Liver Research 3 (2019) 240–249

Contents lists available at ScienceDirect

Liver Research

journal homepage: http://www.keaipublishing.com/en/journals/liver-research

Original Article

LncRNA RP11-307C12.11 promotes the growth of hepatocellular carcinoma by acting as a molecular sponge of miR-138☆

Yinan Deng [a, b], Yusheng Cheng [a, b], Kaining Zeng [a], Haibo Li [a], Yiming Huang [a, b], Yiquan Jiang [a, b], Tingting Xia [c], Tong Zhang [a, b, *], Yang Yang [a, b, *]

[a] Department of Hepatic Surgery and Liver Transplantation Center, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China
[b] Guangdong Provincial Key Laboratory of Liver Disease Research, Guangzhou, China
[c] Center for Reproductive Medicine, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

ARTICLE INFO

Article history:
Received 13 November 2019
Received in revised form
15 November 2019
Accepted 17 November 2019

Keywords:
Long non-coding RNAs (lncRNAs)
LncRNA RP11-307C12.11
Hepatocellular carcinoma (HCC)
Growth
MicroRNA (MiR)-138

ABSTRACT

Background: Abnormal expression of long non-coding RNAs (lncRNAs) has been found in almost all tumors in humans, providing numerous potential diagnostic and prognostic biomarkers, and therapeutic targets.

Materials and methods: The Cancer Genome Atlas (TCGA) database was used to screen potential LncRNAs, and 30 paired hepatocellular carcinoma (HCC) tissues were used to investigate RP11-307C12.11 expression levels by qRT-PCR and another 105 HCC tissues by in situ hybridizsation (ISH). RP11-307C12.11 overexpression and knockdown experiments were performed to investigate the effects of RP11-307C12.11 on HCC growth through *in vitro* and *in vivo* assays (MTT assay, colony formation assay, EdU assay, and xenograft model). The molecular mechanism underlying these effects was confirmed by MS2-RIP-assay, RIP assay, luciferase assay, and rescue experiments.

Results: RP11-307C12.11 expression level was significantly higher in tumor tissues than in the adjacent normal tissues. Elevated RP11-307C12.11 expression level was associated with poor prognosis of HCC patients, and it may be represented as an independent prognostic biomarker in patients with HCC. Functionally, RP11-307C12.11 overexpression promoted HCC growth both *in vitro* and *in vivo*; however, its knockdown reversed these effects. Mechanistically, we found that RP11-307C12.11 expressed predominantly in the cytoplasm and sponged microRNA (miR)-138 to regulate its common target CCND1 and PDK1.

Conclusions: Thus, we found that RP11-307C12.11 acts as an oncogene in HCC by binding to miR-138, which might provide a novel target for HCC therapy.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies, with highest incidence rate worldwide.[1] More than 700,000 new patients are diagnosed with HCC each year.[2] HCC is also an extremely lethal cancer that has exhibited a steady rise in incidence, especially in the East Asia and South Africa.[3] Surgical resection or liver transplantation may provide an opportunity for a cure, but only when the patients are diagnosed at an early stage.[4] For advanced stage patients with HCC, sorafenib may offer only limited survival benefits.[5] Although the prevention, diagnosis, and intervention of HCC have been developed recently, the HCC therapy remains unsatisfactory due to poor prognosis and frequent recurrence.[4] Thus, clarifying the underlying mechanism of HCC growth and metastasis and identifying novel targets for effective intervention are urgently needed.

Long non-coding RNAs (lncRNAs) are a new class of non-protein-coding RNAs with more than 200 bp length and have been reported to regulate the gene expression at the transcriptional, posttranscriptional, and epigenetic levels.[6,7] The abnormal expression of lncRNAs has been found in almost all human tumors and plays a crucial role in multiple malignancies and congenital

☆ Edited by Peiling Zhu and Genshu Wang.

* Corresponding authors. Department of Hepatic Surgery and Liver Transplantation Center, The Third Affiliated Hospital of Sun Yat-sen University, Guangdong Provincial Key Laboratory of Liver Disease Research, Guangzhou, China.
E-mail addresses: zhjeff72@sina.com (T. Zhang), yysysu@163.com (Y. Yang).

https://doi.org/10.1016/j.livres.2019.11.003

diseases, which may provide novel insights and targets for tumor pathogenesis.[7,8] Thus, determining the functions and molecular mechanisms of dysregulated lncRNAs in HCC tumorigenesis and progression is urgently needed.

Here, we identified RP11-307C12.11 using TCGA database and validated that its expression level was significantly upregulated in HCC tissues. Its high expression level was associated with significantly shorter overall survival, and it may represent an independent prognostic biomarker in patients with HCC. We also elucidated its potential oncogenic role in HCC growth both *in vitro* and *in vivo*. Mechanistically, RP11-307C12.11 promoted HCC growth by acting as a sponge of miR-138 to regulate its common target CCND1 and PDK1.

2. Material and methods

2.1. Patients' samples

The samples from the patients clinically diagnosed with HCC were collected during surgical resection at the Third Affiliated Hospital of Sun Yat-sen University. No patients received anticancer treatment before operation. The patients with concurrent HIV, other cancer or autoimmune diseases were excluded. Paired tumor tissues and adjacent normal tissues from 30 patients between August 2017 and May 2018 were used for RT-PCR (Table 1). The tissues from 105 patients between August 2011 and August 2013 were used for In situ hybridization (ISH, Table 1). Written informed consent was signed by each patient, and the protocol was approved by the Review Board of the Third Affiliated Hospital of Sun Yat-sen University.

2.2. Cell culture and transfection

Human HCC cell lines and human normal liver cell lines were obtained from the Cell Bank of Type Culture Collection (Shanghai City, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco) at 37 °C humidified atmosphere with 5% CO_2. RP11-307C12.11 knockdown, RP11-307C12.11 overexpression, and negative control (NC) lentivirus vectors, harboring green fluorescent protein (GFP), were constructed according to the manufacturer's instructions (GeneChem, Shanghai, China). Cell transfection was performed according to the manufacturer's instructions, and stable cells were selected using puromycin. Seventy-two hours post-lentivirus transfection, successful knockdown and overexpression were confirmed by RT-PCR. miR-138 mimics and inhibitors were synthesized by GenePharma (Suzhou, China). The mimics and inhibitor were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Table 1
Characteristics of the study population.

Category	Subcategory	HCC ($N = 30$)[a]	HCC ($N = 105$)[b]
Age(years)	<55	17	63
	≥55	13	42
Gender	Male	21	92
	Female	9	23
HBV	(+)	30	88
	(-)	0	17
TNM stage	I	7	46
	II	11	23
	III	12	36
	IV	0	0
Tumor differentiation	I	3	21
	II	26	69
	III	1	15
Tumor multiplicity	Single	22	79
	Multiple	8	26
Tumor size(cm)	<5	17	45
	≥5	13	60
AFP(μg/L)	<20	10	37
	≥20	20	68

Abbreviations: AFP, alpha-fetoportein; TNM, tumor, node, metastases; HBV, hepatitis B virus.
[a] Paired tumor tissues and adjacent normal tissues from 30 patients between August 2017 and May 2018 were used for RT-PCR.
[b] The tissues from 105 patients between August 2011 and August 2013 were used for In situ hybridization (ISH).

