Long noncoding RNA PURPL promotes cell proliferation in liver cancer by regulating p53

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Abstract. Emerging evidence suggests that long noncoding RNAs (lncRNAs) serve a key role in malignant transformation, tumor progression and metastasis. Increased expression of lncRNA p53 upregulated regulator of P53 levels (PURPL) has been reported to promote tumorigenicity in colorectal cancer; however, the role and potential mechanisms of PURPL in the development of liver cancer remain unclear. We employed reverse transcription-quantitative polymerase chain reaction to detect PURPL and p53 mRNA expression in liver cancer tissues and cell lines. Cell Counting Kit-8 and colony-forming assays were used to examine the cell proliferation; whereas, flow cytometry was applied to detect apoptosis and cell cycle progression. p53 expression was detected by western blotting. The results revealed that PURPL was significantly upregulated in liver cancer tissues compared with in paracancerous tissues, and was associated with tumor differentiation stage and tumor size. PURPL was also upregulated in various liver cancer cell lines. Silencing of PURPL inhibited liver cancer cells proliferation, blocked cell cycle progression, and promoted apoptosis. Most importantly, PURPL expression was negatively correlated with p53 mRNA expression. In summary, lncRNA-PURPL was proposed to promote cell proliferation in liver cancer by regulating the p53 gene. As such, it could serve as a potential therapeutic target for the diagnosis and treatment of liver cancer.

Introduction

Liver cancer is the sixth most common cancer and the third most common cause of cancer-associated mortality worldwide (1,2). Liver resection remains the best therapeutic strategy to treat liver cancer, with a 5-year survival rate of ~30%. Chronic infection with hepatitis B or hepatitis C virus is the most common cause of liver cancer (3); however, the mechanism underlying the development of the disease remain unclear. Advancements in molecular technology have led to a focus on the molecular mechanism of liver cancer progression, and the identification of potential clinically relevant factors which may benefit the diagnosis and treatment of liver cancer.

Long non-coding RNAs (lncRNAs) are long-chain RNA molecules of >200 nucleotides in length that do not encode for proteins; thus, they have been proposed to be ineffective (4). Numerous studies have reported that lncRNAs are closely related to the multi-level regulation of gene expression, particularly the post-transcriptional regulation of the interaction with microRNAs (miRNAs), mRNAs, or proteins (5-8). Recent evidence suggests that lncRNAs are involved in several pathological processes driving cancer, including proliferation, apoptosis, metastasis and metabolism (9-13). In particular, certain lncRNAs have been reported to be critical in the progression of liver cancer. LncRNA p53 upregulated regulator of P53 levels (lncRNA-PURPL), an intergenic lncRNA, has been identified by RNA sequencing in a variety of colorectal cancer cell lines. It has been observed to promote the occurrence of colorectal cancer by preventing the binding of p53 to the small p53-stabilizing protein Myb-binding protein 1A (MYBBP1A), lowering p53 levels (14). However, to the best of our knowledge, no investigations into the role and expression of PURPL in liver cancer have been conducted.

The present study reported that PURPL is significantly upregulated in liver cancer tissues and cell lines. We also demonstrated that PURPL could promote liver cancer cell proliferation and cell cycle progression, while inhibiting apoptosis via regulation of the p53 gene. These findings may provide novel insight into the role of PURPL in the progression of liver cancer.

Materials and methods

Tissue collection and cell culture. Primary tumor tissue and corresponding adjacent tissue were excised from 71 patients (60 males and 11 female; age range: 30-80-years-old) at The First Affiliated Hospital of China Medical University between December 2016 and May 2018. The isolated tissue specimens were placed in a cryotube within 30 min and

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immediately frozen in a -80˚C freezer. All cancerous tissues were pathologically diagnosed as liver cancer. Patients did not receive preoperative radiotherapy or chemotherapy. All individuals provided informed consent and the present study was approved by the Institutional Ethics Committee of China Medical University. Patients were divided into high- and low-PURPL expression groups based on the median value of expression.

A total of five liver cancer cell lines, HepG2, Huh7, HCCLM3, SK-hep1, and PLC/PRF/5, as well as the normal liver cell line, L02, were used in this study. HepG2 cells were obtained from the Chinese Academy of Medical Sciences (Beijing, China). Huh7, SK-hep1, PLC/PRF/5, HCCLM3 and L02 human cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China). SK-hep1 and L02 cells were cultured in RPMI 1640 medium (HyClone; GE Healthcare, Logan, UT, USA). HepG2, Huh7, PLC/PRF/5 and HCCLM3 cells were cultured in Dulbecco's Modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Media were supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 U/ml streptomycin (HyClone; GE Healthcare). All cells were grown at 37˚C in a humidified incubator containing 5% CO_2.

