Original Article

Long non-coding RNA SNHG1 promotes Cyclin D1-mediated proliferation in pancreatic cancer by acting as a ceRNA of miR-195

Dong Li1*, Xuming Zhang1*, Yuxi Yang1, Yi Shen2, Qibing Zhang1, Hong Liu1, Xiaofeng Li1, Chunqiu Zhang1, Pengyu Liu1, Xiangzheng Qin3

1Department of General Surgery, Daqing Oilfield General Hospital, Daqing City, Heilongjiang Province, China; 2Shanghai United International School, Shanghai, China; 3Department of Anatomy, Yanbian University Medical College, Yanji City, Jilin Province, China. *Equal contributors and co-first authors.

Received September 24, 2018; Accepted October 30, 2018; Epub March 1, 2019; Published March 15, 2019

Abstract: Pancreatic cancer (PCa) is one of the most fatal cancers worldwide. Recently, many studies have confirmed that long non-coding RNAs (lncRNAs) play crucial roles in the development of many human cancers, including PCa. The purpose of the present study was to investigate the biological role and underlying mechanisms of lncRNA small nucleolar RNA host gene 1 (SNHG1) in PCa progression. The results demonstrated that the expression levels of SNHG1 were increased in PCa cell lines and human tissue samples. High SNHG1 expression was notably correlated with adverse characteristics and poor survival of PCa patients. Knockdown of SNHG1 suppressed PCa cell proliferation in vitro and PCa tumor growth in vivo, and these effects might be associated with the induction of cell cycle arrest. We further confirmed that, in PCa cells, SNHG1 can negatively regulate miR-195 expression by acting as a ceRNA, and Cyclin D1 is a direct target of miR-195. Overexpression of miR-195 abrogated the oncogenic role of SNHG1 in PCa cells. Collectively, our results identified SNHG1 as a novel oncogenic lncRNA in PCa, and indicated that SNHG1/miR-195/Cyclin D1 axis might be a potential therapeutic target for PCa patients.

Keywords: Long non-coding RNA, SNHG1, pancreatic cancer, cell cycle, competing endogenous RNA, microRNA-195

Introduction

Pancreatic cancer (PCa) remains one of the most aggressive malignancies, with an extremely high mortality rate [1]. By 2030, PCa may become the second leading cause of cancer-related death in the United States [2]. In spite of recent advances in diagnostic and therapeutic methods, the median survival time of PCa patients is still only four months [3]. Accordingly, a better understanding of the molecular mechanisms underlying the development and progression of PCa is of critical importance for the identification of novel therapeutic targets for this fatal malignancy.

More than 90% of the total mammalian genome can be transcribed into non-coding RNAs [4]. Among them, long non-coding RNAs (lncRNAs) are defined as a set of RNA molecules with more than 200 nucleotides and little or no function of protein-coding capacity [5]. Over the years, lncRNAs have gained tremendous attention for their oncogenic or tumor suppressive roles in cancer progression [6]. Among them, small nucleolar RNA host gene 1 (SNHG1), located in the human genomic region of chromosome 11q12.3, was reported to be upregulated in hepatocellular carcinoma [7], non-small cell lung cancer [8], colorectal cancer [9] and many others. In the present study, we aimed to go deeper into SNHG1, to evaluate its functional role in PCa tumor biology, and the underlying mechanisms.

Materials and methods

Patients and tissue samples

PCa and corresponding adjacent non-tumorous pancreatic samples were collected from 76 PCa patients who had undergone surgery at
Daqing Oilfield General Hospital (Daqing City, China). The clinicopathologic characteristics of these patients are listed in Table 1. All patients did not receive any anti-cancer therapies prior to surgical operation. All collected tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until further use. The study was approved by the Ethics Committee of Daqing Oilfield General Hospital, and informed consent was obtained from all patients or their relatives.