2.3. RNA extraction and real-time PCR

Real-time PCR was performed to determine the expression of RP11-307C12.11 in 30 pairs of tumor tissues and adjacent normal tissues and 11 kinds of cell lines. RNA extraction and complementary deoxyribonucleic acid (cDNA) synthesis were performed according to the manufacturer's protocol. Real-time PCR primers and probes for RP11-307C12.11 (Table 2) were obtained from Applied Biosystems (Foster City, CA, USA). The amplification using the conditions of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, was carried out using ViiA 7 Real-time PCR System (Applied Biosystems). The relative expression of RP11-307C12.11 was normalized to that of 18S rRNA as an internal control. $2^{-\Delta\Delta Ct}$ was calculated and used as an indication of the relative expression levels.

2.4. In situ hybridization

The ISH probe used for detecting RP11-307C12.11-labeled digoxin was designed and synthesized by Exiqon (Shanghai, China). ISH was performed using the ISH Kit (Boster Bio-Engineering Company, Wuhan, China) and the stained tissue sections were reviewed and scored separately by two pathologists blinded to the clinical parameters. The score standard for the staining intensity was as follows: 0 (negative), 1 (weak), 2 (medium), and 3 (strong); and 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%) for the staining extent. For statistical analysis, a final staining score of 3 or higher was regarded as high expression.

2.5. Western blot analysis

Equal amounts of protein (30–60 μg) from each sample were separated by 10% SDS-PAGE and transferred to respective PVDF membranes (Millipore, Boston, MA). The membranes were incubated with primary antibodies against human CCND1, HIF1a, SOX4, GAPDH, phospho-AKT, and PDK1 (Cell Signaling Technology, Beverly, MA) overnight at 4 °C. Then, the membranes were washed for 30 min and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The membranes were washed again for 30 min, and then visualized by enhanced chemiluminescence (ECL; Millipore, Billerica, MA) and recorded on Kodak film.

2.6. MTT assay

MTT assay was used to analyze the effect of RP11-307C12.11 on HCC cell proliferation. After 0, 24, 48, 72, and 96 h of transfection, cell proliferation was analyzed by adding 20 μL of 5 mg/mL MTT solution to the culture, followed by incubation for an additional 4 h. The absorbance was measured at 450 nm by a microplate reader (Tecan infinite, Tecan Group Ltd, Switzerland).

Table 2
The list of primers and siRNA sequence.

Gene	Forward primer	Reverse primer
qRT-PCR primer		
RP11-307C12.11	ATCTTACAGGATGAATATCG	ACAGTGGATTAGGTGATT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
clone primer		
pLVX-RP11-307C12.11	GGTACCGCGGGCCCGGGATCCgccAACAAATGTCCACACA GTTAGGAAGCCC	ATGGTGGCGACCGGTGGATCCTTTCACACTTTACAGAGTTTGTTTAATG
siRNA for RP11-307C12.11	**siRNA sequence**	
001	5'AUGCAUCUUACAGGAUGAAUAUCdTdT3'	
002	5'UGCAUCUUACAGGAUGAAUAUCGdTdT3'	
003	5'CCCUACAUAAUGCUUCAGUUUGCdTdT3'	

Abbreviation: siRNA, small interfering RNA.

2.7. Colony formation

HCC cell lines were transfected with the indicated lentivirus, mimics, inhibitor, or siRNA for 48 h in 1×10^3 cells each, followed by incubation in a 6-well plate at 37 °C for 7 d. The medium was changed every two days. On day 7, the cells were fixed in 4% paraformaldehyde and dyed with a crystal violet solution. Cell colonies were then counted and assessed.

2.8. EdU assay

5-ethynyl-2′-deoxyuridine (EdU) assays were carried out using a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. Total 1×10^4 cells were cultured in a 96-well plate. After incubation with 50 μM EdU for 2 h, the cells were fixed in 4% paraformaldehyde and stained with Apollo Dye Solution. Hoechst-33,342 was used to stain the nucleic acids. Images were acquired using an Olympus IX73 microscope (Olympus, Tokyo, Japan), and the percentage of EdU-positive cells was calculated using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.9. Luciferase assay

For the RP11-307C12.11 promoter luciferase reporter assay, different fragment sequences containing predicted SP1 binding sites were synthesized and cloned into the pGL3-basic firefly luciferase reporter (GeneCreat, China). The pRL-TK vector was employed as a control. For the miRNA target gene luciferase reporter assay, target sequences containing the predicted miRNA binding sites (or containing mutations in the predicted miRNA binding sites) were synthesized and inserted into the psiCHECK-2 vector (Promega, USA). Luciferase activity was measured with a Dual Luciferase Assay System (Promega, USA).

2.10. RNA immunoprecipitation (RIP)

Cell lysates were incubated with A/G-plus agarose beads (Santa Cruz, Biotechnology, Inc. CA) together with the antibody or normal mouse or rabbit IgG for 4 h at 4 °C. Beads were subsequently washed and RNAs were then isolated. RT-PCR was performed according to the manufacturer's instructions.

2.11. Tumor xenografts in vivo

The animal studies were approved by the Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China. Male athymic BALB/c nude mice (4–5 weeks old) were used. Stably transfected HCC cells (1×10^6/0.2 ml PBS) were implanted into two sides of the same nude mouse in the armpit. Xenografts were examined every 4 days with digital calipers, and tumor volumes were calculated using the following equation: volume = 1/2(length×width^2). Twenty-three days later, the mice were sacrificed, and volumes of tumors were measured.

2.12. Statistical analysis

All statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL). Data were presented as the mean±standard deviation (SD) unless noted. Predictive factors for recurrence were examined using Cox regression analysis. Factors with a *P*-value <0.05 in univariate analysis were entered into the multivariate model. Significant independent risk factors for recurrence were used to construct a scoring system using the logistic regression model. Overall survival (OS) were compared using the Kaplan-Meier method. Independent samples *t*-test was used to analyze group differences in clinicopathological features. Statistical significance was set at $P < 0.05$.

3. Results

3.1. RP11-307C12.11 expression is upregulated in HCC tissues and high RP11-307C12.11 expression predicates poor prognosis

To analyze the expression pattern of lncRNA in HCC, we compared the profile of lncRNA between tumor tissue and the adjacent normal tissue samples using The Cancer Genome Atlas (TCGA) database. Compared with that in adjacent normal tissues, RP11-307C12.11 expression was remarkably upregulated in tumor tissues (Fig. 1A). Importantly, survival analyses revealed that HCC patients with high RP11-307C12.11 expression had shorter overall survival (OS) times than those with low RP11-307C12.11 expression (Fig. 1B). We further confirmed these results using the samples from the patients of our hospital. RP11-307C12.11 expression was significantly higher in tumor tissues than that in adjacent normal tissues (Fig. 1C). Moreover, we analyzed RP11-307C12.11 expression in human HCC cell lines. As shown in Fig. 1D, RP11-307C12.11 expression was upregulated in 8 HCC cell lines compared with that in the human liver cell line LO2. In addition, RP11-307C12.11 expression was significantly higher in patients with advanced stage HCC than those with early stage (Fig. 1E). Chi-square test showed that the levels of RP11-307C12.11 significantly correlated with Tumor number ($P = 0.020$) (Table 3). Survival analyses revealed that HCC patients with high RP11-307C12.11 expression had shorter overall survival (OS) times than those with low RP11-307C12.11 expression (Fig. 1F). Univariate analysis or Multivariate analysis showed that RP11-307C12.11 level of tumor tissues was an independent prognostic factor of OS (Table 4).

