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ORIGINAL ARTICLE

Overexpressed lncRNA CDKN2B-AS1 is an independent prognostic factor for liver cancer and promotes its proliferation

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Summary

Purpose: *To study the expression of long non-coding RNA (lncRNA) CDKN2B-AS1 in the liver tissue of patients with liver cancer and its effect on the proliferation of liver cancer cell line QGY-7703.*

Methods: *The expression of differentially expressed genes in liver cancer tissues and normal liver tissues was analyzed by bioinformatics. The expressions of differentially expressed genes in clinical samples were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) to analyze whether there is a significant difference of CDKN2B-AS1 expression in clinical features of patients with liver cancer. The clinical data were analyzed to find out the correlation between the expressions of differentially expressed genes and the overall survival, tumor size and TNM staging. The Gene Set Enrichment Analysis (GSEA) enrichment analysis was used to predict the function of CDKN2B-AS1. The cell proliferation was measured by cell counting kit-8 (CCK-8) assay. Cell cycle was measured by flow cytometry. Expressions of proteins were detected by Western blotting.*

Results: *The expression of CDKN2B-AS1 in liver cancer was significantly higher compared to normal tissues. The results of qRT-PCR were consistent with the results of the Cancer Genome Atlas (TGCA). The data of CDKN2B-AS1 showed that the expression of CDKN2B-AS1 was associated with the total survival, tumor size and TNM staging. GSEA results showed that genes are enriched in cell cycle sets and others. Compared with the control, the proliferation of QGY-7703 cells overexpressing CDKN2B-AS1 was significantly increased ($p<0.001$). Western blotting results showed that the expressions of CDK2 and CDK4 were up-regulated and the expression of P16 was decreased after CDKN2B-AS1 was overexpressed.*

Conclusions: *LncRNA CDKN2B-AS1 was highly expressed in liver cancer, and its expression was positively correlated with the overall survival, tumor size and TNM stage. LncRNA CDKN2B-AS1 promoted the proliferation and expression of liver cancer cells.*

Key words: *liver cancer, CDKN2B-AS1, lncRNA, TGCA, GSEA*

Introduction

Liver cancer is now the third leading cause of cancer death [1]. It is also the sixth most common cancer worldwide and has a strong invasion capability and high incidence of metastasis [2]. Therefore, studies of the pathophysiology of liver cancer for early disease detection are helpful for improving effective treatments and overall prognosis of this disease.

Long non-coding RNA (lncRNA) is a class of RNA molecules encoding transcripts with over 200 nucleotides (NT). LncRNAs cannot encode a protein, however they have important regulatory effects on genes [3]. LncRNAs are capable to regulate gene expressions through epigenetic regulation, transcriptional regulation and post-transcriptional regulation [4]. Many abnormal expressions

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of lncRNAs are considered to be associated with recurrence, metastasis and prognosis of liver cancer. The expression of H19 non-coding RNA can be induced by c-Myc, which is frequently overexpressed in hepatocellular carcinoma cells. C-Myc can be used as a transcription factor to bind to a highly conserved E-box near the H19 imprinted regulatory region on the activated maternal chromosome to promote histone acetylation and initiation of H19 expression, thus increasing the proliferation of hepatocellular carcinoma cells [5]. Studies have shown that liver cancer patients with overexpressed HOTAIR were prone to recurrence and lymph node metastasis after liver transplantation, and short relapse-free survival, which was an independent risk factor for liver cancer recurrence after liver transplantation *in vitro* experiments using shRNA for inhibiting the expression of HOTAIR in the hepatocellular carcinoma cells can affect the viability and invasiveness of these cells [6]. In addition, HULC, MEG3, MVIH lncRNA were also related to liver cancer [7-9]. Therefore, a comprehensive understanding of lncRNAs will help elucidate their real effect on liver cancer [10,11].

CDKN2A/B gene is located in the 9p21 region, which is associated with various diseases such as cancer, heart and inflammatory diseases [12], CDKN2A/B includes three tumor suppressor sites, p14ARF, p16INK4A and p15INK4B [12,13]. CDN2B-AS1 (CDN2B antisense RNA 1) is also closely related to many diseases [12,14,15]. However, CDN2B-AS1 has not been reported in the liver cancer.