Cell culture

The human PCa cell lines, including PANC-1, Capan-2, SW1990, BxPC-3, and normal human pancreatic ductal cell line HPDE6-C7 were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). These cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% penicillin/streptomycin in a humidified 5% CO₂ incubator at 37°C.

Table 1. Correlation between clinicopathologic parameters and SNHG1 expression levels in 76 PCa patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total number (n=76)</th>
<th>SNHG1 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low (n=41)</td>
<td>High (n=35)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.403</td>
<td>0.760</td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>33 16 17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>43 25 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>0.138</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>47 26 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>29 15 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor location in the pancreas</td>
<td>0.020</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>43 20 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body-tail</td>
<td>33 21 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>0.217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2</td>
<td>39 16 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2</td>
<td>37 25 12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well + Moderate</td>
<td>49 29 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>27 12 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.090</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II</td>
<td>45 30 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III+IV</td>
<td>31 11 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>42 19 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>34 22 12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The small interfering RNA (siRNA) targeting SNHG1 (si-SNHG1) and negative control siRNA (si-NC) were designed and synthesized by GenePharma (Shanghai, China). The sequence of SNHG1 was synthesized and subcloned into pcDNA3.1 vector (Invitrogen). Overexpression of SNHG1 was achieved by transfection with pcDNA3.1-NC and empty pcDNA3.1 vector was used as control. miR-195 mimics (miR-195) and negative mimics control (miR-NC) were purchased from RiboBio (Guangzhou, China). Cells were seeded into 6-well plates in a density of 1×10⁶ cells per well. When the cells reached 80-90% confluence, the plasmids and oligonucleotides were transfected into the cells using Lipofectamine 2000 (Invitrogen). At 48 h post transfection, cells were harvested for further use.

RNA extraction and RT-qPCR analysis

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen). An aliquot of 1 μg of total RNA was reverse-transcribed into complementary DNA (cDNA) using the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). Thereafter, qPCR analysis was performed using SYBR Green reagent (TaKaRa) on a 7500 Fast Real-Time Sequence detection system (Applied Biosystems, Foster City, CA, USA). Data were analyzed using 2⁻ΔΔCt method [10], and normalized to the expression of GAPDH or U6.

MTT assay

Cell proliferation was measured using 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium
SNHG1 promotes PCa progression

bromide (MTT) assay. Cells were cultured in 96-well plates at 5000 cells/well. At the indicated time points, 20 µl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) solution was added to each well and the plate was then incubated for additional 4 h. After that, culture solution was discarded and 150 µl DMSO (Sigma-Aldrich) was added to each well. The optical density (OD) was determined by measuring the absorbance at 490 nm on a microplate reader (Bio-Tek Company, Winooski, VT, USA).

Cell cycle analysis

For cell cycle analysis, the cells were harvested, washed three times in cold PBS, and then fixed in 70% ice-cold ethanol at 4°C overnight. Finally, the cells were stained with PI using the CycleTEST PLUS DNA Reagent Kit (BD Biosciences, Franklin Lakes, NJ, USA) and then subjected to a FACScan flow cytometer (BD Biosciences).

Western blot analysis

Total protein was extracted from tissues and cells using radioimmunoprecipitation-assay buffer (Beyotime, Shanghai, China), and the protein concentration was determined using a BCA Protein Assay Kit (Solarbio, Beijing, China). Equivalent amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% fat-free milk for 2 h at room temperature, and then incubated with specific primary antibodies at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescent detection kit (Pierce, Rockford, IL, USA), and quantified using Alphalmager 2200 image software (UVP, Upland, CA, USA). GAPDH was considered as the endogenous protein for normalization.

Dual-luciferase reporter assay

The fragment from SNHG1 or Cyclin D1 3'-UTR containing the predicted miR-195 binding site was amplified by PCR and cloned into the psiCHECK2 vector (Promega, Madison, WI, USA). The mutant constructs were generated using the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen). All constructs were verified by DNA sequencing. HEK293T cells were seeded into 24-well plates and co-transfected with the recombinant vectors and miR-195 mimics or mimics control using Lipofectamine 2000. After 48 h of incubation, the Renilla and firefly luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), and the ratio was calculated.