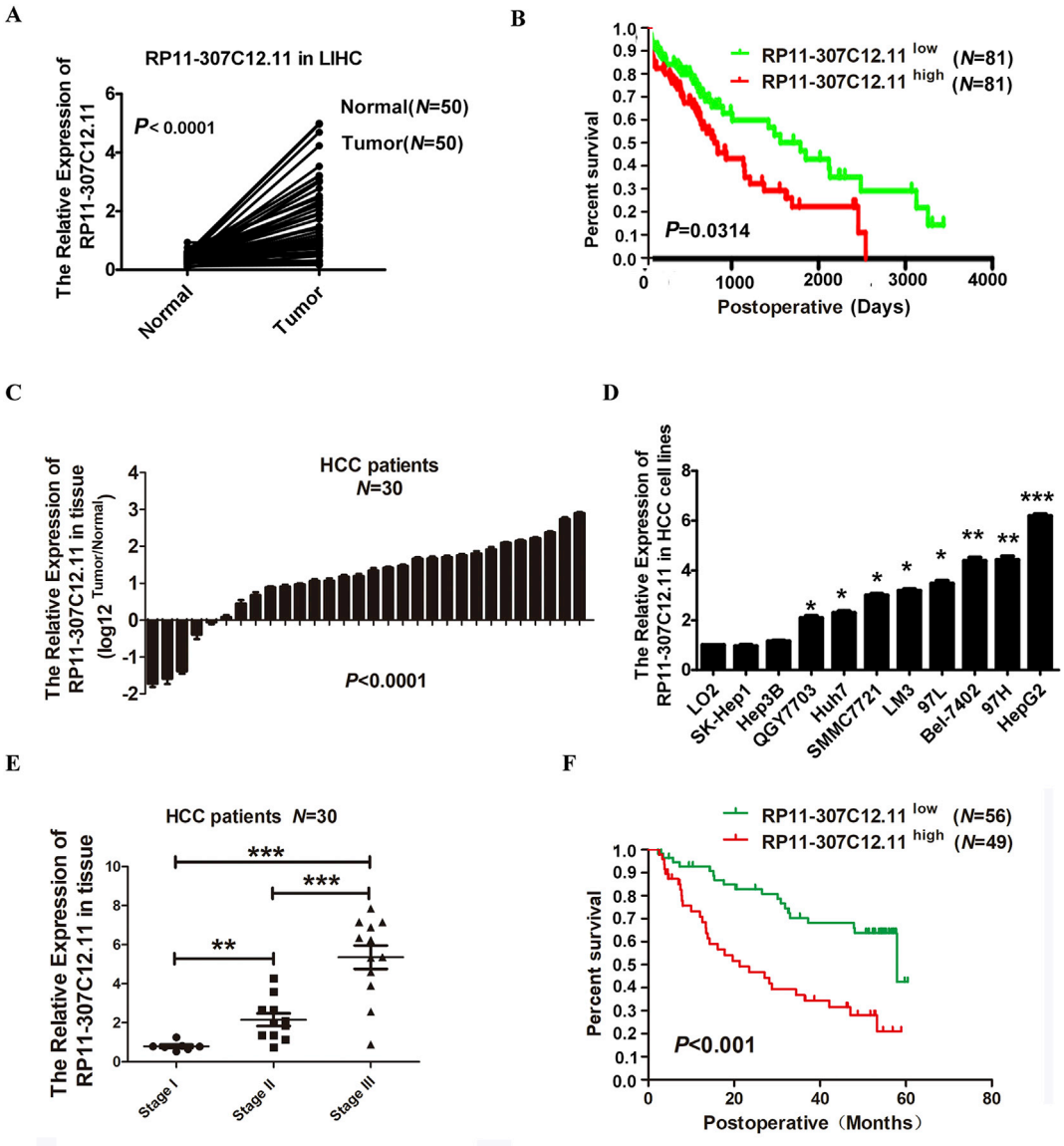

Fig. 1. RP11-307C12.11 functions as an independent prognostic factor for OS in HCC patients. (A) RP11-307C12.11 expression was significantly higher in HCC tissues than in normal adjacent tissues collected from 50 patients from the TCGA database. **(B)** Kaplan-Meier analysis of the association of RP11-307C12.11 expression levels with the OS of HCC patients in TCGA database. **(C)** RP11-307C12.11 expression levels were higher in HCC tissues than in normal tissues. **(D)** RP11-307C12.11 expression was significantly higher in HCC cell lines than in normal liver cell lines. **(E)** RP11-307C12.11 expression levels were higher in advanced stage HCC patients than in early-stage HCC patients. **(F)** Kaplan-Meier analysis of the association of RP11-307C12.11 expression with the OS of HCC patients of our hospital. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3.2. P11-307C12.11 functions as a potential oncogenic lncRNA by promoting the proliferation of HCC cells

As shown in Fig. 1D, Huh7 and QGY7703 cells showed a low expression level of RP11-307C12.11 in all liver cancer cell lines. Thus, we chose Huh7 and QGY7703 cells for RP11-307C12.11 overexpression. We constructed RP11-307C12.11-overexpressing lentiviruses with RP11-307C12.11 sequences and confirmed by RT-PCR that RP11-307C12.11 was significantly overexpressed in Huh7 and QGY7703 cells (Fig. 2A). The roles of RP11-307C12.11 overexpression on cell proliferation were determined by MTT, EdU, and clone formation assays. MTT assays confirmed that RP11-307C12.11 overexpression significantly accelerated the proliferation of Huh7 and QGY7703 cells (Fig. 2B). Similarly, clone formation assays showed that the colony-forming ability of Huh7 and QGY7703 cells was enhanced upon RP11-307C12.11 overexpression (Fig. 2C). EdU incorporation assays also showed that RP11-307C12.11 overexpression promoted Huh7 and QGY7703 cell proliferation (Fig. 2D).

To further confirm the potential oncogenic functions of RP11-307C12.11, we used RP11-307C12.11-knockdown lentiviruses that targeted the back-splice sequence of RP11-307C12.11 in Bel-7402 cells, and the efficiency was confirmed by RT-PCR (Fig. 3A). MTT assays showed that cell proliferation was suppressed upon RP11-307C12.11 silencing (Fig. 3B). Clone formation assays confirmed that RP11-307C12.11 knockdown inhibited the colony-forming ability of Bel-7402 cells (Fig. 3C). EdU incorporation assays revealed that cell proliferation was suppressed upon RP11-307C12.11 knockdown (Fig. 3D).

3.3. RP11-307C12.11 accelerates the HCC growth in vivo

To further explore whether RP11-307C12.11 influences tumor growth and metastasis *in vivo*, we constructed RP11-307C12.11

Table 3
Correlation between RP11-307C12.11 expression and clinicopathological characteristics in HCC patients.