Small interfering RNA (siRNA) and transfection. A total of three siRNAs for the silencing of PURPL and a negative control siRNA (si-NC) were designed and synthesized by Shanghai GenePharma Co., Ltd. (Suzhou, China). Cell lines cultured in six-well plates were transfected with 20 μM siRNAs dissolved in ddH_2O using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and mixed with siRNA-PURPL or si-NC according to the manufacturer's protocols. After 48 h, cells were collected for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting and other experiments. The sequences of the siRNAs were as follows: 1#PURPLsiRNA, 5'-CCACAUACAGGGUUCUUAATT UUAAGAACCCUGUAUGUGGTT-3'; 2#PURPLsiRNA, 5'-GGAAUCGAUCUGUGAGCAUTTAUGCUCACAGAU CGAUUCCTT-3'; 3#PURPLsiRNA, 5'-GGCCUACGUGAA UAAUAAUTTAUUAUUAUUCACGUAGGCCTT-3'; and si-NC sense, 5'-UUCUCCGAACGUGUCACG-3' and si-NC antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Among them, 3#PURPLsiRNA with the strongest silencing efficiency, was used in subsequent experiments.

RNA extraction and RT-qPCR. Total RNA was extracted from clinical patient tissue samples and cultured cells using Total RNA Extractor (Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using the GoScript™ Reverse Transcription Mix and Random Primers (Promega Corporation, Madison, WI, USA) according to the the manufacturer's instructions. The cDNA was then amplified by qPCR using the GoTaqR qPCR Master Mix (Promega Corporation) according to the manufacturer's instructions on an ABI PRISMR 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) instrument using a two-step standard PCR amplification procedure. The first step (pre-denaturation) included a cycle of 2 min at 95˚C; the second step (PCR reaction) included 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. Finally, the relative expression of PURPL and p53 mRNA in tissue and cell samples was determined by the $2^{-\Delta\Delta Cq}$ method (15). GAPDH was used as a control. The nucleotide sequences of the primers for qPCR were as follows: PURPL forward, 5'-TTCTACCGC AATTCGATGGAGTCTTG-3', reverse, 5'-GAGGCAGGA GAATGGCGTGAAC-3'; p53 forward, 5'-ACCGGCGCACAG AGGAAGAG-3', reverse, 5'-GCCTCATTCAGCTCTCGG AACATC-3'; and GAPDH forward, 5'-CGGAGTTGTTCG TATTCGG-3' and reverse, 5'-TACTAGCCGATGATGGC ATT-3'.

Western blotting. Transfected cells and frozen liver tissues, including liver cancer and corresponding adjacent tissues, were subjected to western blotting to evaluate p53 protein content. Cells and tissue samples were lysed for 30 min in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) on ice, the resulting lysates were centrifuged at 10,000 x g for 15 min at 4˚C, and the protein concentration was measured using a Bicinchoninic Acid kit (FDbio Science, Hangzhou, China). Protein samples (30 μg) were then separated by conventional electrophoresis at 70 V, transferred under constant pressure, blocked in 5% skim milk, and finally incubated with anti-p53 (cat. no. 60283-l-lg, 1:1,000) or anti-GAPDH (cat. no. 60004-l-lg, 1 mg/ml), ProteinTech Group, Chicago, IL, USA) primary antibodies at 4˚C overnight. The following day, membranes were washed with PBS, incubated with a secondary antibody (Goat anti-mouse IgG, horseradish peroxidase, cat. no. ZB-2305, 1:10,000, ZSGB-BIO) for 1 h at room temperature, and proteins were visualized by chemiluminescence using an ECL kit and an ECL western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.). Densitometry analysis was performed using ImageJ software version 1.8.0 (National Institutes of Health, Bethesda, MD, USA).

Cell proliferation assays. Transfected HepG2 and Huh7 cells were seeded in 96-well microtiter plates at a density of $5x10^3$ cells/well. At 24, 48, 72 and 96 h post-transfection, 10 μl Cell Counting Kit-8 (CCK-8) solution (Beyotime Institute of Biotechnology) was added to each well and the plates were incubated in a 37˚C incubator for 1.5 h. Then, the absorbance per well was measured at 450 nm using an automatic microplate reader (BGI, Hong Kong, China).

For the colony-forming assay, each group of cells was seeded on a six-well plate at $1x10^3$ cells/well and incubated for 10 days at 37˚C in 5% CO_2. Then, the cells were washed with PBS, fixed with paraformaldehyde for 15 min then stained with 0.5% crystal violet at room temperature and colonies were then counted.