In vivo tumor formation assay

A total of 12 male nude mice (18-20 g, 5-6 weeks old), purchased from the SLAC Laboratory Animal Company (Shanghai, China), were housed under SPF condition. These mice were allocated into two groups (n=6/group), and 1×10^6 Capan-2 cells stably transfected with sh-SNHG1 or sh-NC were resuspended in 150 µl of culture medium and injected subcutaneously into the left flank of nude mice. The maximum (L) and minimum (W) length of tumors were measured every three days, and tumor volume was calculated using the following formula: volume = (L×W^2)/2. Twenty days after cell inoculation, the mice were euthanized by cervical dislocation, and the tumors were precisely excised and weighed. All experiments involving animals were by the Ethics Committee of Daqing Oilfield General Hospital.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) and SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA). The significance of differences between groups was estimated by Student's t-test or one-way analysis of variance. The association between SNHG1 expression and clinicopathological features of PCa patients was evaluated using the chi-square test. Survival curves were plotted using the Kaplan-Meier method and analyzed using the log-rank test. A P value<0.05 was considered significant.

Results

SNHG1 is upregulated in PCa tissues and cell lines

First, we found that the expression levels of SNHG1 in four PCa cell lines (PANC-1, Capan-2, SW1990, BxPC-3) were all dramatically higher
SNHG1 promotes PCa progression

than in normal human pancreatic ductal cell line HPDE6-C7 (Figure 1A). We then investigated the clinical significance of SNHG1 in a cohort of 76 PCa patients. As shown in Figure 1B, the average expression of SNHG1 was significantly higher in PCa tissues than in adjacent normal tissues. Subsequently, according to relative SNHG1 expression in tumor tissues, these PCa patients were classified into two groups: the high SNHG1 expression group (n=35; >median) and the low SNHG1 expression group (n=41; ≤median). As listed in Table 1, SNHG1 expression was notably correlated with tumor size (P=0.020) and TNM stage (P=0.007) of PCa patients. In addition, we also found that PCa patients in the high SNHG1 expression group presented a significant poorer overall survival compared to those in the low SNHG1 expression group (Figure 1C).

SNHG1 promotes PCa cell proliferation in vitro and PCa tumor growth in vivo

To further evaluate the functional role of SNHG1 in PCa cells, we transfected BxPC-3 cells who had lowest SNHG1 expression with pcDNA3.1-SNHG1 and transfected Capan-2 cells whose SNHG1 expression was highest with si-SNHG1. After transfection, the levels of SNHG1 in these two cells were validated by RT-qPCR analysis (Figure 2A). Through MTT assay, we observed that cell proliferation was significantly inhibited when SNHG1 was knocked down in Capan-2 cells (Figure 2B). In contrast, overexpression of SNHG1 could promote the proliferation of BxPC-3 cells. To further study the effects of SNHG1 on PCa tumor growth in vivo, Capan-2 cells stably transfected with sh-SNHG1 or sh-NC were inoculated into nude mice. As demonstrated in Figure 2C, the growth rate of tumors formed by Capan-2/sh-SNHG1 cells was significantly attenuated than that formed by Capan-2/sh-NC cells. Moreover, the average tumor weight at the end of the experiment was also markedly lower in the sh-SNHG1 group compared with the sh-NC group (Figure 2D).

