was not expressed to the same extent as indicated in the TCGA database, the levels of expression were reasonable given the differences in clinical samples and cell lines and differences in genetic backgrounds.

3.3. *lncRNA expression was markedly up-regulated in HCC cell lines infected with HBV*

In addition to the high level of expression of the five lncRNAs in 10 HCC cell lines, results also indicated that HCC cell lines that were infected with HBV or that were positive for its protein or DNA seemed to have a higher level of expression of lncRNAs than other HCC cell lines that were negative for HBV protein or DNA (Figure 3A). In addition, the average level of expression of each lncRNA was compared in normal cell lines, HCC cell lines not infected with HBV, and HCC cell lines infected with HBV. Results revealed significant differences in expression of PRC1-AS1, LINC00665, and AC092171.4 (Figure 3B). There were no significant differences in expression of CRNDE and RP11-334E6.12 in all HCC cell lines not infected with HBV and all HCC cell lines infected with HBV. In contrast, expression of CRNDE differed significantly in HCC cell lines not infected with HBV and the PLC/PRF/5 cell line. Expression of RP11-334E6.12 differed significantly in HCC cell lines not infected with HBV and the HepG2.2.15 cell line. These results suggest that the five lncRNAs might interact with HBV or its protein or DNA. Thus, future research should involve study of the potential interaction between candidate lncRNAs and HBV.

In summary, the current study suggested that five lncRNAs were substantially up-regulated in 10 HCC cell lines in comparison to two normal liver cell lines. HCC cell lines that were infected with HBV or that were positive for its protein or DNA had a higher level of expression of lncRNAs than HCC cell lines that were not infected with HBV or that were negative for