Cell cycle analysis. To investigate the effects of PURPL silencing on cell cycle progression, we analyzed the proportion of cells in G1, S, and G2 phase using a BD FACSCanto™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). We routinely detached cells with trypsin (0.25%) for 5 min at 37˚C, centrifuged at 850 x g at 4˚C for 5 min, removed the supernatant, resuspended the cells in pre-cooled PBS, and centrifuged them again. The supernatant was aspirated, and the cells were fixed at 4˚C by adding 1 ml of pre-cooled 70% ethanol and subsequent incubation for 12 h. The next day, following

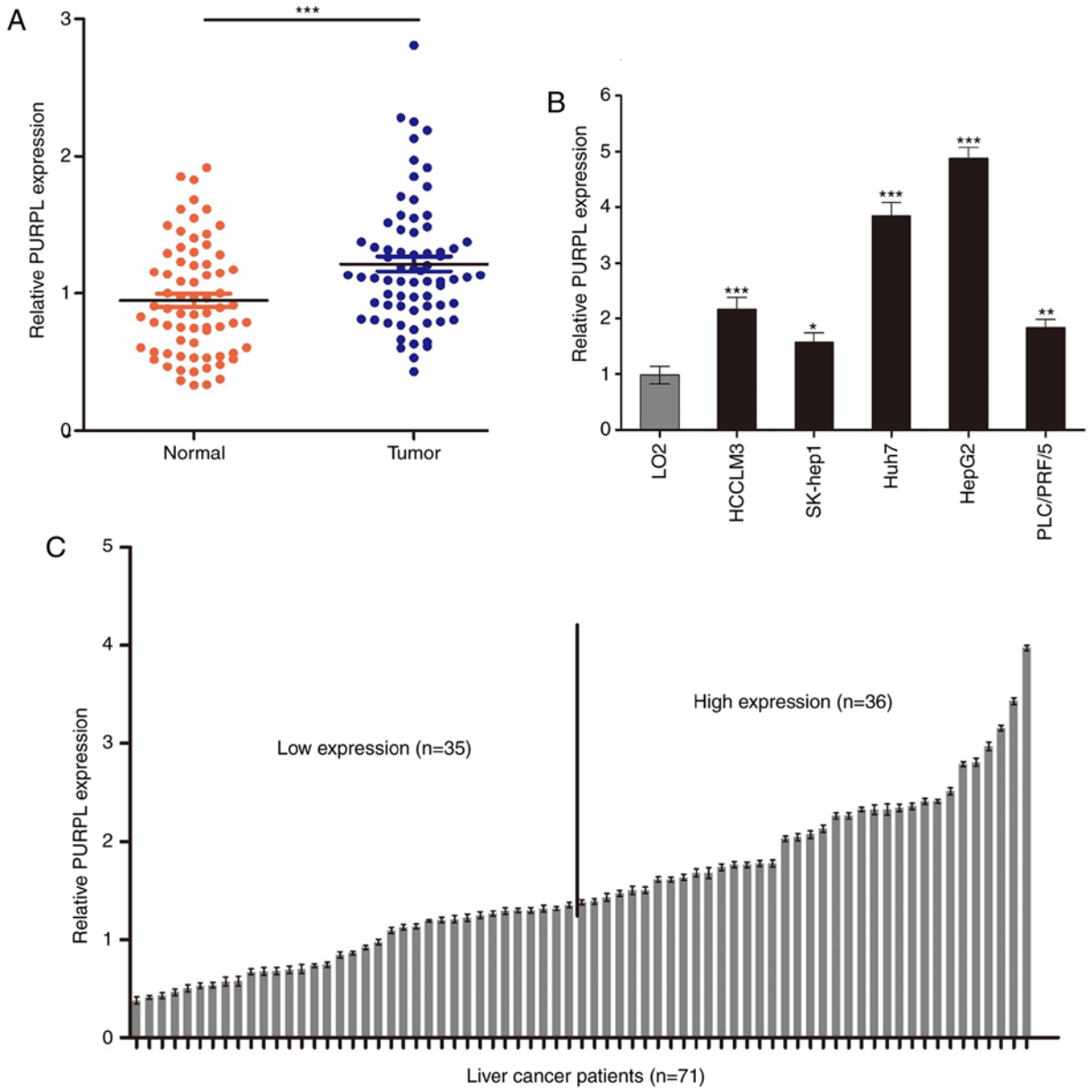

Figure 1. LncRNA-PURPL is upregulated in liver cancer tissues and cell lines. (A) Reverse transcription-quantitative polymerase chain reaction analysis of PURPL expression in 71 pairs of liver cancer and corresponding adjacent non-tumor control tissues. The results were analyzed using via a Student's t-test. ***P<0.001. (B) Relative expression of PURPL in five liver cancer cell lines (HepG2, Huh7, HCCLM3, SK-hep1, PLC/PRF/5) and a normal liver cell line (L02). The results were analyzed using one-way analysis of variance followed by the Least Significant Difference test. (C) Division of the 71 patients with liver cancer into a high-expression PURPL group (n=36) and a low-expression PURPL group (n=35) based upon levels of median expression. *P<0.05, **P<0.01 and ***P<0.001 vs. L02. Error bars correspond to the standard deviation. PURPL, long noncoding RNA p53 upregulated regulator of P53 levels.

centrifugation (850 x g, 4˚C, 5 min) and washing(PBS), 0.5 ml of propidium iodide (PI) staining solution (Beyotime Institute of Biotechnology) was added to each group of cell samples, which were incubated at 37˚C for 30 min in the dark. Samples were then stored on ice until they were detected on the flow cytometer (BD FACSCanto™).