SNHG1 promotes PCa cell cycle progression

We further determined whether the oncogenic role of SNHG1 in PCa cells is associated with the accelerated cell cycle progression. As demonstrated in Figure 3A, Capan-2 cells transfected with si-SNHG1 had an obvious cell cycle arrest in the G0/G1 phase, accompanied by the decrease of cells in S phase, whereas SNHG1 overexpression in BxPC-3 cells led to a significant decrease of cells in the G0/G1 phase and a remarkable accumulation of cells in the S phase. Moreover, western blot analysis showed that p21 expression was increased, whereas Cyclin D1 expression was decreased in Capan-2 cells after SNHG1 knockdown. In contrast, SNHG1 overexpression decreased p21 expression and increased Cyclin D1 expression in BxPC-3 cells (Figure 3B).

SNHG1 acts as a ceRNA to regulate miR-195 expression in PCa cells

We first searched for the potential miRNAs that can be regulated by SNHG1 through starBase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php), and we found that, miR-195, which has
SNHG1 promotes PCa progression

Figure 2. SNHG1 promotes PCa cell proliferation in vitro and PCa tumor growth in vivo. A. RT-qPCR analysis of SNHG1 expression levels in Capan-2 and BxPC-3 cells after transfection. B. The proliferation of Capan-2 and BxPC-3 cells after transfection was detected by MTT assay. C. The volume of PCa tumors was calculated every 3 days. D. 20 days after implantation, PCa tumors were excised and weighed. * P<0.05 vs. si-NC-transfected cells, # P<0.05 vs. empty vector (NC)-transfected cells, ^ P<0.05 vs. sh-NC group.
SNHG1 promotes PCa progression

been frequently regarded as a tumor suppressor, could bind to SNHG1 (Figure 4A). To further validate the direct binding relationship between SNHG1 and miR-195, dual-luciferase reporter assay was then conducted, and the results showed that co-transfection of miR-195 mim-
SNHG1 promotes PCa progression

SNHG1 acts as a ceRNA to regulate miR-195 expression in PCa cells. A. The binding sites of miR-195 on SNHG1. B. Dual-luciferase reporter assay was performed to validate the binding relationship between SNHG1 and miR-195. C. RT-qPCR analysis of miR-195 expression levels in Capan-2 and BxPC-3 cells after transfection. *P<0.05 vs. si-NC-transfected cells, #P<0.05 vs. empty vector (NC)-transfected cells, ^P<0.05 vs. miR-NC-transfected cells. D. RT-qPCR analysis of miR-195 expression levels in PCa and adjacent normal pancreas tissues. E. Association between SNHG1 and miR-195 expression in PCa tissues.

Figure 4. SNHG1 acts as a ceRNA to regulate miR-195 expression in PCa cells. A. The binding sites of miR-195 on SNHG1. B. Dual-luciferase reporter assay was performed to validate the binding relationship between SNHG1 and miR-195. C. RT-qPCR analysis of miR-195 expression levels in Capan-2 and BxPC-3 cells after transfection. *P<0.05 vs. si-NC-transfected cells, #P<0.05 vs. empty vector (NC)-transfected cells, ^P<0.05 vs. miR-NC-transfected cells. D. RT-qPCR analysis of miR-195 expression levels in PCa and adjacent normal pancreas tissues. E. Association between SNHG1 and miR-195 expression in PCa tissues.

ics and SNHG1-WT reporter significantly suppressed the luciferase activity in HEK293T cells (Figure 4B). Besides, SNHG1 overexpression decreased, whereas SNHG1 knockdown increased miR-195 expression in PCa cells (Figure 4C). We also confirmed that the expression of miR-195 was markedly decreased in PCa tissues (Figure 4D), and Pearson correlation analysis revealed that SNHG1 expression is inversely correlated with miR-195 expression in PCa tissues (Figure 4E).

