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

Jufeng Xia¹, Yoshinori Inagaki¹, Tatsuo Sawakami¹, Peipei Song², Yulong Cai¹,³, Kiyoshi Hasegawa¹, Yoshihiro Sakamoto¹, Nobuyoshi Akimitsu⁴, Wei Tang¹,* Norihiro Kokudo¹

¹Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;
²Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa-shi, Chiba, Japan;
³Department of Bile Duct Surgery, West China Hospital, Sichuan University, Chengdu, Sichuan Province, China;
⁴Radioisotope Center, The University of Tokyo, Tokyo, Japan.

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
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 IncRNA, 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 IncRNAs 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 IncRNAs 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 IncRNAs could affect virus infection and replication. For example, EGOT affects the antiviral response to HCV, IncRHOXF1 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 IncRNAs from the Cancer Genome Atlas database. IncRNAs 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, hNHEP 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% CO₂.

2.2. Identification of candidate IncRNAs from the TCGA database

The Cancer Genome Atlas (TCGA) database was searched to identify candidate IncRNAs. As a result, several IncRNAs with abnormal levels of expression were identified. Five of these IncRNAs, 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 IncRNAs were selected for further research (Figure 1).

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 IncRNAs from the TCGA database

The TCGA database was searched to identify candidate IncRNAs. As a result, several IncRNAs with abnormal levels of expression were identified. Five of these IncRNAs, 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 IncRNAs were selected for further research (Figure 1).
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 not infected with HBV or 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

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>PRC1-AS1</th>
<th>CRNDE</th>
<th>RP11-334E6.12</th>
<th>LINC00665</th>
<th>AC092171.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNHEPS</td>
<td>1.00000</td>
<td>1.00000</td>
<td>1.00000</td>
<td>1.00000</td>
<td>1.00000</td>
</tr>
<tr>
<td>L02</td>
<td>1.10022</td>
<td>1.024768</td>
<td>1.03541</td>
<td>0.923571</td>
<td>1.04776</td>
</tr>
<tr>
<td>BEL7402</td>
<td>4.486377</td>
<td>2.243313</td>
<td>2.72536</td>
<td>2.257758</td>
<td>1.971264</td>
</tr>
<tr>
<td>HLF</td>
<td>2.005248</td>
<td>3.922631</td>
<td>12.25951</td>
<td>2.496699</td>
<td>1.528039</td>
</tr>
<tr>
<td>HLE</td>
<td>2.663165</td>
<td>6.190015</td>
<td>4.084482</td>
<td>2.900079</td>
<td>2.565964</td>
</tr>
<tr>
<td>HepG2</td>
<td>2.10022</td>
<td>2.422593</td>
<td>9.781447</td>
<td>7.655874</td>
<td>2.639765</td>
</tr>
<tr>
<td>Huh-7</td>
<td>2.389504</td>
<td>2.326131</td>
<td>1.974695</td>
<td>1.532592</td>
<td>1.890742</td>
</tr>
<tr>
<td>SK-Hep-1</td>
<td>2.366716</td>
<td>1.351478</td>
<td>2.367365</td>
<td>2.316163</td>
<td>1.339115</td>
</tr>
<tr>
<td>HepG2.2.15*</td>
<td>6.918878</td>
<td>4.072771</td>
<td>14.71719</td>
<td>5.375211</td>
<td>1.716978</td>
</tr>
<tr>
<td>Huh-1*</td>
<td>4.242769</td>
<td>2.820624</td>
<td>10.59926</td>
<td>12.87392</td>
<td>3.794856</td>
</tr>
<tr>
<td>PLC/PRF/5*</td>
<td>2.700666</td>
<td>1.570577</td>
<td>3.211381</td>
<td>3.346897</td>
<td>2.299993</td>
</tr>
<tr>
<td>Hep3B*</td>
<td>6.204433</td>
<td>2.204943</td>
<td>2.686601</td>
<td>11.57074</td>
<td>2.91626</td>
</tr>
</tbody>
</table>

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

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

Acknowledgements

This study was funded by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (26462037).

References


(Received July 9, 2016; Revised July 30, 2016; Accepted August 1, 2016)