LncRNA UCHL1-AS1 prevents cell mobility of hepatocellular carcinoma: a study based on in vitro and bioinformatics

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Abstract: We set out to investigate biological functions and potential molecular mechanisms of long non-coding RNA (lncRNA) in hepatocellular carcinoma (HCC). HCC cell line Bel-7404 was cultured and transfected with antisense to the ubiquitin carboxyl-terminal hydrolase L1 (UCHL1-AS1). Viability and mobility were detected by MTT and wound healing assays. Additionally, enrichment analysis and functional networks of UCHL1-AS1 related genes in HCC were performed. Results showed that high level UCHL1-AS1 could effectively inhibit HCC cell migration. However, there was no significant correlation between overexpressed UCHL1-AS1 and HCC proliferation. Meanwhile, BMP4, CALM3, and HRAS were selected from 204 genes that related to UCHL1-AS1. All of these hub genes play critical roles in HCC occurrence and development. Thus, underlying molecular mechanisms among hub genes and UCHL1-AS1 in HCC might be valuable for prognosis and treatment.

Keywords: Long non-coding RNAs, UCHL1-AS1, hepatocellular carcinoma, mobility, bioinformatics

Introduction

As the most common liver cancer worldwide, hepatocellular carcinoma (HCC) ranks highly in overall cancer-related deaths [1, 2]. A variety of main factors causing HCC have been reported such as hepatitis B or C infections, alcohol abuse, and aflatoxin B1 [3]. Currently, surgical resection, liver transplantation, and chemotherapy are considered as available treatment strategies for HCC [4]. However, frequent metastasis and recurrence have induced a lower 5-year overall survival of HCC patients [5, 6]. Moreover, high resistance to chemotherapy has led to poor prognosis of HCC [7, 8]. Dysregulation of growth factors and receptors and signaling pathways could influence HCC’s development and prognosis [9]. Therefore, it is necessary to investigate potential molecular targeting mechanisms for enhancing treatment efficiency and prognostic conditions of HCCs.

Long non-coding RNAs (lncRNAs) are a novel group of non-coding RNAs > 200 nucleotides, which play pivotal roles in different processes of cell biology and pathology. LncRNAs have been broadly examined in tumors and some new functions of lncRNAs have updated our awareness of their roles in tumor incidence, development, and distinct therapeutic options. Deregulation of some lncRNAs in cancers has potential to act as a favorable marker for cancer surveillance, early diagnosis, and postoperative monitoring.

Some studies have determined that aberrant expression levels of certain lncRNAs have been observed in HCC, one of the most common cancers all over the world. Previously, expression levels of a spliced lncRNA antisense to the ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), namely, UCHL1-AS1, has been detected by our group to be clearly downregulated in HCC tissues. Moreover, we found that downregulation of UCHL1-AS1 was evidently related to progression of HCC, as reflected by its correlation with the status of portal vein tumor thrombus and distant metastasis [10]. However, the biological function of UCHL1-AS1 on HCC cells remains
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Expression level of UCHL1-AS1 in HCC from Gene Expression Profiling Interactive Analysis (GE-PIA). Transcripts Per Million (TPM) was calculated and expression level of a gene was presented by using Log₂ (TPM+1) Scale. Cutoff value was 1 for |Log₂ FC| and 0.05 for P-value. Due to the small sample size, no possible statistical analysis could be conducted.

Materials and methods

Expression levels of UCHL1-AS1 in HCC tissues from TCGA project

Expression levels of UCHL1-AS1 in HCC and non-HCC controls were achieved from Gene Expression Profiling Interactive Analysis (GE-PIA), which re-analyzed data from The Cancer Genomic Atlas (TCGA). Transcript Per Million (TPM) was calculated and expression level of a gene was presented by using Log₂ (TPM+1) Scale. Cutoff value was 1 for |Log₂ FC| and 0.05 for P-value. Meanwhile, expression levels of UCHL1-AS1 in 21 HCC cell lines and normal tissues were achieved from Cancer Cell Line Encyclopedia (CCLE) and three BioProjects: Illumina bodyMap2 transcriptome (https://www.ncbi.nlm.nih.gov/bioproject/PRJEB24-45/), total RNA sequencing from 20 human tissues [11], and HPA RNA-seq normal tissues [12].

