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Long non-coding RNA APTR promotes the activation of hepatic stellate cells and the progression of liver fibrosis

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ABSTRACT

In this study, we aimed at assessing a role of *Alu*-mediated p21 transcriptional regulator (APTR) in hepatofibrogenesis. APTR was upregulated in fibrotic liver samples and activated hepatic stellate cells (HSCs). Knockdown of APTR inhibited the activation of HSCs *in vitro* and mitigated the accumulation of collagen *in vivo*. Importantly, APTR silencing could abrogate TGF-β_1-induced upregulation of α-SMA in HSCs. In addition, inhibition of cell cycle and cell proliferation by APTR knockdown was attenuated by p21 siRNA1 in primary HSCs. Finally, serum APTR levels were increased in patients with liver cirrhosis, indicating a potential biomarker for liver cirrhosis. Collectively, evidence is proposed for a new biological role of APTR in hepatofibrogenesis.

1. Introduction

Liver fibrosis is the common outcome in response to chronic hepatic diseases [1,2]. Liver cirrhosis is the late stage of liver fibrosis. It is one of the primary causes of mortality worldwide [3–5]. Hepatic stellate cells (HSCs) have emerged as the key cell type responsible for extracellular matrix (ECM) production during liver fibrogenesis [6,7]. TGF-β_1 is one potent factor stimulating the activation of HSCs [8]. Therefore, an ideal therapeutic strategy for liver fibrosis is to inhibit HSC activation and TGF-β_1 synthesis.

Recently, the deregulation of long non-coding RNAs (lncRNAs) has been found to be associated with human liver diseases [9,10]. LncRNA lncTCF7 promotes tumorigenic activity in liver cancer stem cells through Wnt signaling regulatory circuit [11]. The long intergenic ncRNA UFC1 accelerates proliferation and inhibits apoptosis in liver cancer cells, thereby increasing growth of xenograft tumors in mice [12]. The expression of lncRNA HOXA transcript at the distal tip and HOXA13 is associated with metastasis in patients with hepatocarcinoma [13]. LncRNA-activated by TGF-β upregulates ZEB1 and ZEB2 by competitively binding the miR-200 family and then induces epithelial–mesenchymal transition in liver cancer [14]. These findings have attracted much interest in targeting deregulated lncRNAs to treat liver diseases.

Negishi and colleagues [15] have identified a new lncRNA *Alu*-mediated p21 transcriptional regulator (APTR) related with cell proliferation. Depletion of APTR in glioblastoma multiforme cells inhibits cell growth. However, little is known about the biological role of APTR in liver fibrosis. Here, we examined whether APTR plays a pivotal role in the progression of liver fibrosis and elucidated the possible mechanism.

2. Materials and methods

2.1. Liver fibrosis model

Male 8-week-old C57BL/6J mice were given carbon tetrachloride (CCl_4) at 7 μl (10% CCl_4 in olive oil)/g body weight, by a biweekly

Abbreviation: ECM, extracellular matrix; HSCs, hepatic stellate cells; lncRNAs, long non-coding RNAs; APTR, *Alu*-mediated p21 transcriptional regulator; CCl_4, carbon tetrachloride; BDL, bile duct ligation; EdU, 5-ethynyl-2-deoxyuridine; IHC, Immunohistochemistry; qRT-PCR, quantitative real-time PCR.

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intraperitoneal injection for 8 weeks. Control mice were treated with olive oil. For bile duct ligation (BDL), mice were anaesthetized and received midline laparotomy. The common bile duct was identified and cut between ligatures before abdominal closure. The sham operation mice were taken as the control. The Masson-positive area were quantified in at least 10 independent fields by histomorphometry using Olympus Cell (Olympus Soft Imaging Solution GmbH, Münster, Germany) and Image J software (Macbiophotonics, McMaster University, Hamilton, ON, Canada), respectively. All mice were in compliance with the criteria outlined in the Animal Experimentation Ethics Committee Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Fudan University.

2.2. Human specimens

Written informed consent was obtained from all patients prior to liver tissues and blood collection. Nine control patients and 15 liver cirrhosis patients undergoing partial liver resection or liver biopsy were selected from the First Affiliated Hospital of Wenzhou Medical University and Ningbo Yinzhou Second Hospital. To detect serum APTR levels, peripheral blood was drawn from 34 liver cirrhosis patients and 24 control patients. Liver cirrhosis was diagnosed according to the typical morphological findings using computed tomography or ultrasound analysis. This study was performed in compliance with the Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University and Ningbo Yinzhou Second Hospital Ethics Committee, respectively. The characteristics of the patients are summarized in Supplementary Tables S1 and S2.