The purpose of this study was to investigate the expression of lncRNA CDKN2B-AS1 in the liver tissue of patients with liver cancer and its effect on the proliferation of liver cancer cell lines.

Methods

Expression of lncRNA CDKN2B-AS1

The data of liver cancer were downloaded from TCGA database. The expression of CDKN2B-AS1 in patients with liver cancer and its relationship with prognosis were analyzed.

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) version 2.2.1 software was used to analyze genes function. GSEA website MSIGDB database was used to obtain the data set; then, the default weighted enrichment method was applied for enrichment analysis. The random combination was set for 1000 times.

Cell culture and plasmid transfection

Hepatocellular carcinoma cells QGY-7703, PLC/PRF/5, HB611 and MHCC97 were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA). The cells were placed in 6-well plates at 37°C, in 5% CO_2 incubator. Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the cells reached 80% confluence. The culture medium was replaced 6 h after transfection.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

After 24 h of transfection, the total RNA was extracted by 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was reverse transcripted according to the instructions of the reverse transcription Kit (TaKaRa, Tokyo, Japan). qRT-PCR was used to detect the expression level of CDKN2B-AS1. Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) gene was used as the internal reference in the quantitative analysis of CDKN2B-AS1 expression. Measurements were performed in triplicate.

Cell proliferation assay by cell counting kit-8 (CCK-8) method

The transfection time point was 0 h, RPMI 1640 medium containing 10% FBS was changed 6 h later, cells were inoculated into 96-well plates 24 h later and CCK8 assay (Dojindo, Kumamoto, Japan) was performed after culture for 24, 48, 72 and 96 h. The serum-free medium was replaced at the time of detection. Ten μL of CCK8 were added to each well. After incubation at 37°C and 5% CO_2 for 1 h, the optical density (OD) value was measured at 450 nm by a microplate reader (BIO-RAD, Hercules, CA, USA). Each measurement was performed in quintuplicate.

Cell cycle assay

Cells were plated in the 6-well plates and were divided into the plasmids transfection pcDNA-NC and pcDNA-CDN2B-AS1 groups.

After 24 h of incubation, $1x10^6$ cells were collected in each group, washed twice with PBS, and the supernatant was discarded. 70% ethanol (-20°C pre-cooling) was used for fixation and placed at -20°C overnight. Then, the cells were washed with pre-cooling PBS twice, incubated with Rnase for 15 min and 50 μg/mL PI (propidium iodide) were added in each well for 30-min incubation. The PI-stained cells were detected by flow cytometry. Each group contained four samples.

Western blotting

The RIPA (radioimmunoprecipitation assay) protein lysate (Beyotime, Shanghai, China) was used to extract the total protein in each group of cells. The BCA (Bicinchoninic acid) method was performed to quantitate the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (MERCK Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (ABCAM, Cambridge, MA, USA) at 4°c overnight. The membrane was incubated with the secondary goat anti-rabbit (HRP) IGG antibody after

rinsing with the buffer solution (TBST). Chemiluminescence was used to expose the protein bands on the membrane.

Statistics

SPSS 22.0 (SPSS IBM, Armonk, NY, USA) statistical software was used for data analysis, and GraphPad Prism 5.0 (Version X; La Jolla, CA, USA) was used for picture editing. Kaplan-Meier method was used for survival analysis and log-rank test was used for comparisons between groups. Quantitative data were compared with *t*-test and presented as mean ± standard deviation (x±s), and categorical data were compared with chi square test. P<0.05 indicated significant difference.