Cell culture and transfection

HCC Bel-7404 cell line was achieved from Shanghai Institute of Cell Biology (Shanghai, China) and incubated in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Wisent, Nanjing, China) supplemented with 10% fetal bovine serum (Si-Ji-Qing, Hangzhou, China) in an atmosphere with 5% CO₂ at 37°C. Bel-7404 cells were stably transfected with Lentiviral Vector (LV) to establish the cell model of over-expressed UCHL1-AS1. The current study design included three groups: blank control (BC), negative control (NC), and experimental group (LV-UCHL1-AS1). Bel-7404 cells were plated on six-well plates and transfected using 8 μL Lipofectamine 2000 (Invitrogen, CA), following manufacturer instructions. The medium was replaced at 8-12 hours post transfection and culturing proceeded. Finally, cells were collected at 48 hours post-transfection for further research.

RNA isolation and RT-qPCR for UCHL1-AS1

Total RNA was extracted from cultured cells using TRizol Reagent (Invitrogen, USA). Reverse transcription of isolated RNA was performed using PrimeScript RT reagent kit (Takara, China), according to manufacturer instructions. RT-qPCR was performed by Light Cycler 480 (Roche, Switzerland) with a SYBR Green Master Mix (Roche, Switzerland). The primers were designed by our group and synthesized by Invitrogen as follows: UCHL1-AS1, the forward primer 5’-AGCGTGAGCAAGGAGAA-3’, the reverse primer 5’-GGAAGAGGGAAATCAGCAA-3’; internal controller β-actin, the forward primer 5’-GGACGGAGGGAAATCAGCAA-3’, the reverse primer 5’-GGACGAACGGGAAATCAGCAA-3’, the reverse primer 5’-GGACGGAGGGAAATCAGCAA-3’, the reverse primer 5’-GGACGGAGGGAAATCAGCAA-3’, the reverse primer 5’-GGACGGAGGGAAATCAGCAA-3’, the reverse primer 5’-GGACGGAGGGAAATCAGCAA-3’. The steps of PCR were set up as follows: an initial denaturation at 95°C for 15 minutes, denaturation step lasted 10 seconds 95°C, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Finally, UCHL1-AS1 levels were expressed by the 2⁻ΔΔCq comparative method [13-17].
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MTT assay

Viability of HCC cells overexpressing UCHL1-AS1 was detected by MTT assay. HCC cells in logarithmic phase were digested with enzymes and turned into a single cell liquid. We plated $4 \times 10^3$ cells per well in 96-well plates and cultured in an environment with 5% CO$_2$ at 37°C. Each well would be added 20 µl MTT (Sigma, USA) at 24 hours, 48 hours, 72 hours and 96 hours, respectively. After 4 hours of incubation of cells, medium was aspirated and 150 µl DMSO (Solarbio, Beijing, China) was added. Subsequent to cells shaking for 10 minutes, values of optical density (OD) for each sample were measured at 570 nm by
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A microplate reader. Three experimental groups were performed in triplicate and cell viability was achieved and calculated.

Scratch testing

Three groups of cells growing in logarithmic phase were cultured in 6-well cell plates. We drew a trace on the surface of cultured cells using a pipette tip when the density of cells reached 90%. After culturing in high glucose DMEM (Wisent, Nanjing, China) without serum for 24 hours, the healing area of scratch was observed and pictured at 0 hours, 24 hours, 48 hours, 72 hours and 96 hours. Image J was used for determination of scratch testing [18]. Scratching width among 3 groups was calculated using Student’s t-test and one-way Analysis of variance (ANOVA) by SPSS version 22.0 (Chicago, IL, USA).