Features	*N*	RP11-307C12.11 expression		*P*-value
		Low	High	
Total number(*N*)	105	57	48	
Age(years)				0.937
>55	42	23	19	
≤55	63	34	29	
Gender				0.534
Male	92	51	41	
Female	13	6	7	
Liver cirrhosis				0.800
with	67	37	30	
without	38	20	18	
HBsAg				
with	88	49	39	0.518
without	17	8	9	
AFP(μg/L)				
>20	68	36	32	0.711
≤20	37	21	16	
Tumor size (cm)				0.727
>5	60	32	28	
≤5	45	25	20	
Tumor number				**0.020**
solitary	79	48	31	
multiple	26	9	17	
Edmondson grade				0.051
I+II	83	41	42	
III	22	16	6	
TNM stage				0.113
I+II	57	35	22	
III+IV	48	26	22	

Abbreviations: HBsAg, hepatitis B surface antigen; AFP, alpha-fetoportein; TNM, tumor, node, metastases.

knockdown or negative control (NC) transfected stable HCC cells that were subcutaneously or intravenously injected into nude mice. RP11-307C12.11 knockdown significantly suppressed tumor growth (Figs. 4A–C) in nude mice.

3.4. RP11-307C12.11 is a cytoplasm lncRNA that sponges miR-138 to upregulate its common target

Subcellular fractionation assays indicated that RP11-307C12.11 was predominately located in the cytoplasm (Fig. 5A), suggesting that RP11-307C12.11 might play a role in the sponge effect of miRNA. To explore this possibility, we predicted the potential binding sites of miRNA on RP11-307C12.11 using miRcode (http://www.mircode.org/) and StarBase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php), and the results revealed that the binding site of miR-138 existed in RP11-307C12.11 (Fig. 5B). The role of RP11-307C12.11 on miR-138 sponging was determined by luciferase assay, MS2-RIP-assay, and RIP assay. The results confirmed that RP11-307C12.11 could bind to miR-138 (Figs. 5C–E). Since the proliferation genes CCND1 and PDK1 have been reported as the targets of miR-138, we analyzed the relation between those genes and RP11-307C12.11. Luciferase assay revealed that RP11-307C12.11 could upregulate the expression of CCND1 and PDK1 (Fig. 5F). The results were further confirmed by western blot analysis (Fig. 5G).

3.5. RP11-307C12.11 promotes HCC cell proliferation by binding to miR-138

MTT assays confirmed that RP11-307C12.11 overexpression significantly accelerated the proliferation of Huh7 and QGY7703

Table 4
Univariate and multivariate analysis of overall survival in HCC patients cox regression analysis.

Variables	*N*	Univariated analysis			Multivariate analysis		
		HR	95%*CI*	*P*-value	*HR*	95%*CI*	*P*-value
Age(years)		1.139	0.649–1.999	0.650			
>55	42						
≤55	63						
Gender		0.648	0.257–1.635	0.330			
Male	92						
Female	13						
Liver cirrhosis		0.663	0.374–1.175	0.159			
with	67						
without	38						
HBsAg		0.655	0.327–1.315	0.234			
with	88						
without	17						
AFP(μg/L)		1.334	0.736–2.419	0.342			
>20	68						
≤20	37						
Tumor size (cm)		1.879	1.057–3.34	**0.032**	1.63	0.76–3.493	0.209
>5	60						
≤5	45						
Tumor number		1.761	0.912–3.401	0.092			
solitary	79						
multiple	26						
Edmondson grade		1.315	0.639–2.707	0.457			
I+II	83						
III	22						
TNM stage		2.237	1.276–3.923	**0.005**	1.518	0.724–3.184	**0.269**
I+II	57						
III+IV	48						
RP11-307C12.11		3.137	1.759–5.594	**<0.001**	3.217	1.776–5.829	**<0.001**
Low	57						
High	48						

Abbreviations: HBsAg, hepatitis B surface antigen; AFP, alpha-fetoportein; TNM, tumor, node, metastases; *HR*, hazard ratio; *CI*, confidence interval.

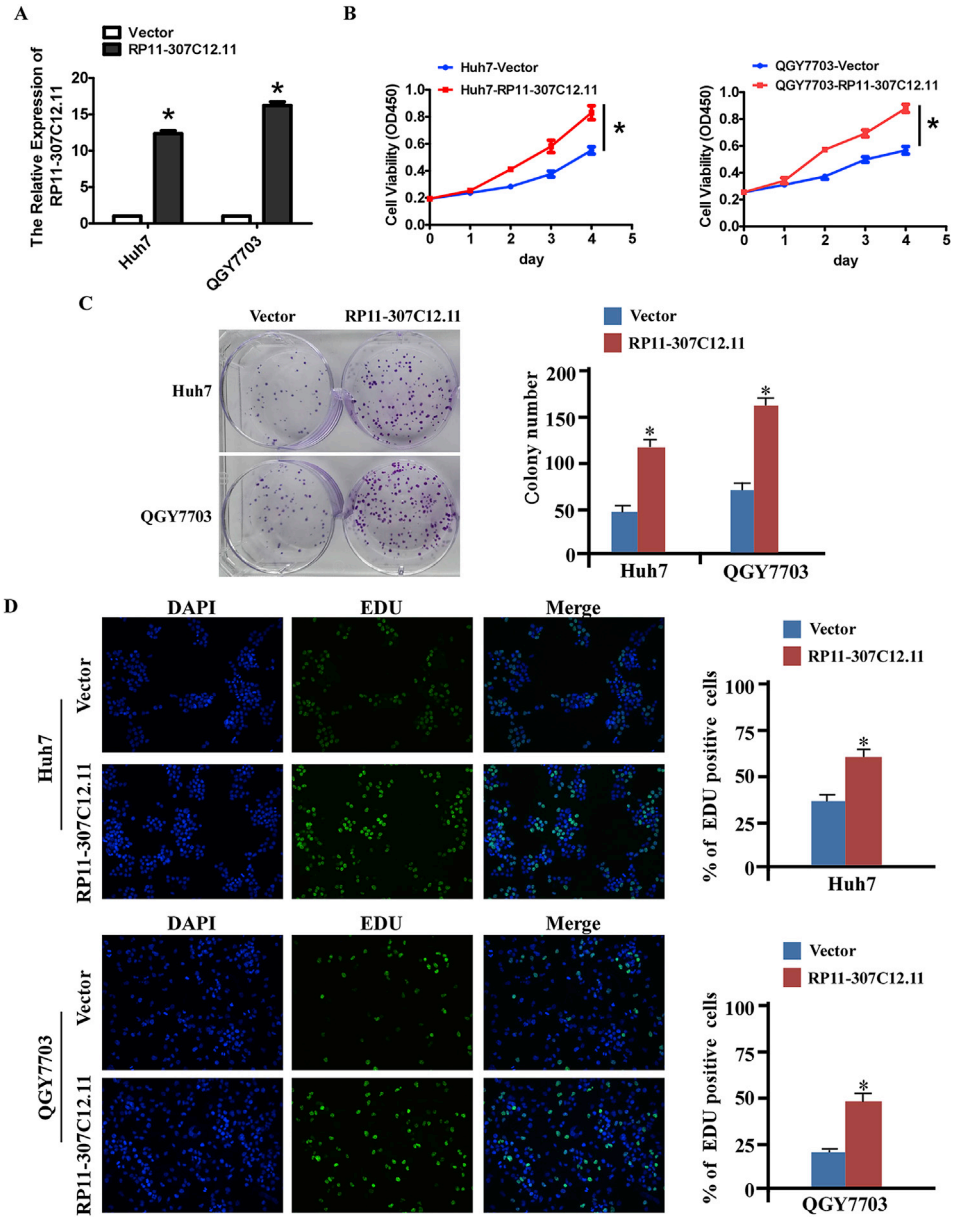

Fig. 2. RP11-307C12.11 overexpression promotes HCC cell proliferation. (A) Overexpression of RP11-307C12.11 in Huh7 and QGY7703 was confirmed by RT-PCR. **(B)** MTT assays showed that overexpression of RP11-307C12.11 promoted the proliferation of Huh7 cells (left panel) and QGY7703 cells (right panel). **(C)** Clone formation assays showed that overexpression of RP11-307C12.11 promoted the proliferation of Huh7 cells and QGY7703 cells. **(D)** EdU assays showed that overexpression of RP11-307C12.11 promoted the proliferation of Huh7 cells (upper panel) and QGY7703 cells (down panel). $^{*}P < 0.05$ and $^{**}P < 0.01$.