Results

Expression of CDKN2B-AS1 in patients with liver cancer and its relationship with clinical data and functional gene enrichment analysis of CDKN2B-AS1

TCGA data showed that the CDKN2B-AS1 expression was significantly increased in patients with liver cancer (Figure 1A and 1B, p<0.001). The overall survival time of the CDKN2B-AS1-overexpression group was significantly lower in patients with liver cancer than in the low-expression group in TCGA (Figure 1C, p=0.048). The qRT-PCR performed on liver cancer tissues and adjacent tissues of the selected 48 patients further confirmed that the CDKN2B-AS1 was overexpressed in the liver cancer tissues (Figure 2A, p<0.001). The analyzed clinical data of patients showed that the expression level of CDKN2B-AS1 was conversely correlated with overall survival (Figure 2B, p<0.01), which was consistent with those of TCGA data. In addition, the expression of CDKN2B-AS1 was increased in patients with advanced tumor stage and large tumor volumes (Figure 2C and Figure 2D). Based on the median expression of CDKN2B-AS1, patients were divided into high expression group and low expression group. Chi square test showed that tumor stage in the CDKN2B-AS1 high expression group was higher, and the tumor volume was larger (Table 1). GSEA results showed that CDKN2B-AS1 was enriched in the cell cycle (Figure 3).