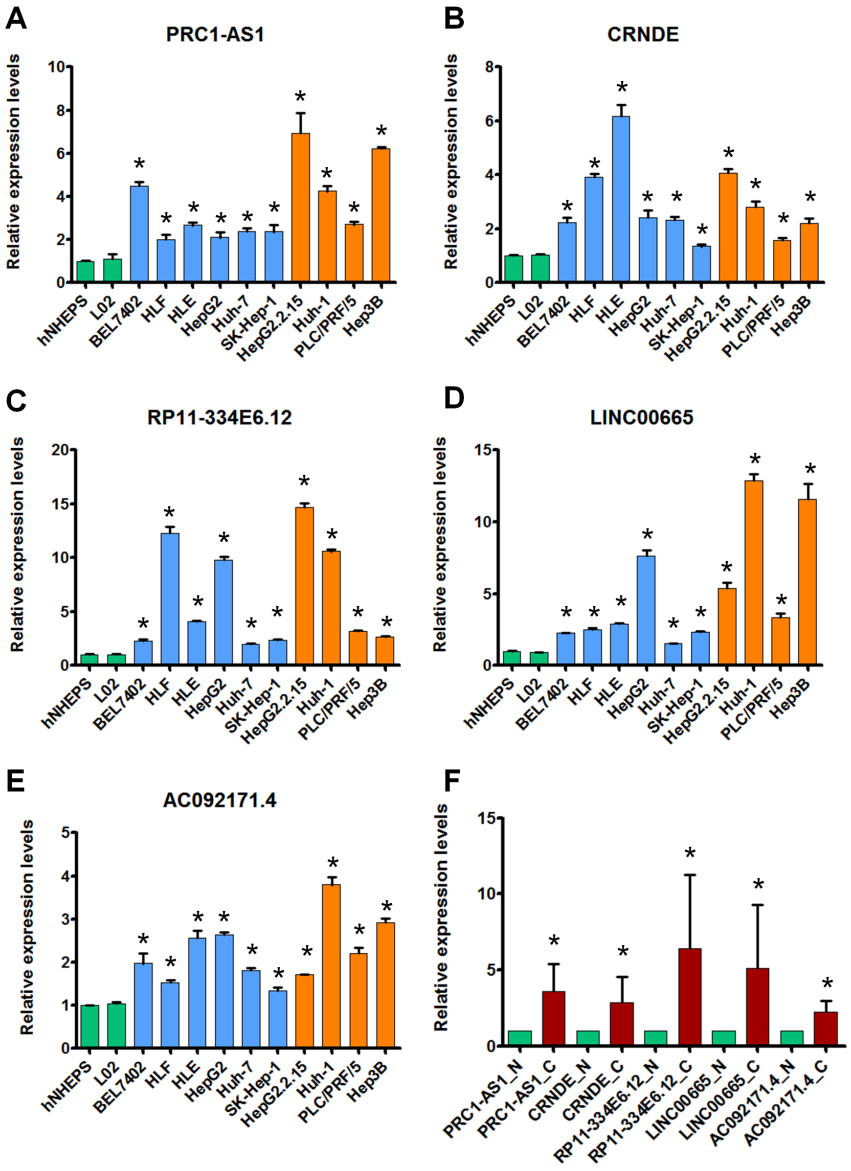

Figure 2. Relative expression of five lncRNAs in 12 cell lines. (A) The level of PRC1-AS1 expression in 12 cell lines. **(B)** The level of CRNDE expression in 12 cell lines. **(C)** The level of RP11-334E6.12 expression in 12 cell lines. **(D)** The level of LINC00665 expression in 12 cell lines. **(E)** The level of AC092171.4 expression in 12 cell lines. **(F)** Comparison of the expression of five lncRNAs in normal liver cell lines and HCC cell lines. N: normal liver cell lines; C: HCC cancer cell lines. Green bar: normal liver cell lines; blue bar: HCC cell lines not infected with HBV; yellow bar: HCC cell lines infected with HBV; wine red bar: HCC cell lines. Data are expressed as the mean ± S.D. ($n = 3$). $^*p < 0.05$ *vs*. normal liver cell lines.

Table 2. Relative average level of expression of five lncRNAs in liver cell lines

Cell lines	Long non-coding RNAs				
	PRC1-AS1	CRNDE	RP11-334E6.12	LINC00665	AC092171.4
hNHEPS	1.000000	1.000000	1.000000	1.000000	1.000000
L02	1.100222	1.024768	1.03541	0.923571	1.044776
BEL7402	4.486377	2.243313	2.272536	2.257758	1.971264
HLF	2.005248	3.922631	12.25951	2.496699	1.528039
HLE	2.663165	6.190015	4.084482	2.900079	2.565964
HepG2	2.100222	2.422593	9.781447	7.655874	2.639765
Huh-7	2.389504	2.326131	1.974695	1.532592	1.809742
SK-Hep-1	2.366716	1.351478	2.367365	2.316163	1.339115
HepG2.2.15*	6.918878	4.072771	14.71719	5.375211	1.716978
Huh-1*	4.242769	2.820624	10.59926	12.87392	3.794856
PLC/PRF/5*	2.700666	1.57057	3.211381	3.346897	2.209993
Hep3B*	6.204443	2.204493	2.686601	11.57074	2.91626

*HCC cell lines that were infected with HBV or that were positive for its protein or DNA.

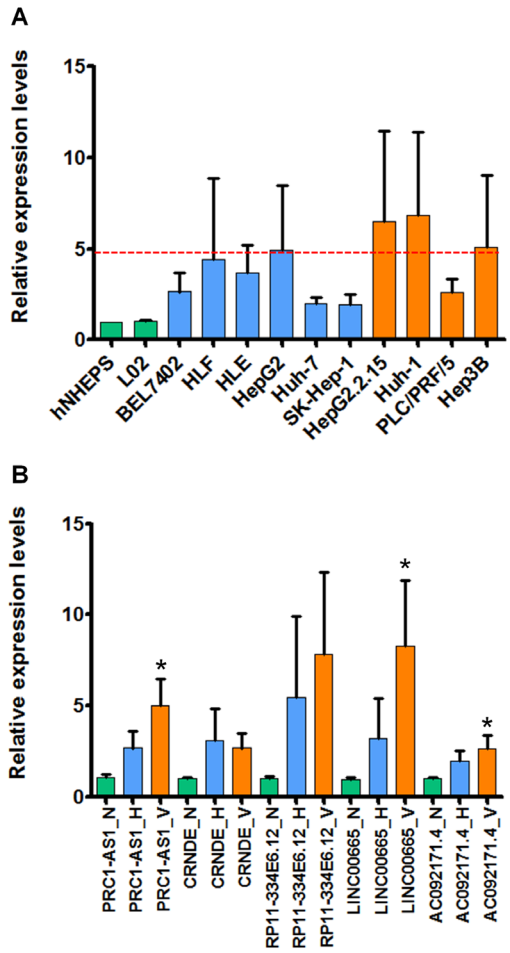

Figure 3. Comparison of HCC cell lines infected or not infected with HBV. (A) The average level of lncRNA expression in 12 cell lines. The dotted line represents the highest average level of expression in HCC cell lines not infected with HBV. **(B)** Comparison between HCC cell lines infected with HBV and HCC cell lines not infected with HBV. Cell lines expressing lncRNAs were divided into three groups: normal liver cell lines (N), HCC cell lines not infected with HBV (H), and HCC cell lines that were infected with HBV or positive for its protein or DNA (V). Green bar: normal liver cell lines; blue bar: HCC cell lines not infected with HBV; yellow bar: HCC cell lines infected with HBV. Data are expressed as the mean ± S.D. (n = 3). *p < 0.05 *vs.* HCC cell lines not infected with HBV.

its protein or DNA. These results indicate that these lncRNAs might play important roles in the progression of HCC and/or HBV infection.

Acknowledgements

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