Cell apoptosis analysis. In accordance with the manufacturer's protocols of an apoptosis kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), cells were trypsinized, collected (~5x10^5 cells/group), washed twice with PBS, centrifuged at 850 x g at 4˚C for 5 min, and then 100 μl of Annexin V buffer was added. Then, 5 μl of PI and 5 μl of Annexin V-FITC solution were added to each tube. The tubes were mixed by pipetting and incubated at room temperature for 15 min, after which 400 μl of Annexin V buffer (included in kit) was added to each tube and mixed by pipetting. Readings were obtained on a flow cytometer (BD FACSCanto™) as aforementioned.

Statistical analysis. The results were analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard deviation. Differences between two groups were assessed using a Student's t-test, whereas differences among multiple groups were analyzed using one-way analysis of variance followed by the Least Significant Difference test. The relationship between PURPL and p53 expression was analyzed by

Table I. Association between lncRNA-PURPL expression according to reverse transcription-quantitative polymerase chain reaction and conventional clinicopathological parameters in 71 patients with liver cancer.

Characteristics	Number of patients	lncRNA-PURPL Low expression (%)	lncRNA-PURPL High expression (%)	P-value
Total cases	71	35	36	
Age (years)				0.111
≥55	29	11 (37.93)	18(62.07)	
<55	42	24 (57.14)	18 (42.86)	
Sex				0.705
Male	60	29 (48.33)	31 (51.67)	
Female	11	6 (54.56)	5 (45.44)	
Tumor size				0.013[a]
≥5cm	39	14 (35.90)	25 (64.10)	
<5cm	32	21 (65.63)	11 (34.37)	
HBsAg status				0.591
Positive	57	29 (50.88)	28 (49.12)	
Negative	14	6 (42.86)	8 (57.14)	
Tumor differentiation				0.002[b]
High	21	17 (80.95)	4 (19.05)	
Moderate	30	12 (40.00)	18 (60.00)	
Poor	20	6 (30.00)	14 (70.00)	
PVTT				0.097
Yes	10	2 (20.00)	8 (80.00)	
No	61	33 (54.10)	28 (45.90)	
Serum AFP				0.288
<20 ng/ml	30	17 (56.67)	13(43.33)	
≥20 ng/ml	41	18 (43.90)	23(56.10)	
Liver cirrhosis				0.350
Yes	56	26 (46.43)	30 (53.57)	
No	15	9 (60.00)	6 (40.00)	
Metastasis				0.999
Yes	7	3 (42.86)	4 (57.14)	
No	64	32 (50.00)	32 (50.00)	

[a]P<0.05, [b]P<0.01.

Pearson correlation analysis. The association between PURPL expression and the clinicopathological parameters of patients was assessed via a c^2 test. All experiments were performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

LncRNA PURPL is upregulated in liver cancer tissues and cell lines. Using RT-qPCR, we examined the expression of PURPL in 71 patients with liver cancer and several liver cancer cell lines. The expression of PURPL was significantly increased in cancer tissues than in adjacent tissues (P<0.001; Fig. 1A). In addition, PURPL was highly expressed in HepG2 and Huh7 cells compared with the three other liver cancer cell lines and L02 cells (P<0.05; Fig. 1B). To investigate the association of PURPL expression with clinicopathological factors of patients, the patients were divided into high- (n=36) and low-expression (n=35) groups based on PURPL median expression levels (Fig. 1C). As presented in Table I, PURPL expression levels in patients with liver cancer were closely associated with tumor size (P<0.05) and tumor differentiation (P<0.01). These data indicated that PURPL is significantly elevated in liver cancer patients and may be closely related to the progression of liver cancer.

Depletion of lncRNA PURPL suppresses liver cancer cell proliferation. To determine whether PURPL regulates the proliferative capacity of liver cancer cells, we used three different siRNAs to silence the PURPL gene in liver cancer cells. #3PURPLsiRNA exhibited the strongest silencing efficiency (P<0.05; Fig. 2A and B); thus, #3PURPLsiRNA was selected for subsequent experiments. To determine the effect of PURPL on proliferation of liver cancer cells, we performed