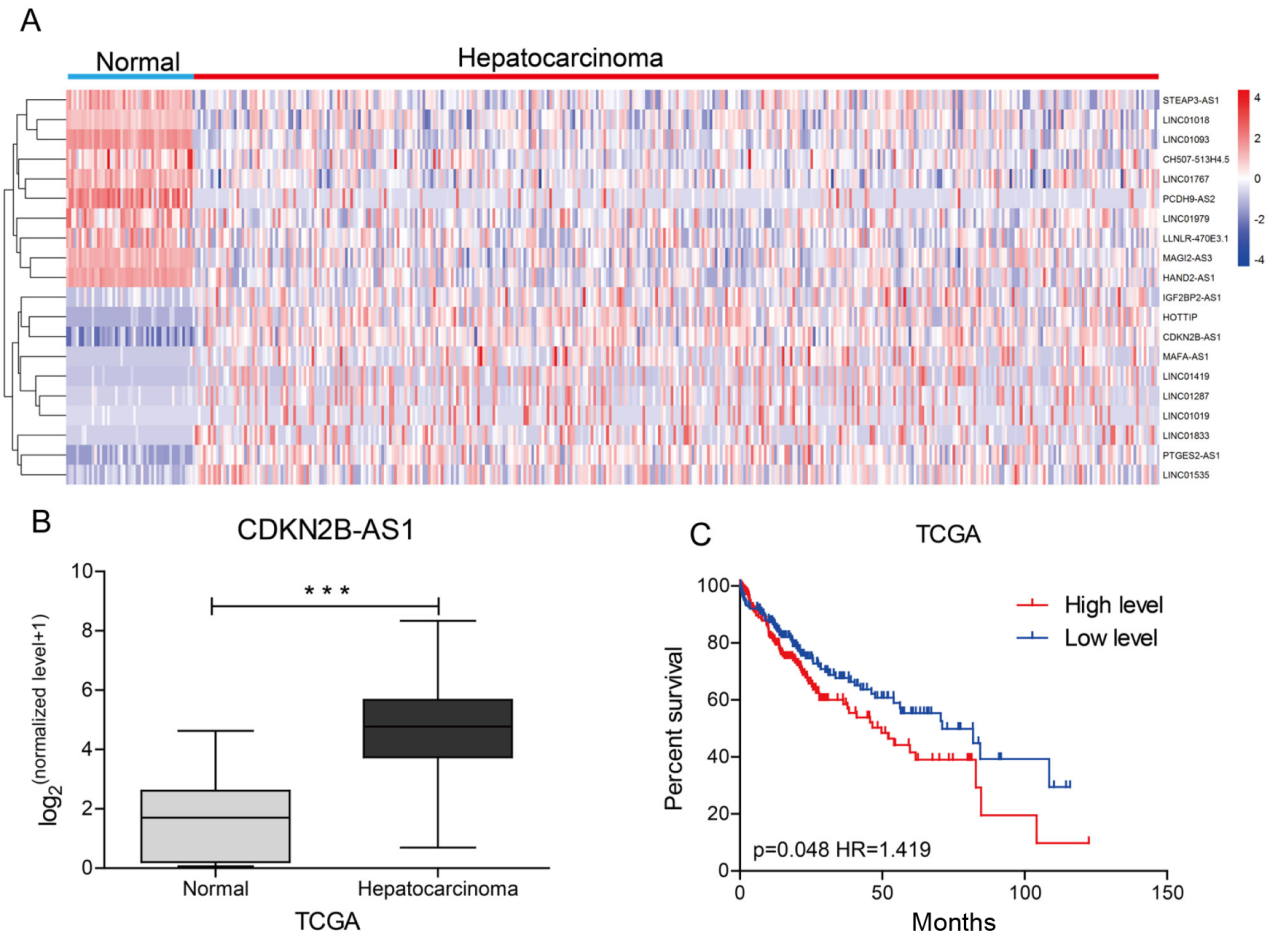

Figure 1. Database analysis showed that lncRNA CDKN2B-AS1 was highly expressed in liver cancer. **A:** Differential expressions of liver cancer tissues and normal liver tissues. **B:** TCGA data analysis showed that CDKN2B-AS1 was highly expressed in liver cancer tissues. **C:** TCGA data showed that the overall survival rate of liver cancer patients with overexpressed CDKN2B-AS1 was significantly lower than that of CDKN2B-AS1 low expression group. ***p<0.001.

Screening cell lines

The normal cell line L-02 was used as control, the total RNA was extracted from the cell lines QGY-7703, PLC/PRF/5, HB611, MHCC97, and then the relative expression of CDKN2B-AS1 in these cells was detected by qRT-PCR. As shown in Figure 4A, the expression of CDKN2B-AS1 was slightly lower in the QGY-7703 cell line, so we selected this cell line for subsequent overexpression experiments. The corresponding plasmids were constructed and transfected into the corresponding hepatocarcinoma cell lines and the transfection results are shown in Figure 4B.

Effects of CDKN2B-AS1 overexpression on the proliferation of QGY-7703 cells

CCK8 assay showed that overexpression of CDKN2B-AS1 promoted the proliferation of QGY-7703 cells after transfection of these cells with pcDNA-CDKN2B-AS1. The difference was statistically significant (Figure 4C).

Effects of CDKN2B-AS1 overexpression on the cell cycle of QGY-7703 cells

In QGY-7703 cells, cell cycle progression was accelerated after pcDNA-CDKN2B-AS1 transfection compared to the pcDNA-NC negative control. The difference was statistically significant (Figure 4D).

Effect of CDKN2B-AS1 overexpression on the regulation of cyclins in QGY-7703 cells

Western blotting results showed that the expressions of CDK2 and CDK4 proteins were increased and the expression of p16 protein was decreased after transfection of QGY-7703 cells with pcDNA-CDKN2B-AS1, which indicated that CDKN2B-AS1 promoted the cell cycle from G0/G1 phase to S phase in QGY-7703 cells (Figure 4E).