cells (Figs. 6A and 6B). However, the effects of cell proliferation upregulation by RP11-307C12.11 were ablated when the cells were co-transfected with miR-138 mimics (Figs. 6A and 6B). MTT assays showed that the cell proliferation was suppressed upon RP11-307C12.11 knockdown and these effects could be impaired by the co-transfected miR-138 inhibitors (Fig. 6C). EdU incorporation assay revealed that RP11-307C12.11 overexpression significantly promoted Huh7 and QGY7703 cell proliferation. However, the effects of cell proliferation upregulation by RP11-307C12.11 were ablated when the cells were co-transfected with miR-138 mimics (Figs. 6D and 6E). Moreover, EdU incorporation assay revealed that RP11-307C12.11 knockdown significantly inhibited Bel-7402 cell proliferation and these effects could be impaired by the co-transfected miR-138 inhibitors (Figs. 6F and 6G).

4. Discussion

In the present study, we identified RP11-307C12.11 using TCGA database, and validated that its RP11-307C12.11 expression level was significantly upregulated in HCC tissues. Its high expression level was associated with significantly shorter overall survival, and it may represent an independent prognostic biomarker in patients with HCC. LncRNAs with abnormal expression were correlated with cancer prognosis, providing numerous potential prognostic biomarkers.[9–11] For example, MIR22HG was significantly downregulated in gastric cancer, thyroid cancer, lung cancer, and hepatocellular carcinoma, and low expression of MIR22HG predicted tumor progression and poor prognosis of patients with HCC.[12–15] CDKN2B-AS1 was highly expressed in HCC which

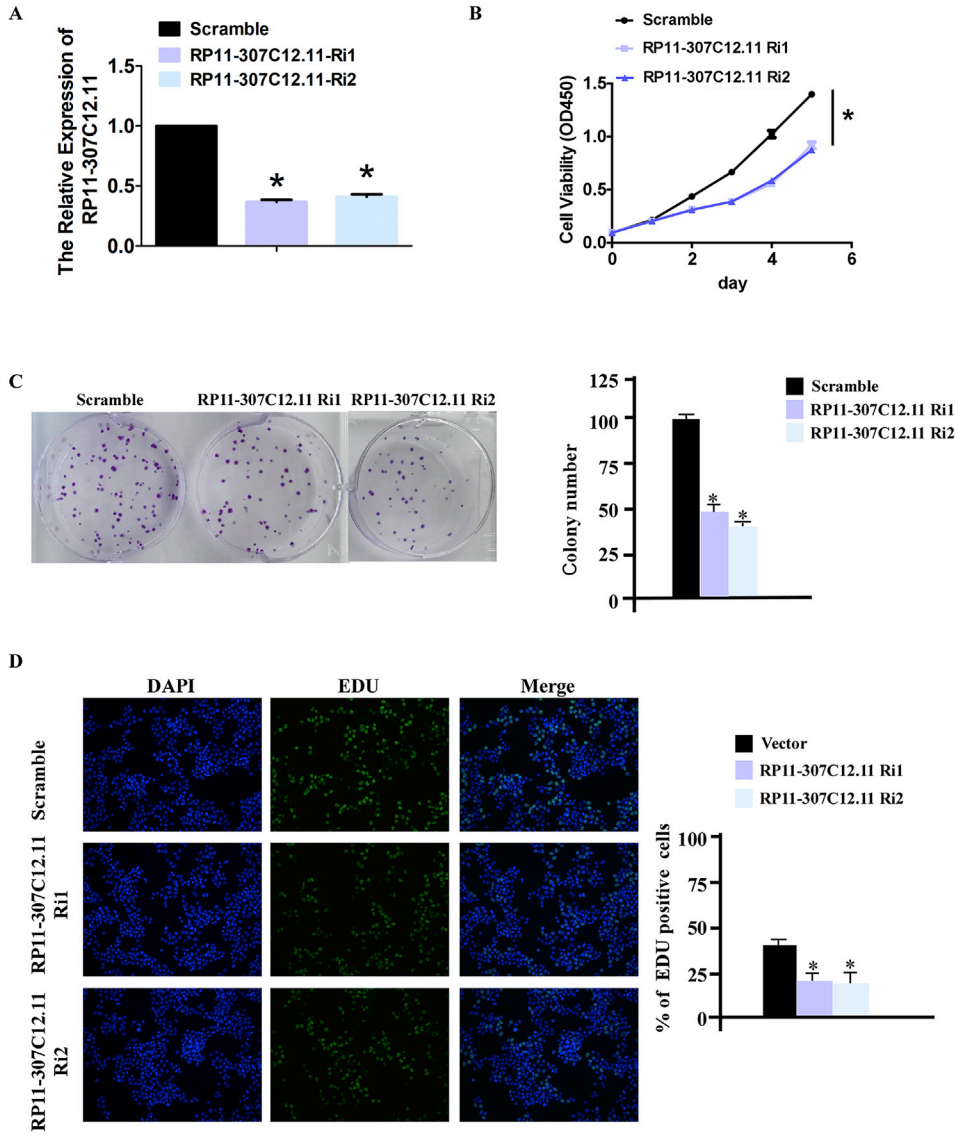

Fig. 3. RP11-307C12.11 knockdown inhibits the HCC cell proliferation. (A) Knockdown of RP11-307C12.11 in Bel-7402 was confirmed by RT-PCR. **(B)** MTT assays showed that knockdown of RP11-307C12.11 inhibited the proliferation of Bel-7402 cells. **(C)** Clone formation assays showed that knockdown of RP11-307C12.11 inhibited the proliferation of Bel-7402 cells. **(D)** EdU assays showed that knockdown of RP11-307C12.11 promoted the proliferation of Bel-7402 cells. $^{*}P < 0.05$ and $^{**}P < 0.01$.

predicted poor prognosis of HCC patients.[16] HOXD-AS1 was significantly upregulated in several cancers, which predicted poor prognosis of patients with HCC.[17–20] LINC01138, PTTG3P, NEAT1 and miR503HG were associated with the malignant features and poor outcomes of HCC patients.[21–24] These results indicate that lncRNAs have considerable prognostic potential in HCC.