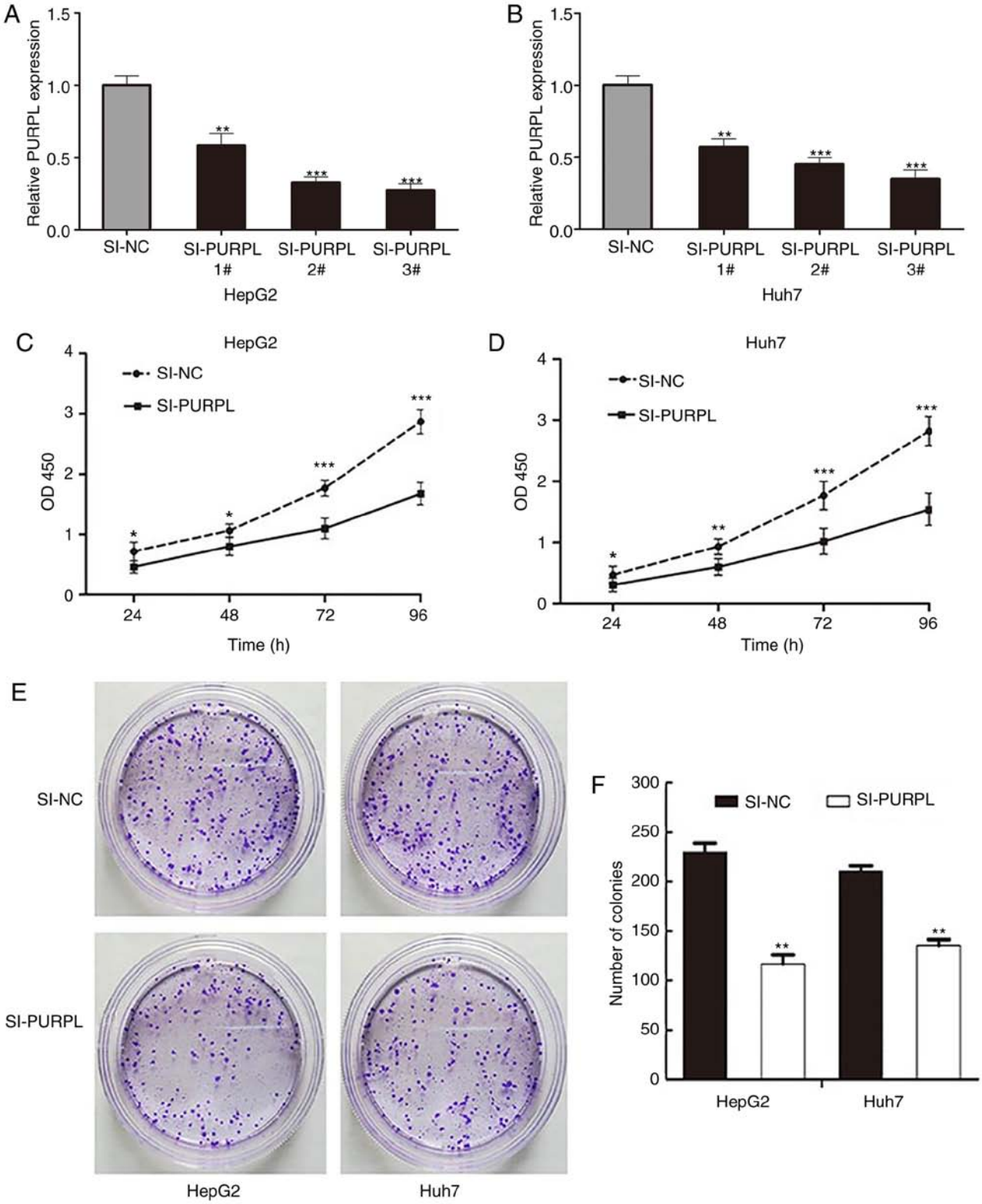

Figure 2. Long noncoding RNA-PURPL promotes the proliferation of HepG2 and Huh7 cells. (A and B) Efficiency of PURPL knockdown 24 h after transfecting cells with three SI-PURPL or SI-NC, as verified by reverse transcription-quantitative polymerase chain reaction. The results were analyzed using one-way analysis of variance followed by the Least Significant Difference test. (C and D) Effects of PURPL silencing on cell proliferation, as detected by a Cell Counting Kit-8 assay and absorbance measurements. (E and F) Effects of PURPL silencing on cell proliferation, as detected by colony-forming assays. The results were analyzed using a Student's t-test. *P<0.05, **P <0.01 and ***P<0.001 vs. SI-NC. Error bars correspond to the standard deviation. NC, negative control; PURPL, p53 upregulated regulator of P53 levels; SI, small interfering RNA.

a CCK-8 assay. Silencing of PURPL significantly inhibited the proliferation of HepG2 and Huh7 cells compared with the control (P<0.05; Fig. 2C and D). In addition, the number of clones formed by PURPL-silenced cells was significantly decreased compared with the corresponding controls (P<0.01; Fig. 2E and F).