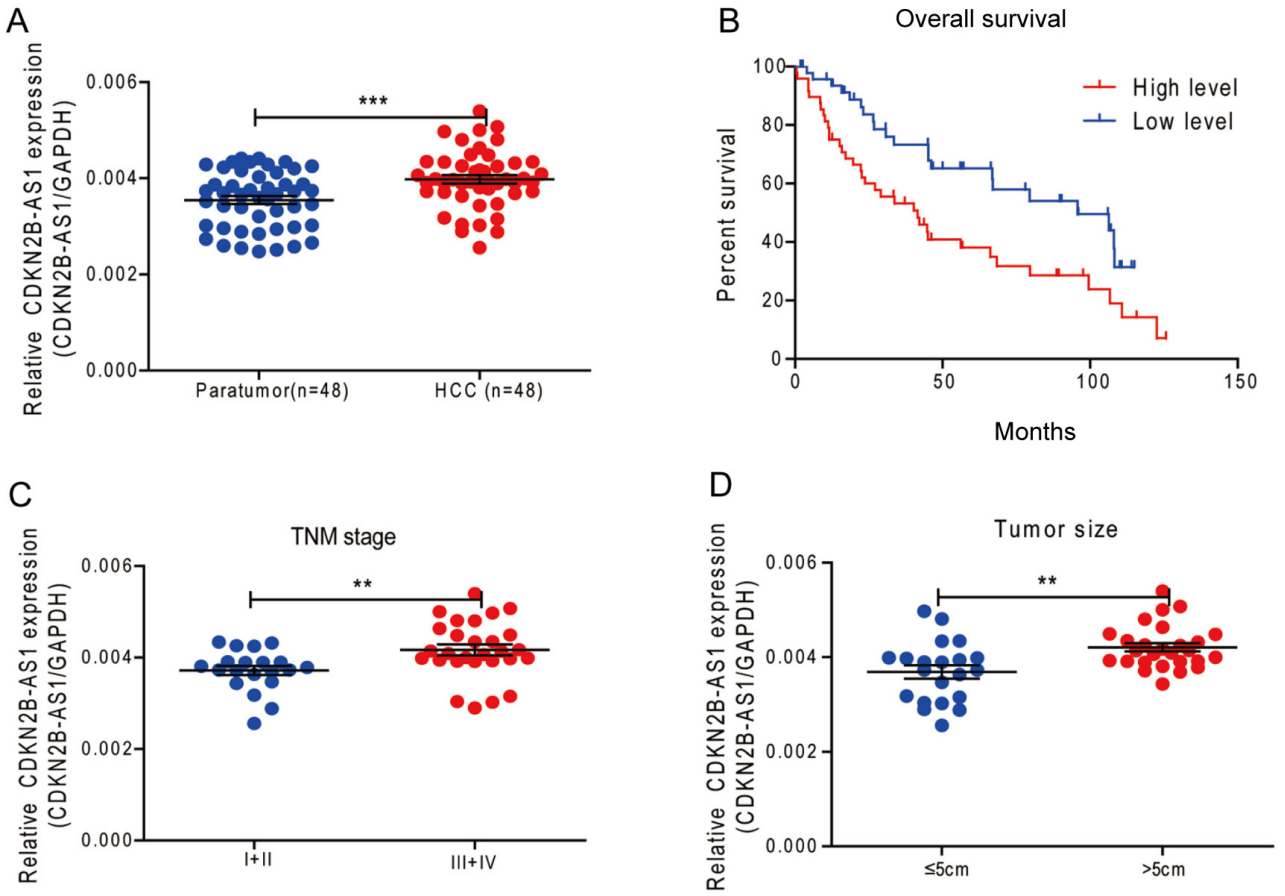

Figure 2. Clinical sample analysis showed that lncRNA CDKN2B-AS1 was highly expressed in the liver cancer. **A:** The expression of lncRNA CDKN2B-AS1 in 48 liver cancer tissues was significantly higher than that in adjacent tissues. **B:** The overall survival rate of liver cancer patients with CDKN2B-AS1 overexpression was significantly lower than that of CDKN2B-AS1 low expressing group ($p<0.05$). **C:** The expression of CDKN2B-AS1 was positively correlated with FIGO staging. D: The expression of CDKN2B-AS1 was positively correlated with tumor size. **$p<0.01$ and ***$p<0.001$.

Table 1. Correlation between lncRNA CDKN2B-AS1 expression and clinicopathological features in patients with liver cancer (n = 48)

Clinicopathologic features	*Number of cases*	*LncRNA CDKN2B-AS1 expression*		*p value*
		Low (n=22)	*High (n=26)*	
Age (years)		22	26	0.7151
≤60	21	9	12	
>60	27	13	14	
Gender				0.1746
Male	19	11	8	
Female	29	11	18	
Tumor size, cm				0.0280
≤5	29	17	12	
>5	19	5	14	
TNM stage				0.0176
I-II	26	16	10	
III-IV	22	6	16	
Grade				0.2466
Low	24	13	11	
Medium and high	24	9	15	
Lymph node metastasis				0.2029
Absent	28	15	13	
Present	20	7	13	