Related gene collection and pathways annotation

Genes correlated to UCHL1-AS1 in HCC were collected from Tanric (http://ibl.mdanderson.org/tanric/_design/basic/index.html). The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) was used for Gene Oncology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) investigation. All significant pathways related to UCHL1-AS1 in HCC were presented by ImageGP (http://www.ehbio.com/ImageGP/) and integrated by the plugin of ClueGO from Cytoscape (v 3.5.1), which helped analyze prospective functions of cross-talk genes. Meanwhile, protein-protein interaction (PPI) of these UCHL1-AS1-related genes was outlined by STRING. Expression data of selected hub genes in HCC were downloaded from TCGA database (level 3).

Results

Expression levels of UCHL1-AS1 in HCC tissues from TCGA project

Previously, we reported downregulation of UCHL1-AS1 in HCC tissues detected by real time RT-qPCR with clinical samples from our
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Table 1. Width of wound healing assay in LV-UCHL1-AS1 group, blank control group, and negative control group

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>LV-UCHL1-AS1 (Mean ± SD)</th>
<th>Blank controls (Mean ± SD)</th>
<th>Negative controls (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00 ± 3.00</td>
<td>1.00 ± 3.05</td>
<td>1.00 ± 1.73</td>
</tr>
<tr>
<td>24</td>
<td>81.07 ± 1.22</td>
<td>68.90 ± 1.39</td>
<td>65.63 ± 0.75</td>
</tr>
<tr>
<td>48</td>
<td>80.27 ± 0.46</td>
<td>53.80 ± 1.20</td>
<td>56.53 ± 0.75</td>
</tr>
<tr>
<td>72</td>
<td>73.07 ± 0.46</td>
<td>45.80 ± 0.69</td>
<td>47.43 ± 1.99</td>
</tr>
<tr>
<td>96</td>
<td>72.00 ± 0.80</td>
<td>26.70 ± 0.69</td>
<td>30.00 ± 2.60</td>
</tr>
</tbody>
</table>

P value

Note: SD, standard deviation.

To achieve more information on expression levels of UCHL1-AS1 in HCC, we found that different expression levels could
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be found in various cell lines including 21 HCC cell lines from CCLE (Figure 3). But none of the three BioProjects revealed that UCHL1-AS1 mRNA could be detected in non-HCC liver tissues (data not shown). Thus, the expression status of UCHL1-AS1 in HCC and normal liver tissues still needs to be investigated with a larger sample size in a new independent cohort.

Transfection of UCHL1-AS1 into HCC Bel-7404 cells

Transfection efficiency was monitored via fluorescence observed by microscope (Figure 4A-F) and real time RT-qPCR (Figure 4G) 48 hours post transfection, which indicated a successful transfection of UCHL1-AS1 into HCC Bel-7404 cells.

Proliferation of overexpressed UCHL1-AS1 HCC cells

We further investigated the influence of UCHL1-AS1 on proliferation of HCC cells by MTT assay. Cell viability was intuitively revealed via cell growth rate curves (Figure 5). The results revealed that compared with the blank control and negative control groups, overexpressed UCHL1-AS1 insignificantly inhibited cell proliferation (P > 0.05).

Overexpression of UCHL1-AS1 inhibited HCC cell mobility

Scratch wound test was used for investigating effects of overexpressed UCHL1-AS1 on cell migration in HCC cases. The number of migrated cells in wound areas of BC and NC groups increased quickly from 24 hours to 96 hours.
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Additionally, compared with BC and NC groups separately, the width of UCHL1-AS1 group was significantly wider \((P < 0.01)\) (Table 1). These evidences manifested that overexpression UCHL1-AS1 could suppress migration of Bel-7404 HCC cells in vitro.