2.3. Isolation and culture of primary HSCs

Primary HSCs were isolated as previously described [16]. Isolated cells were plated in culture dish in DMEM supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin. In the case of TGF-β_1 treatment , recombinant TGF-β_1 (Sigma–Aldrich) was added at a final concentration of 5 ng/mL.

2.4. Plasmid transfection

The *APTR and p21* siRNA sequences are listed in Supplementary Tables S3. The scrambled siRNA was used as the negative control. The transfection of siRNA was performed using Lipofectamine reagent (Invitrogen Carlsbad, CA, USA) at 50 nM final concentration. Twenty mice were randomly divided into four groups as follows: olive oil (control, $n = 5$), model (CCL_4, $n = 5$), CCL_4 combined with Adenovirus vector (Ad-NC, $n = 5$), and CCL_4 combined with adenovirus expressing APTR siRNA3 (Ad-APTR siRNA3, $n = 5$). Ad-APTR siRNA3 was obtained from Shanghai GenePharma Corporation. Ad-APTR siRNA3 (1×10^9 pfu/100 μL) were injected every two weeks by way of the tail vein. After four weeks, these mice were sacrificed.

2.5. Cell proliferation assay

The proliferation of primary HSCs was determined by 5-ethynyl-2-deoxyuridine (EdU) incorporation kit (RiboBio, Guangzhou, China). Cells were incubated with PDGF-bb (20 ng/mL; R&D Systems, Minneapolis, MN, USA). After EdU was labeled for 24 h, cells were formalin-fixed and stained. The proliferation rate was determined by normalizing the number of EdU-positive cells to Hoechst 33342-stained cells.

2.6. Cell cycle

Cells were trypsinized and fixed in 75% ethanol overnight at −20 °C. Then cells were treated with 0.01% RNase for 30 min at 25 °C and 500 μg/mL of propidium iodide was added just before the analysis. The DNA content was detected using a FACS Calibur flow cytometer (Becton, Dickinson and Company, Colombia, USA).

2.7. Hepatic hydroxyproline content

The content of hydroxyproline was measured colorimetrically. Briefly, liver tissue was homogenized and then hydrolyzed at 110 °C for 2 h. After the filtered supernatant was treated with chloramine T/citrate-acetate buffer, we dissolved Ehrlich's reagent in 60% perchloric acid and then the mixture was incubated at 60 °C for 25 min. The absorbance was read at a wave length of 558 nm.

2.8. Immunohistochemistry (IHC)

IHC analysis was performed using the standard biotin-peroxidase complex method as previously described [17]. Briefly, the slides were incubated overnight at 4 °C with primary antibody against α-SMA (Abcam, Cambridge, MA, USA). Biotinylated goat anti-mouse secondary antibody (Abcam) was incubated for 40 min at room temperature. α-SMA was quantified by histomorphometry using Olympus Cell. A minimum of 15 fields per sample was quantified.

2.9. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from liver tissues and HSCs using Trizol Reagent (Invitrogen). To quantify serum APTR expression, total RNA was isolated using an miRNeasy Serum/Plasma Mini kit (Qiagen, Valencia, CA, USA). For cDNA synthesis, reverse transcription reaction was performed using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, China). qRT-PCR was performed using KAPA SYBR FAST qPCR kit master mix (2×) universal (KapaBiosystems, Boston, MA, USA). All primers used in this study are listed in Supplementary Table S4. Relative mRNA and APTR expression was calculated using GAPDH as internal control.

2.10. Western blotting analysis

Total proteins were separated by SDS-PAGE and blotted onto PVDF membranes (Millipore, Billerica, MA, USA). After blockade, nitrocellulose blots were incubated overnight with primary antibodies (β-actin, Col1A1, α-SMA and p21; Abcam). Then the secondary IRdye800-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Rockland, Limerick, PA, USA) were incubated at 37 °C for 1 h. Antibody binding was examined using an Odyssey infrared scanner (**Li-Cor Biosciences Inc**., Lincoln, NE, USA).

2.11. Statistical analysis

Data were expressed as mean ± SD. Statistical comparisons between multiple groups were made using one-way analysis of variance. Differences between two groups were compared using a Student's *t*-test. $P < 0.05$ was considered significant. All statistical analyses were performed with the SPSS 13.0 (IBM, Armonk, NY, USA).