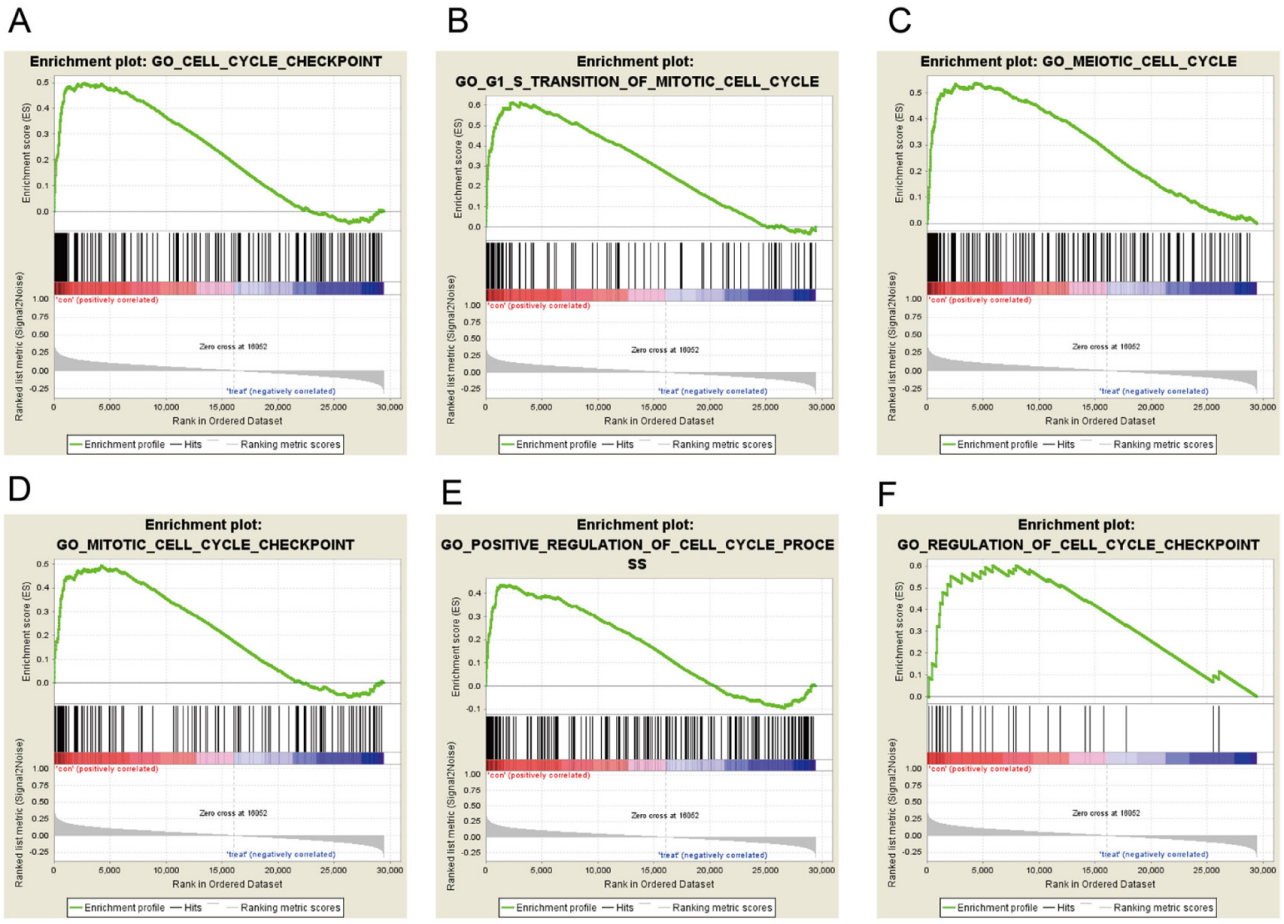

Figure 3. The GO analysis of CDKN2B-AS1 in TCGA datasets. **A-F:** The GO analysis showed that CDKN2B-AS1 mainly regulated cell cycle. The results of these figures showed that CDKN2B-AS1 could promote the proliferation of liver cancer cells by regulating the cell cycle.