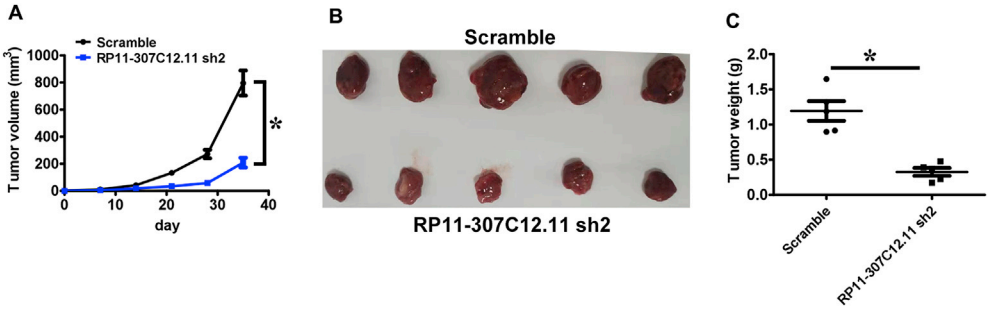

Fig. 4. RP11-307C12.11 promotes HCC growth *in vivo*. (A) Tumor volumes in nude mice were calculated in Bel-7402 cells transfected with the RP11-307C12.11 knockdown vector. **(B)** Images of the tumors removed once the mice were killed. **(C)** Tumor weights in nude mice were calculated in Bel-7402 cells transfected with the RP11-307C12.11 knockdown vector. $^{*}P < 0.05$ and $^{**}P < 0.01$.

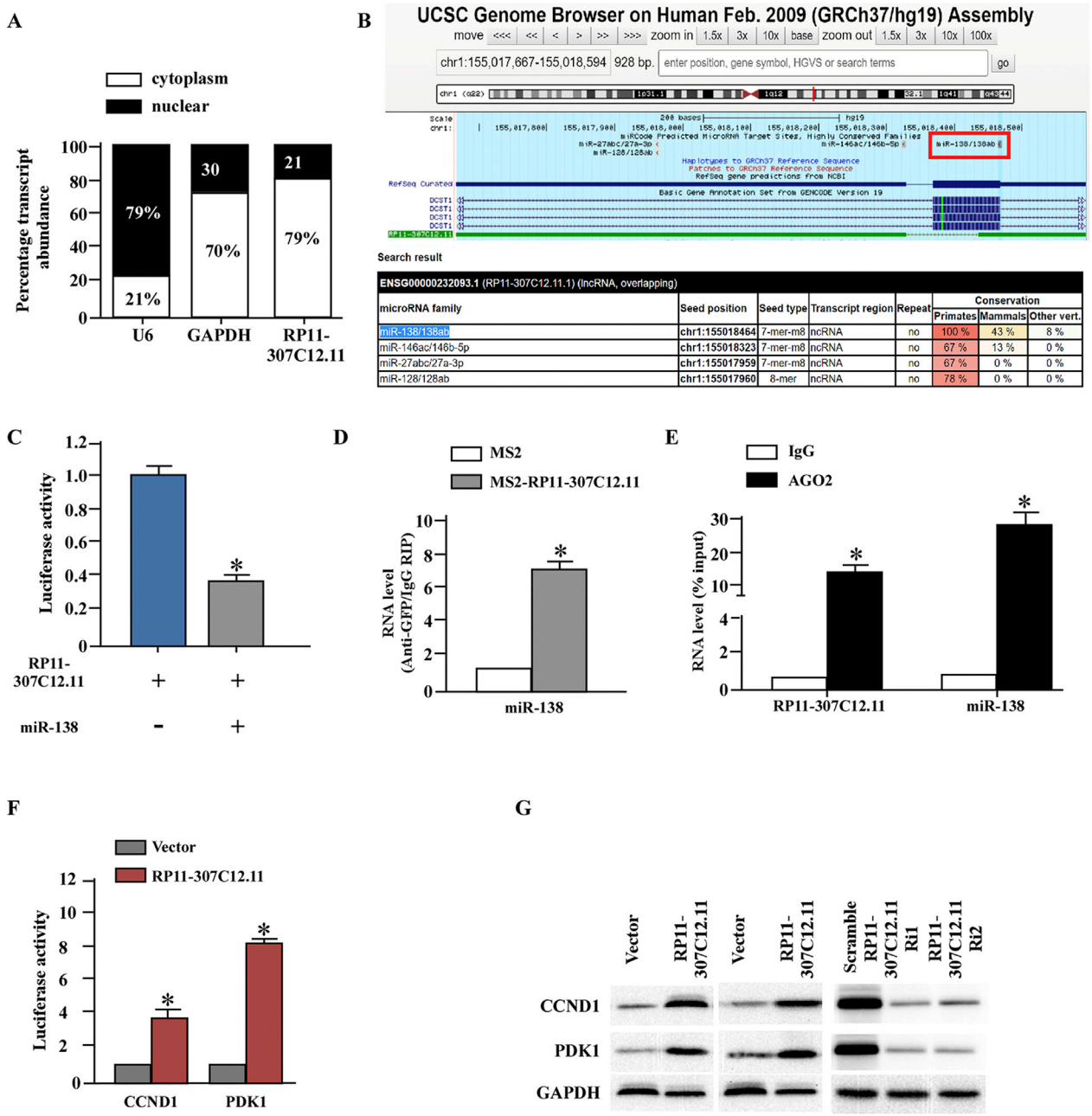

Fig. 5. RP11-307C12.11 is a cytoplasm lncRNA that sponges miR-138 and up-regulates its common target. **(A)** Subcellular fractionation assays indicated that RP11-307C12.11 was predominately located in the cytoplasm. **(B)** miRcode and StarBase v2.0 predicted that miR-138 was bound to RP11-307C12.11. **(C)** Luciferase assay showed that RP11-307C12.11 directly binds to miR-138. **(D)** MS2-RIP-assay showed that RP11-307C12.11 directly binds to miR-138. **(E)** RIP assay showed that RP11-307C12.11 directly binds to miR-138. **(F)** Luciferase assay showed that RP11-307C12.11 regulated the target of miR-138. **(G)** Western blot assay showed that RP11-307C12.11 regulated the target of miR-138. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Emerging evidence indicates that lncRNAs can participate in numerous biological processes and play important roles in the genesis and development of diseases.[25,26] Dysregulation of lncRNAs is commonly investigated in digestive system malignancies including HCC.[16,19,24] In this study, we have confirmed that RP11-307C12.11 expression was remarkably upregulated in HCC tissues. Functionally, RP11-307C12.11 can promote the proliferation of HCC cells *in vitro* and contribute to the growth of HCC *in vivo*. To our knowledge, this is the first study to report that RP11-307C12.11 promoted the tumor growth and metastasis of HCC.