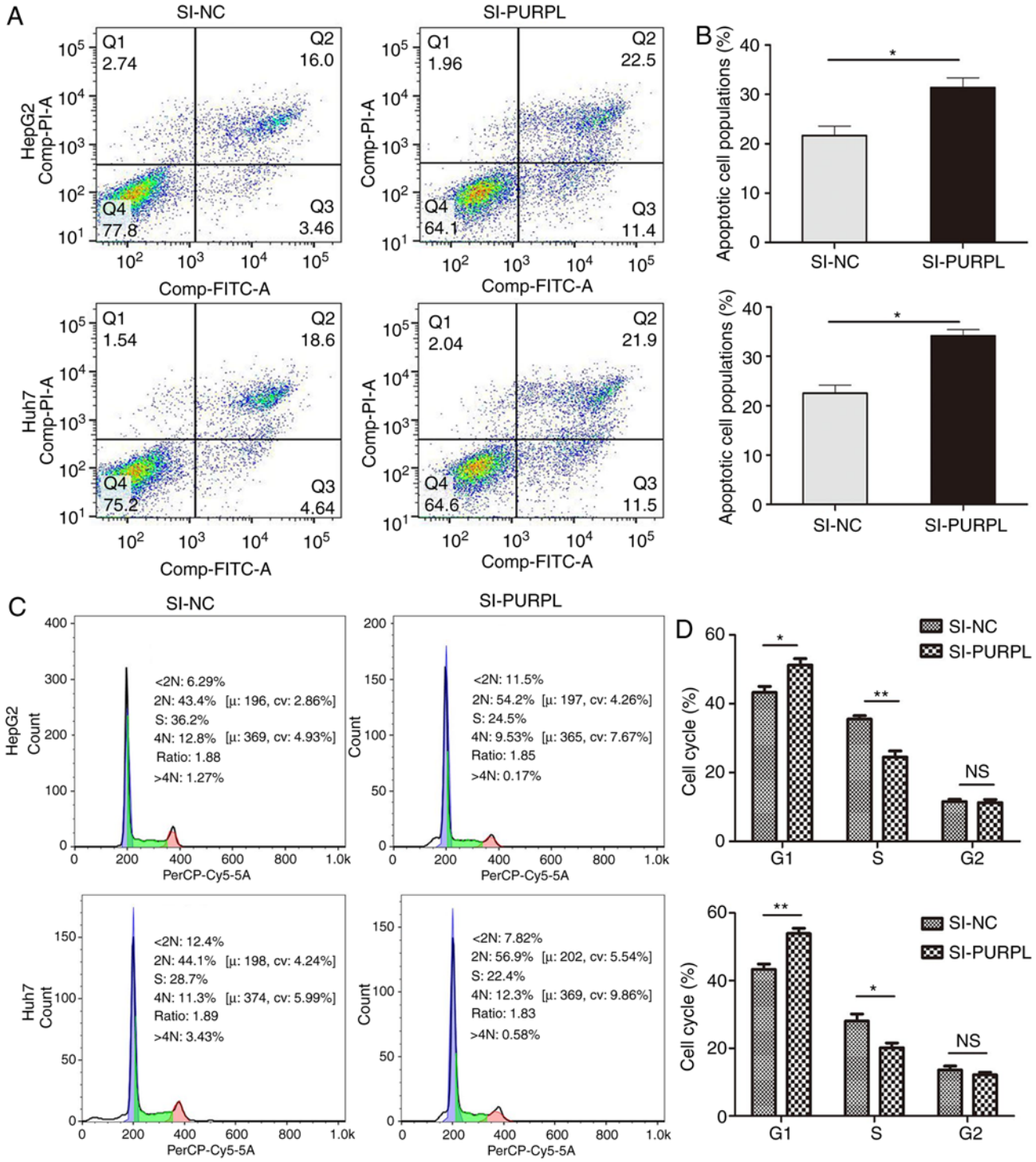

Figure 3. Long noncoding RNA-PURPL inhibits HepG2 and Huh7 apoptosis and promotes cell cycle progression. Effect of PURPL silencing on apoptosis and cell cycle progression of HepG2 and Huh7 cells, as determined by flow cytometry. (A and C) Representative flow cytometry plots and (B and D) corresponding quantification are presented. All results were analyzed using a Student's t-test. NS, not significant. *P<0.05 and **P<0.01. Error bars correspond to the standard deviation. NC, negative control; PURPL, p53 upregulated regulator of P53 levels; SI, small interfering RNA.

Depletion of lncRNA PURPL promotes liver cancer cell apoptosis and leads to liver cancer cell cycle arrest. Cell apoptosis was monitored by flow cytometry. The number of apoptotic cells was significantly increased following transfection with siRNA-PURPL than those transfected with si-NC (P<0.01; Fig. 3A and B). Regarding cell cycle progression, PURPL knockdown induced G1 phase arrest of liver cancer cells; siRNA-PURPL transfection resulted in a significant decrease in the number of cells in S phase compared with the control (P<0.05; Fig. 3C and D). Collectively, these results suggested that PURPL knockdown may induce G1 phase arrest and promote liver cancer cell apoptosis.