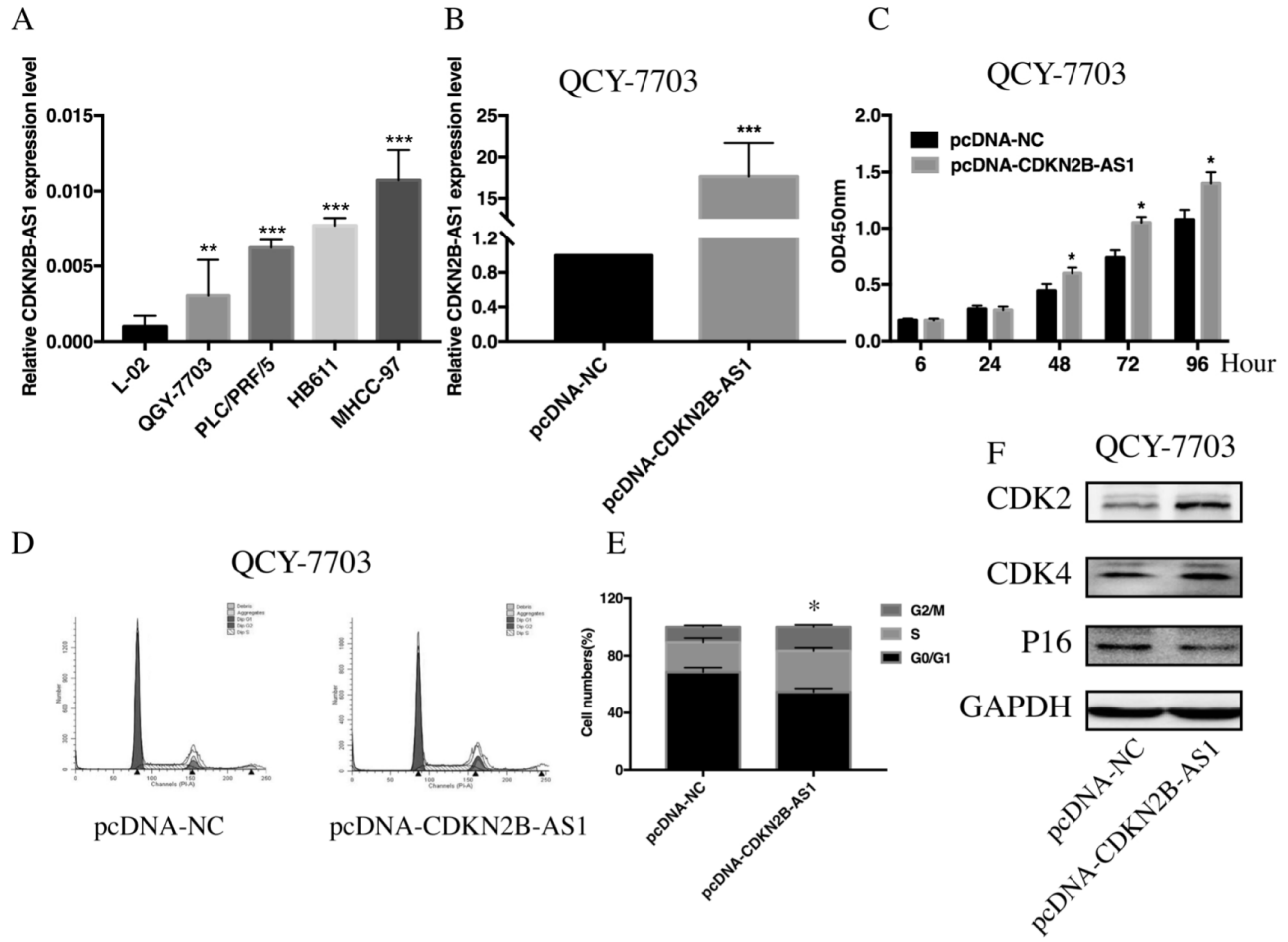

Figure 4. LncRNA CDKN2B-AS1 promoted the proliferation of HCC. **A:** Expression of lncRNA CDKN2B-AS1 in hepatocytes and liver cancer cell lines (L-02, QGY-7703, PLC/PRF/5, HB611, MHCC97). **B:** Transfection of pcDNA-CDKN2B-AS1 in QGY-7703. **C:** CCK8 assay showed that overexpression of CDKN2B-AS1 in QGY-7703 cells promoted the cell viability. **D and E:** Overexpression of CDKN2B-AS1 promoted the cell cycle of QGY-7703 cells. **F:** Expressions of CKD2 and CKD4 were increased and the expression of cell cycle inhibitor protein p16 was decreased after CDKN2B-AS1 was overexpressed in liver cancer cell line QGY-7703. *p<0.05, **p<0.01, ***p<0.001.