Accumulating evidence confirmed that cytoplasmic lncRNAs mainly act as ceRNAs or molecular sponges to competitively inhibit several miRNAs, and then participate in the carcinogenesis.[27,28] It has been reported that LINC01939 function as ceRNA regulated EGR2 by competitively binding to miR-17-5p in gastric cancer.[29] LncRNA PTAR promoted EMT and invasion-metastasis in serous ovarian cancer by acting as a molecular sponge of miR-101-3p to regulate ZEB1 expression.[30] Our results showed that the majority of RP11-307C12.11 are located in cytoplasm. Thus, we speculated that RP11-307C12.11 might be a ceRNA required for HCC growth and metastasis. We predicted the potential binding sites of miRNA on RP11-307C12.11 using miRcode and StarBase v2.0, and the results revealed that RP11-307C12.11 could directly bind to miR-138, which has been reported to be a tumor suppressor in various

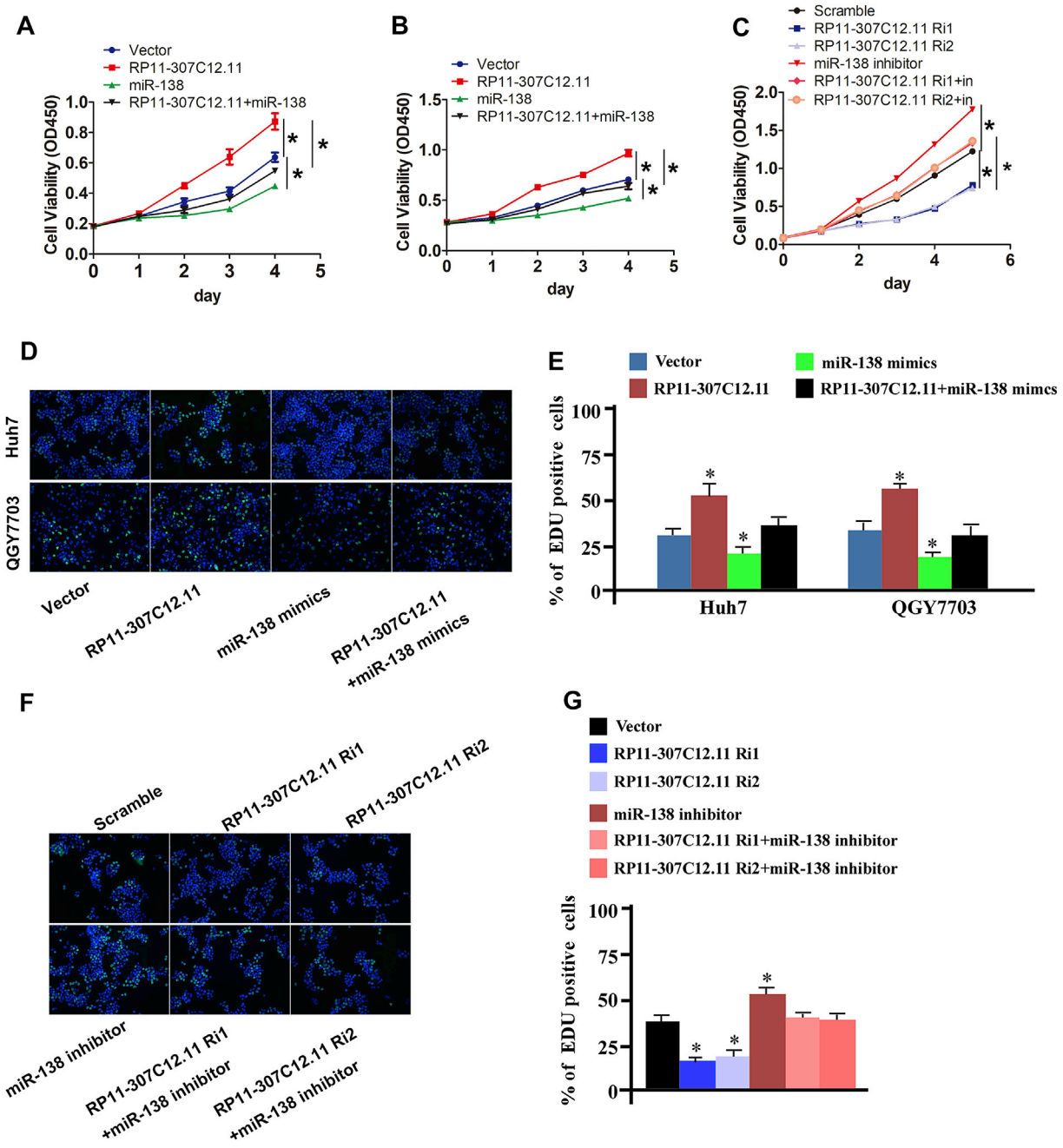

Fig. 6. RP11-307C12.11 promotes HCC cell proliferation by binding to miR-138. **(A)** and **(B)** MTT assays of Huh7 and QGY7703 cells transfected with the RP11-307C12.11 overexpression vector, miR-138 mimics, or the RP11-307C12.11 overexpression vector + miR-138 mimics. **(C)** MTT assays of Bel-7402 cells transfected with the RP11-307C12.11 knockdown vector, miR-138 inhibitor, or the RP11-307C12.11 knockdown vector + miR-138 inhibitor. **(D)** and **(E)** EdU assays of Huh7 and QGY7703 cells transfected with the RP11-307C12.11 overexpression vector, miR-138 mimics, or the RP11-307C12.11 overexpression vector + miR-138 mimics. **(F)** and **(G)** EdU assays of Bel-7402 cells transfected with the RP11-307C12.11 knockdown vector, miR-138 inhibitor, or the RP11-307C12.11 knockdown vector + miR-138 inhibitor. $^{*}P < 0.05$ and $^{**}P < 0.01$.

cancer.[31–33] Luciferase reporter assay, MS2-RIP-assay and RIP assay confirmed that RP11-307C12.11 directly binds to miR-138. Overexpression of RP11-307C12.11 promotes HCC cell proliferation. This effect was reversed by co-transfection with the miR-138 mimics, indicating that RP11-307C12.11 promotes HCC cell proliferation by competitively binding to miR-138.

It has been shown that miR-138 suppressed nasopharyngeal carcinoma growth and tumorigenesis by targeting the oncogene CCND1 and PDK1.[34,35] miRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1α.[36] In the present study, we observed that RP11-307C12.11 upregulates the expression of miR-138 target CCND1 and PDK1, suggesting that RP11-307C12.11 promotes HCC growth by acting as a molecular sponge of miR-138 to regulate CCND1 and PDK1 expression.

In conclusion, RP11-307C12.11 functions as a ceRNA that competitively binds to miR-138, then upregulates CCND1 and PDK1, and then promotes HCC growth. These findings provide a new mechanism for understanding HCC metastasis and RP11-307C12.11 may be a potential candidate in the prevention and treatment of HCC.

5. Conclusions

Here, we identified that RP11-307C12.11 expression level was significantly upregulated in HCC tissues, its high expression level was associated with significantly short overall survival, and it may represent an independent prognostic biomarker in patients with HCC. We also elucidated its potential oncogenic role in HCC growth both *in vitro* and *in vivo*. Mechanistically, RP11-307C12.11 promoted HCC growth through a sponge of miR-138 to regulate its common target CCND1 and PDK1. RP11-307C12.11 is a prognostic marker for HCC patients, and acted as an oncogene in HCC by targeting the miR-138, which might provide a novel target for HCC therapy.

Authors' contributions

Y. Deng and Y. Cheng contributed to the conception and design of the study, acquisition of the data, and analysis and interpretation of the data. Y. Deng participated in critical drafting and revising of the article for important intellectual content. K. Zeng, H. Li, Y. Huang, Y. Jiang, and T. Xia contributed substantially to the conception and design of the study and acquisition of the data. T. Zhang and Y. Yang contributed to the conception and design of the study and provided final approval of the version to be submitted and any revised versions.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

Our original work described in this review article was supported by: the National Natural Science Foundation of China (81702393, 81770648, 81670601, 81570593); Key Scientific and Technological Projects of Guangdong Province (2015B020226004, 2017A020215178); Guangdong Natural Science Foundation (2017A030310373, 2015A030312013); Science and Technology Planning Project of Guangdong Province (2017B030314027, 2017B020209004, 2015B020226004); Science and Technology Planning Project of Guangzhou (2014Y2–00544); Guangzhou Science and Technology Huimin Special Project (2014Y2–00200); and Sun Yat-sen University Young Teacher Training Project (17ykpy47).