Enrichment analysis and potential hub genes of UCHL1-AS1

A total of 204 genes that related to UCHL1-AS1 in HCC were extracted. KEGG pathways and GO enrichment terms were analyzed, respectively.

Figure 8. Functional networks of UCHL1-AS1 related genes in HCC. A. Relations network of enriched pathways and genes by ClueGO; B. Protein-Protein Interaction (PPI) network.
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Table 2. The top 10 protein-protein interactions

<table>
<thead>
<tr>
<th>Node 1</th>
<th>Node 2</th>
<th>Coexpression</th>
<th>Experimentally determined interaction</th>
<th>Database annotated</th>
<th>Automated textmining</th>
<th>Combined score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSMB5</td>
<td>PSMD8</td>
<td>0.705</td>
<td>0.994</td>
<td>0.9</td>
<td>0.365</td>
<td>0.999</td>
</tr>
<tr>
<td>GNA1</td>
<td>GNB4</td>
<td>0.164</td>
<td>0.827</td>
<td>0.9</td>
<td>0.512</td>
<td>0.992</td>
</tr>
<tr>
<td>CXCL9</td>
<td>CCR1</td>
<td>0</td>
<td>0</td>
<td>0.9</td>
<td>0.626</td>
<td>0.961</td>
</tr>
<tr>
<td>CALM3</td>
<td>PTP3CC</td>
<td>0.056</td>
<td>0.904</td>
<td>0</td>
<td>0.587</td>
<td>0.959</td>
</tr>
<tr>
<td>HRAS</td>
<td>IQGAP1</td>
<td>0.053</td>
<td>0.178</td>
<td>0.9</td>
<td>0.546</td>
<td>0.959</td>
</tr>
<tr>
<td>HDAC3</td>
<td>RARA</td>
<td>0</td>
<td>0.367</td>
<td>0.9</td>
<td>0.358</td>
<td>0.955</td>
</tr>
<tr>
<td>GNA1</td>
<td>HRAS</td>
<td>0.105</td>
<td>0.173</td>
<td>0.9</td>
<td>0.456</td>
<td>0.954</td>
</tr>
<tr>
<td>HRAS</td>
<td>FGF19</td>
<td>0</td>
<td>0.097</td>
<td>0.9</td>
<td>0.358</td>
<td>0.936</td>
</tr>
<tr>
<td>CSF1</td>
<td>HRAS</td>
<td>0</td>
<td>0</td>
<td>0.9</td>
<td>0.357</td>
<td>0.933</td>
</tr>
<tr>
<td>IL12RB1</td>
<td>CXCL9</td>
<td>0</td>
<td>0</td>
<td>0.9</td>
<td>0.323</td>
<td>0.929</td>
</tr>
</tbody>
</table>

Figure 9. The 3 hub genes levels in HCC from TCGA database.

Discussion

RNA sequences that transcribe from the opposite direction at the same locus of DNA strands leads to natural antisense transcripts. Sense-antisense pairs might be formed from over 20% and is a usual manifestation in the human genome [19, 20]. However, a mass of endogenous RNA antisense transcripts has been found to be in a wide variety of eukaryotic organisms [21, 22]. Previously, studies have demonstrated that natural antisense transcripts carry out a role of regulator in gene levels by affecting mRNA transcription, translation, and processing [20, 23]. Dysfunction of natural antisense transcripts could be closely related with human diseases. Furthermore, as a type of endogenous cellular RNAs with no open reading frame, IncRNAs function both in nuclear architecture and gene expression modulation [24, 25]. Moreover, a similar structure exists between IncRNAs and mRNAs such as 5' capped, 3' polyadenylated, and multi-exonic [26]. Varying cellular locations of IncRNAs may reflect heterogenous functions and mechanisms in diseases.