Discussion

Abnormal expressions of lncRNAs play an important role in the development and progression of tumors. Studies of lncRNAs are expected to provide new targets for the diagnosis and treatment of tumors. It has been found that lncRNA CDNK2B-AS1 was associated with the incidence of POAG (primary open angle glaucoma) within the genome [16,17]. In addition, CDNK2B-AS1 was associated with the development of cardiovascular disease [18], diabetes [19], intracranial aneurysms [20] and gastric cancer [21], however, its research in tumors was less and no report on its involvement in liver cancer has been reported.

With the arrival of big data age, the Cancer Genome Atlas (TCGA) has been increasingly used in medical research because of its large sample size, complete gene expression spectrum and comprehensive prognostic information of patients. Therefore, in this study, the expression of CDKN2B-AS1 in liver cancer and its effect on prognosis through TCGA database was analyzed to provide evidence and ideas for further studies on the mechanism of CDKN2B-AS1 in tumor genesis and progression. TCGA analysis showed that CDKN2B-AS1 was significantly overexpressed in liver cancer and was associated with the overall survival, suggesting that CDKN2B-AS1 was a risk factor for poor prognosis in patients with liver cancer. Then, we further validated that CDKN2B-AS1 was highly expressed in liver cancer in clinical samples. The results were consistent with TCGA analysis. Clinical data showed that CDKN2B-AS1 was positively correlated with overall survival, tumor size and TNM staging, but not with age, sex, grade, lymph node involvement and distant metastasis. Therefore, we speculated that CDKN2B-AS1 was a cancer-promoting agent of liver cancer, which could be expected to become a biomarker of liver cancer.

To further study the role of overexpressed CDKN2B-AS1 in the development and progression of liver cancer, we analyzed the data in the TCGA database by gene set enrichment analysis (GSEA). GSEA results suggested that CDKN2B-AS1 was connected with the cell cycle and that CDKN2B-AS1 may play a role in the proliferation of liver cancer cells though affecting the cell cycle.

Cell cycle is a very complex and a basic regulatory process. A number of molecules has been involved in cell cycle regulation, including three major categories: cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors, which are collectively called cell cycle genes, of which, CDKs are the core of the regulatory network. Cyclins have a positive regulatory effect on the CDKs, and a negative regulation on the CKIs, and altogether these constitute the molecular basis of cell cycle regulation [22-24]. Western blotting results showed that the expression of cyclin-dependent kinase 2 was increased after overexpression of CDKN2B-AS1, and the expression of p16 in CKIs family decreased. This suggested that CDKN2B-AS1 regulated cell cycle through a cyclin-regulated protein. Cell function experiments showed that CDKN2B-AS1 overexpression promoted the proliferation of hepatocellular carcinoma cells and promoted the cell cycle from G0/G1 phase to S phase.

In summary, this study suggested that the development of hepatocellular carcinoma was closely related to the expression of CDKN2B-AS1, which could promote the proliferation of hepatocarcinoma cells, and its expression could be used as a prognostic independent factor. Exploring the mechanism of CDKN2B-AS1 in liver cancer is expected to provide potential target support for the clinical treatment of liver cancer.

Conclusions

LncRNA CDKN2B-AS1 was highly expressed in the liver cancer, which can promote the proliferation of HCC cells. The expression of CDKN2B-AS1 was positively correlated with overall survival, tumor size and TNM stage of patients, and is expected to be a target of liver cancer therapy.

Conflict of interests

The authors declare no conflict of interests.

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