LncRNA PURPL inhibits p53 expression in liver cancer. p53 can be modulated by lncRNAs to regulate cancer cell progression and PURPL has been reported to inactivate p53 protein in

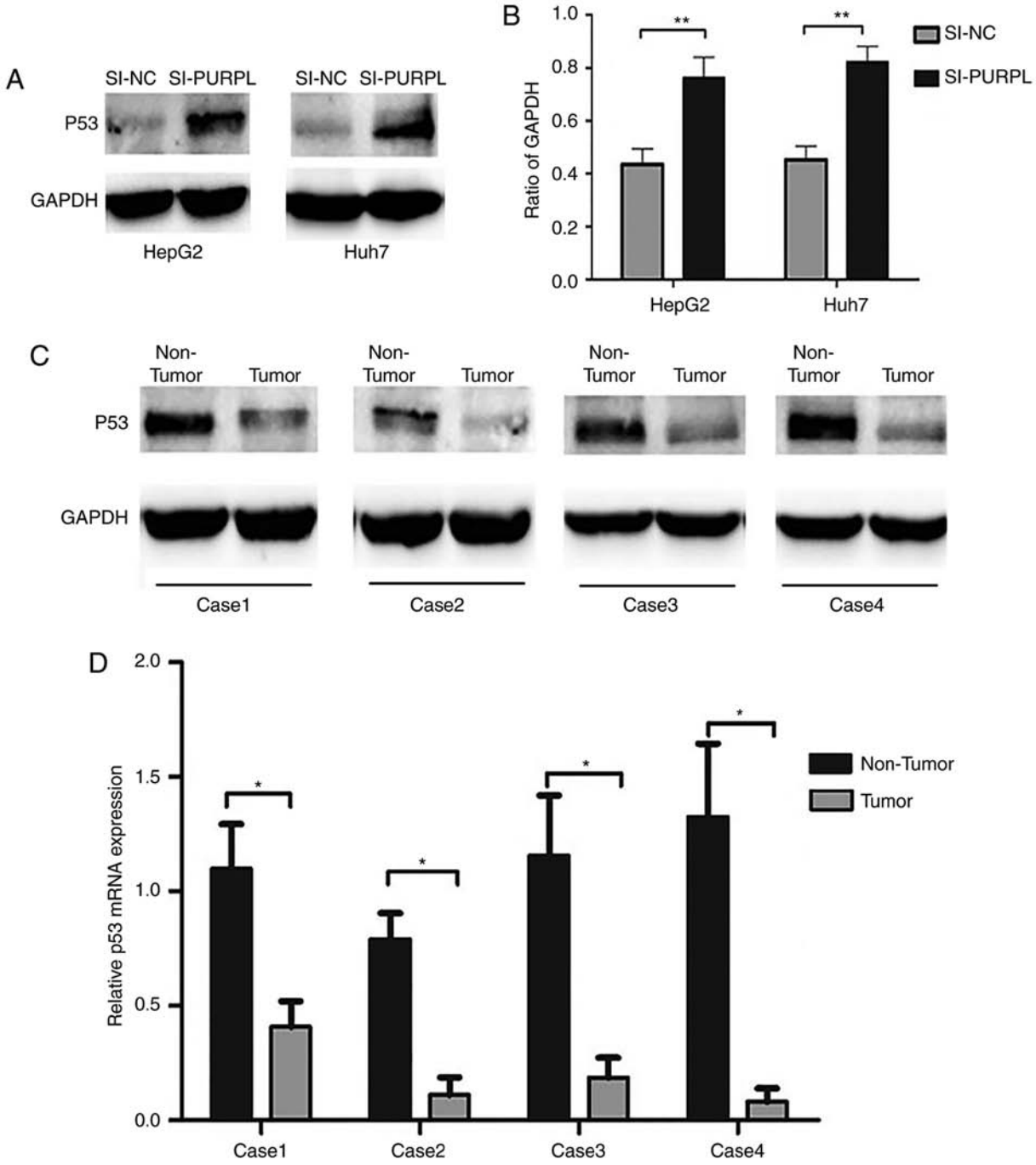

Figure 4. Long noncoding RNA-PURPL inhibits p53 expression. (A and B) Expression of p53 protein in HepG2 and Huh7 cells transfected with SI-PURPL or SI-NC. GAPDH was used as internal control. (C) p53 protein expression in liver extracts obtained from four patients with advanced liver cancer. (D) p53 mRNA expression, as determined reverse transcription-quantitative polymerase chain reaction, in liver extracts from the same four advanced liver cancer patients. All results were analyzed using a Student's t-test. *P<0.05 and **P<0.01. Error bars correspond to the standard deviation. NC, negative control; PURPL, p53 upregulated regulator of P53 levels; SI, small interfering RNA.

colorectal cancer (14). To improve understanding of the role of PURPL in liver cancer, we used western blotting to measure the expression levels of p53 in liver cancer cells. The results revealed that p53 was significantly upregulated in HepG2 and Huh7 cells transfected with siRNA-PURPL compared with si-NC (P<0.01; Fig. 4A and B). Western blotting and RT-qPCR of liver cancer tissues demonstrated downregulation of p53 in tumor tissue compared with paracancerous tissues in four randomly selected late-stage liver cancer patients, at the protein and mRNA levels (P<0.05; Fig. 4C and D). Additionally, relative p53 mRNA expression was negatively correlated with PURPL expression in all 71 patients (P<0.001; Fig. 5). These results indicate the role of PURPL as an oncogene that may be partially responsible for inducing p53 in liver cancer.