Protein ubiquitin plays important roles in different biological processes including cell growth and cycling, embryonic development, tumorigenesis, and protein degradation [27, 28]. UCHL1 has been indicated as a functional ubiquitin balancer and has appeared as a tumor suppressor in HCC, probably via stabilizing p53.
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Table 3. Expression data of hub genes in HCC tissues and normal controls

<table>
<thead>
<tr>
<th>Hub gene symbol</th>
<th>HCC tissues</th>
<th>Normal tissues</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean value</td>
<td>SD</td>
<td>n</td>
</tr>
<tr>
<td>HRAS</td>
<td>10.0094</td>
<td>0.8335</td>
<td>374</td>
</tr>
<tr>
<td>BMP4</td>
<td>7.9313</td>
<td>1.8434</td>
<td>374</td>
</tr>
<tr>
<td>CALM3</td>
<td>13.1920</td>
<td>0.7412</td>
<td>374</td>
</tr>
</tbody>
</table>

Note: SD, standard deviation.

[29, 30]. UCHL1 is also beneficial to cell homeostasis of either normal growth or under oxidative stress inner-environments by accumulating p27 [31]. However, as a spliced antisense IncRNA to the UCHL1 gene, no related studies about IncRNA UCHL1-AS1 in HCC have been reported.

In our earlier study, decreased expression of UCHL1-AS1 in HCC cells was proven [10]. In this current study, proliferation and migration of overexpressed UCHL1-AS1 in HCC cells was further detected. We found that high levels of UCHL1-AS1 could effectively inhibit HCC cell mobility. This result implies that IncRNA UCHL1-AS1 might also play a similar inhibitory role in HCC as protein UCHL1. Interestingly, researchers have found antisense IncRNA to UCHL1 in mice could increase UCHL1 synthesis, which means that antisense IncRNAs functioned as a SINEUP, upregulating translation of partially intersected protein-coding mRNAs. Based on these, Aleks Schein et al. first pointed out that natural antisense IncRNAs can also enhance protein translation in human and SINEUPs may exist in a wide variety of mammalian species [32]. Thus, we speculate that IncRNA UCHL1-AS1 may work as a SINEUP to increase UCHL1 expression to further suppress HCC by p53, p27, or other factors.

Meanwhile, genes related to IncRNA UCHL1-AS1 significantly participated in cAMP signaling pathway and Ras signaling pathway. Gene transcription has been principally regulated by cAMP and dysregulated cAMP signaling, closely correlating to hepatic cysto-genesis and steatosis [33-35]. In addition, Ras proteins could adjust proliferation, apoptosis, differentiation, and senescence and aberrant Ras GTPase signal has been well known in tumorigenesis [36, 37]. In HCC, inhibitory effects of V-ATPase could restrain proliferation of tumor cells by hindering membrane related Ras signaling pathways [38].

Compared to normal liver tissues, all hub genes (HRAS, BMP4 and CALM3) were significantly higher in HCC tissues. As a member of Ras oncogene family, HRAS can bind GTP and GDP and they have inherent GTPase activity. GTPases have been identified as intracellular signaling molecules that play crucial roles in regulating cytoskeleton recombination and cell migratory ability [39]. GTPases are widespread in different oncogenes among various cancers and could induce metastasis, invasion, and poor prognosis [40, 41]. Importantly, the overexpressed hub gene BMP4 facilitates HCC cells proliferation, invasion, and metastasis via ID2 [42, 43]. HRAS and BMP4 might be valuable prognosis biomarkers and therapeutic targets for HCC patients. Protein CALM3 regulates cell cycle and cytokinesis, as a key role, and contains multiple alternative splicing transcriptions. All of these evidences hint that hub genes strongly mediate occurrence, progression, and prognosis of HCC. However, the specific molecular mechanisms among 3 hub genes and IncRNA UCHL1-AS1 still require future experimental verification.

Conclusion

Our results suggest that IncRNA UCHL1-AS1 could inhibit HCC cell migration. Moreover, enrichment analysis reveals that HRAS, BMP4, and CALM3 are hub genes of HCC and are closely correlated with HCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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References


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