Cyclin D1 is a direct target of miR-195 in PCa cells

Through TargetScan database (http://www.targetscan.org/vert_71/), we observed the potential miR-195 binding sites in the 3’-UTR of Cyclin D1 (Figure 5A), and co-transfection of miR-195 mimics could markedly reduce the luciferase activity of Cyclin D1-WT, but had no obvious effect on the luciferase activity of Cyclin D1-MUT in HEK293T cells (Figure 5B).

miR-195 abrogates the oncogenic role of SNHG1 in PCa cells

Next, rescue experiments were performed to investigate whether SNHG1 serves its oncogenic role in PCa cells partly through the miR-195/Cyclin D1 axis. As demonstrated in Figure 6A, 6B, co-transfection with miR-195 mimics reduced the mRNA and protein levels of Cyclin D1 in SNHG1 overexpressing BxPC-3 cells.
SNHG1 promotes PCa progression

Furthermore, the enhanced proliferation and cell cycle progression of SNHG1 overexpressing BxPC-3 cells were blocked by co-transfection with miR-195 mimics (Figure 6C, 6D).

Discussion

The molecular mechanisms underlying the development of PCa remain poorly understood. A number of recent papers have demonstrated the critical roles of IncRNAs in the pathologic process of human cancers. In PCa, many IncRNAs have been found as either oncogenic IncRNAs, including UCA1 [11] and ADPGK-AS1 [12], or tumor suppressive IncRNAs, such as MEG3 [13]. SNHG1 is a valuable cancer-related IncRNA [14], and in this research, the biological functions and underlying regulatory mechanisms of SNHG1 in PCa were studied.

In line with the findings of Zhang et al. [15], our team also identified that SNHG1 was significantly upregulated in clinical PCa tissues, and high SNHG1 expression was notably associated with adverse characteristics and unfavorable prognosis of PCa patients. To highlight the
SNHG1 promotes PCa progression

function of SNHG1 overexpression in PCa, we further investigated the biological role of SNHG1 in the regulation of PCa cell phenotypes through loss-of-function and gain-of-function experiments, and the results demonstrated that SNHG1 overexpression promoted, whereas SNHG1 knockdown inhibited PCa cell proliferation in vitro. It was additionally shown that SNHG1 knockdown inhibited PCa tumor growth in vivo. Cell cycle deregulation is also a common factor leading to the malignant cellular behaviors [16], and in this study, we also found that SNHG1 knockdown induced cell cycle arrest at the G0/G1 phase, thereby inhibiting the proliferation of PCa cells.

Non-coding RNAs are generally divided into miRNAs and lncRNAs. Recent studies have shown that lncRNAs can serve as competing endogenous RNA (ceRNA) of miRNAs to protect downstream mRNAs from repression [17]. miR-195 is extensively established as a tumor suppressor in various kinds of human cancers, including PCa [18], and through bioinformatics prediction and experimental analysis, we confirmed that SNHG1 could directly bind to miR-195 in PCa, and this binding relationship was also reported in HCC [7]. miRNAs exert their functions by negative regulation of their target genes [19], and among hundreds of potential target genes for miR-195, we selected Cyclin D1 for further investigation. Cyclin D1 is a well-known oncogene by inducing the transition from G0/G1 to S phase [20]. Here, our study verified that Cyclin D1 is a direct functional target of miR-195 in PCa, and more importantly, we speculated that, as a ceRNA of miR-195, SNHG1 can relieve the expression and function of Cyclin D1, thereby functioning as an oncogene in PCa.

In summary, the present study provided more evidence that SNHG1 was upregulated in PCa and promoted proliferation and cell cycle progression of PCa cells via acting as a ceRNA of miR-195. Our findings revealed that the SNHG1/miR-195/Cyclin D1 axis may be considered a promising biomarker and therapeutic target for PCa patients in the future.

Acknowledgements

The work of our research was supported by grant from National Natural Science Foundation of China (grant number: 81270563) and State Key Project specialized for HBV-related severe hepatitis of China (grant number: 2012ZX10-002004).

Disclosure of conflict of interest

None.

Address correspondence to: Xiangzheng Qin, Department of Anatomy, Yanbian University Medical College, No. 977 Park Road, Yanji 133002, Jilin Province, China. E-mail: qinxz888@163.com

References

[10] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time
SNHG1 promotes PCa progression