Discussion

Aggressive tumor invasion, metastatic dissemination, recurrence, and drug resistance are characteristic of patients with liver cancer (16-19). Thus, research on liver cancer has focused

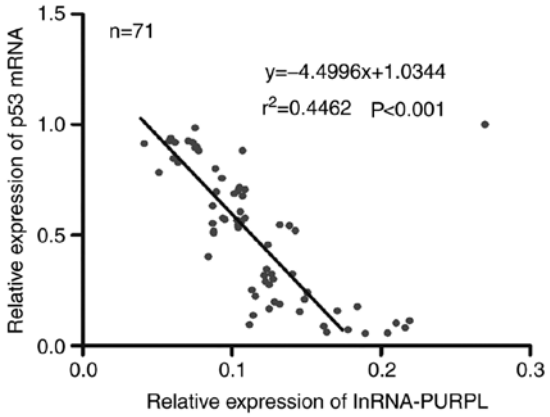

Figure 5. p53 expression is negatively correlated with PURPL expression levels. Negative correlation between relative p53 mRNA expression and PURPL mRNA expression in 71 patients with liver cancer (r^2=0.4462, P<0.001). NC, negative control; lncRNA-PURPL, long noncoding RNA p53 upregulated regulator of P53 levels.

on identifying genes that induce hepatocarcinogenesis and promote the development of liver cancer, for which targeted drugs and molecular markers capable of early diagnosis could be developed. Increasing evidence suggests that aberrant expression of lncRNA serves a key role in the development and progression of liver cancer. For example, lncRNA-CCAL promotes liver cancer progression by modulating AP-2α and Wnt/β-catenin pathways, lncRNA-HULC promotes liver cancer by increasing the expression of the high mobility group AT-hook 2 oncogene and sequestration of miRNA-186; lncRNA-TSLNC8 serves as a tumor suppressor that inactivates the interleukin-6/signal transducer and activator or transcription 3 signaling pathway in liver cancer (20-22).

LncRNA-PURPL is highly expressed in colorectal cancer and serves an important role in carcinogenesis. The present study analyzed the expression of PURPL by RT-qPCR and revealed that PURPL was highly expressed in liver cancer tissues and numerous cell lines. Furthermore, the upregulated expression of PURPL was positively associated with tumor size and tumor differentiation in patients with liver cancer. In addition, silencing of PURPL expression could inhibit liver cancer cell proliferation, arrest cell cycle progression and promote cell apoptosis.

The p53 gene was first described in 1979 (23). It serves an important role in the occurrence and development of various tumors via the control of DNA damage repair, apoptosis and cell cycle regulation (23,24). Several proteins, miRNAs and lncRNAs have been reported to affect the occurrence and development of liver cancer by regulating the p53 gene (25-29).

In recent years, numerous studies have investigated on the role of lncRNAs in liver cancer and p53 gene regulation. For example, Zhou *et al* (30) reported that p53 regulation associated lncRNA inhibited liver cancer growth and induced apoptosis *in vivo* and *in vitro* via p53. Ren *et al* (31) reported that lncRNA prostate-cancer-associated ncRNA transcript-1 expression could be regulated by miR-215, a p53-inducible miRNA in liver cancer, and this post-transcriptional regulation significantly affected a variety of malignant phenomena of liver cancer cells. Additionally, Zhang *et al* (32) revealed that lncRNA-small nucleolar RNA host gene was a predictor of poor prognosis and promoted liver cancer tumorigenesis by regulating p53. In colorectal cancer, PURPL inhibits p53 gene expression by blocking the interaction between p53 and MYBBP1A (14). Whether PURPL inhibits the p53 gene via MYBBP1A or through other mechanisms of action requires further investigation.

In conclusion, our data demonstrated that PURPL is upregulated in liver cancer tissues, and is associated with tumor size and tumor differentiation. Silencing of PURPL could inhibit the proliferation of liver cancer cells, block cell cycle progression and promote apoptosis. Furthermore, PURPL may affect these malignant features of liver cancer cells by regulating p53. In this regard, we elevated the expression of PURPL in liver cancer cell lines, however this failed to alter the malignant characteristics of liver cancer cells (data not shown). Our future studies aim to investigate the specific mechanisms whereby PURPL affects the development of liver cancer. Collectively, lncRNA-PURPL was proposed to exert an important carcinogenic effect on liver cancer, and may be a potential predictor and a novel therapeutic target for the treatment of this disease.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GW made substantial contributions to the design of the present study. XF conducted all the experiments and wrote the manuscript. YW, WZ, SX and WW made substantial contributions to the analysis of the data and discussed the results. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

All patients provided written informed consent for the use of tissues. The present study was approved by the institutional ethics committee of China Medical University (Shenyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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