References

1. Laursen L. A preventable cancer. *Nature*. 2014;516:S2–S3.
2. Maluccio M, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin*. 2012;62:394–399.
3. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394–424.
4. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet*. 2012;379:1245–1255.
5. Worns MA, Galle PR. HCC therapies–lessons learned. *Nat Rev Gastroenterol Hepatol*. 2014;11:447–452.
6. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet*. 2009;10:155–159.
7. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. *Cell*. 2009;136:629–641.
8. Fatica A, Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. *Nat Rev Genet*. 2014;15:7–21.
9. Yarmishyn AA, Kurochkin IV. Long noncoding RNAs: a potential novel class of cancer biomarkers. *Front Genet*. 2015;6:145.
10. Huo X, Han S, Wu G, et al. Dysregulated long noncoding RNAs (lncRNAs) in hepatocellular carcinoma: implications for tumorigenesis, disease progression, and liver cancer stem cells. *Mol Cancer*. 2017;16:165.
11. Yao J, Wu L, Meng X, et al. Profiling, clinicopathological correlation and functional validation of specific long non-coding RNAs for hepatocellular carcinoma. *Mol Cancer*. 2017;16:164.
12. Li H, Wang Y. Long noncoding RNA (lncRNA) mIR22HG suppresses gastric cancer progression through attenuating NOTCH2 signaling. *Med Sci Monit*. 2019;25:656–665.
13. Qin L, Luo JZ, Tang XL, et al. Identification of long noncoding RNA mIR22HG as a novel biomarker in thyroid cancer. *Pathol Oncol Res*. 2018. https://doi.org/10.1007/s12253-018-0521-6.
14. Su W, Feng S, Chen X, et al. Silencing of long noncoding RNA mIR22HG triggers cell survival/death signaling via oncogenes YBX1, MET, and p21 in lung cancer. *Cancer Res*. 2018;78:3207–3219.
15. Zhang DY, Zou XJ, Cao CH, et al. Identification and functional characterization of long non-coding RNA MIR22HG as a tumor suppressor for hepatocellular carcinoma. *Theranostics*. 2018;8:3751–3765.
16. Huang Y, Xiang B, Liu Y, et al. LncRNA CDKN2B-AS1 promotes tumor growth and metastasis of human hepatocellular carcinoma by targeting let-7c-5p/NAP1L1 axis. *Cancer Lett*. 2018;437:56–66.
17. Chen Y, Zhao F, Cui D, et al. HOXD-AS1/miR-130a sponge regulates glioma development by targeting E2F8. *Int J Cancer*. 2018;142:2313–2322.
18. Wang H, Huo X, Yang XR, et al. STAT3-mediated upregulation of lncRNA HOXD-AS1 as a ceRNA facilitates liver cancer metastasis by regulating SOX4. *Mol Cancer*. 2017;16:136.
19. Lu S, Zhou J, Sun Y, et al. The noncoding RNA HOXD-AS1 is a critical regulator of the metastasis and apoptosis phenotype in human hepatocellular carcinoma. *Mol Cancer*. 2017;16:125.
20. Gu P, Chen X, Xie R, et al. lncRNA HOXD-AS1 regulates proliferation and chemo-resistance of castration-resistant prostate cancer via recruiting WDR5. *Mol Ther*. 2017;25:1959–1973.
21. Li Z, Zhang J, Liu X, et al. The LINC01138 drives malignancies via activating arginine methyltransferase 5 in hepatocellular carcinoma. *Nat Commun*. 2018;9:1572.
22. Liu X, Liang Y, Song R, et al. Long non-coding RNA NEAT1-modulated abnormal lipolysis via ATGL drives hepatocellular carcinoma proliferation. *Mol Cancer*. 2018;17:90.
23. Wang H, Liang L, Dong Q, et al. Long noncoding RNA miR503HG, a prognostic indicator, inhibits tumor metastasis by regulating the HNRNPA2B1/NF-kappaB pathway in hepatocellular carcinoma. *Theranostics*. 2018;8:2814–2829.
24. Huang JL, Cao SW, Ou QS, et al. The long non-coding RNA PTTG3P promotes cell growth and metastasis via up-regulating PTTG1 and activating PI3K/AKT signaling in hepatocellular carcinoma. *Mol Cancer*. 2018;17:93.
25. Bergmann JH, Spector DL. Long non-coding RNAs: modulators of nuclear structure and function. *Curr Opin Cell Biol*. 2014;26:10–18.
26. Ernst C, Morton CC. Identification and function of long non-coding RNA. *Front Cell Neurosci*. 2013;7:168.
27. Zhan Y, Chen Z, Li Y, et al. Long non-coding RNA DANCR promotes malignant phenotypes of bladder cancer cells by modulating the miR-149/MSI2 axis as a ceRNA. *J Exp Clin Cancer Res*. 2018;37:273.
28. Liu B, Wu S, Ma J, et al. lncRNA GAS5 reverses EMT and tumor stem cell-mediated gemcitabine resistance and metastasis by targeting miR-221/SOCS3 in pancreatic cancer. *Mol Ther Nucleic Acids*. 2018;13:472–482.
29. Chen M, Fan L, Zhang SM, et al. LINC01939 inhibits the metastasis of gastric cancer by acting as a molecular sponge of miR-17-5p to regulate EGR2 expression. *Cell Death Dis*. 2019;10:70.
30. Liang H, Yu T, Han Y, et al. LncRNA PTAR promotes EMT and invasion-metastasis in serous ovarian cancer by competitively binding miR-101-3p to regulate ZEB1 expression. *Mol Cancer*. 2018;17:119.
31. Tsukamoto S, Lovendorf MB, Park J, et al. Inhibition of microRNA-138 enhances bone formation in multiple myeloma bone marrow niche. *Leukemia*. 2018;32:1739–1750.
32. Hong Z, Chen KH, DasGupta A, et al. MicroRNA-138 and microRNA-25 down-regulate mitochondrial calcium uniporter, causing the pulmonary arterial hypertension cancer phenotype. *Am J Respir Crit Care Med*. 2017;195:515–529.
33. Wei J, Nduom EK, Kong LY, et al. MiR-138 exerts anti-glioma efficacy by targeting immune checkpoints. *Neuro Oncol*. 2016;18:639–648.
34. Liu X, Lv XB, Wang XP, et al. MiR-138 suppressed nasopharyngeal carcinoma growth and tumorigenesis by targeting the CCND1 oncogene. *Cell Cycle*. 2012;11:2495–2506.
35. Wang Y, Zhang Z, Wang H, et al. miR-138-1* regulates aflatoxin B1-induced malignant transformation of BEAS-2B cells by targeting PDK1. *Arch Toxicol*. 2016;90:1239–1249.
36. Yeh YM, Chuang CM, Chao KC, et al. MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1alpha. *Int J Cancer*. 2013;133:867–878.