3. Results

3.1. APTR expression is upregulated in liver fibrosis

It has been reported that APTR acts as a proliferative factor in glioblastoma [15]. Here we explored whether APTR was involved in the progression of liver fibrosis. The degree of liver fibrosis was evaluated by HE staining and Masson staining (Fig. 1A, B). The expression of APTR was significantly upregulated in fibrotic liver tissues (Fig. 1C). We next established a second mouse liver fibrosis model of BDL (Supplementary Fig.S1A, B). The expression of APTR was increased in BDL mice compared with the control (Supplementary Fig. S1E). Finally, we analyzed these findings in human cirrhotic liver tissues (Supplementary Fig. S1C, D). The expression of APTR in cirrhotic liver tissues was also increased compared with normal liver tissues (Supplementary Fig. S1F).

The activation of HSCs is the pivotal step in the initiation and progression of liver fibrosis. Therefore, we determined the expression of APTR in primary HSCs. The expression of APTR was elevated in primary HSCs upon culture-induced activation (Fig. 1D). Compared with oil treatment, the expression of APTR was also increased in HSCs isolated from mice, 8 weeks following CCL_4 treatment (Fig. 1E).

Collectively, our data demonstrated that the expression of APTR is upregulated in liver fibrosis, independently of the type of liver fibrosis model.

3.2. Knockdown of APTR attenuates the activation of HSCs

We next analyzed the effect of APTR knockdown on primary HSCs. We constructed three pairs of APTR siRNAs, of which APTR siRNA3 presented the strongest inhibitory effect on APTR mRNA expression and was therefore selected for further experiments (Fig. 2A). As indicated in Fig. 2B and C, knockdown of APTR reduced the mRNA and protein expression of α-SMA and Col1A1.

TGF-β_1 can mediate the expression of many non-coding genes, such as the miR-29 family and miR-133a [18,19]. Here we examined whether TGF-β_1 could mediate the expression of APTR in HSCs. As expected, TGF-β_1 led to an increase of APTR expression (Fig. 2D). Importantly, APTR siRNA3 abrogated TGF-β_1-induced upregulation of α-SMA expression in HSCs (Fig. 2E).

In summary, our data suggested a critical role of APTR silencing in inhibiting the activation of HSCs in a TGF-β_1-dependent manner.

3.3. APTR silencing ameliorates mouse liver fibrosis in vivo

We investigated whether decreased APTR expression could ameliorate CCL_4-induced liver fibrosis. As shown in Fig. 3A, APTR siRNA3 delivery by adenovirus effectively lowered the level of APTR. Compared with CCL_4, the hepatic hydroxyproline content was reduced in Ad-APTR siRNA3 (Fig. 3B). The decrease of α-SMA and Col1A1 expression was observed in mice treated with Ad-APTR siRNA3 (Fig. 3C, D). Histochemical analysis showed that CCL_4-induced liver fibrosis was alleviated by APTR silencing (Fig. 3E, G). IHC results showed that CCL_4-induced up-regulation of α-SMA protein was down-regulated by APTR knockdown (Fig. 3F, H).

Taken together, our data showed that the progression of liver fibrosis can be attenuated by APTR down-expression *in vivo*.

3.4. Silencing of APTR increases the expression of p21 in primary HSCs

APTR can recruit the polycomb repressive complex 2 complex to the p21 promoter, thereby inhibiting the expression of p21 [15]. Here we investigated whether APTR functioned through this mechanism in HSCs. The expression of p21 was downregulated in fibrotic livers upon CCL_4 treatment (Fig. 4A). In addition, the expression of APTR and p21 was inversely correlated (Fig. 4B). Furthermore, silencing of APTR increased the mRNA and protein expression of p21 in primary HSCs (Fig. 4C, D).

3.5. Downregulation of APTR inhibits cell cycle and proliferation of HSCs through increasing p21

we next investigated whether p21 was required for inhibitory effect of APTR silencing on the cell cycle. p21 siRNA1 exerted the strongest inhibitory effect on the mRNA expression of p21 and was therefore selected for further experiments (Fig. 4E). Compared with the control, APTR siRNA3 decreased S phase population from 33.23% to 16.73%, while G1 phase population increased from 49.85% to 64.82%. When we added p21 siRNA1 to APTR siRNA3 group, the S

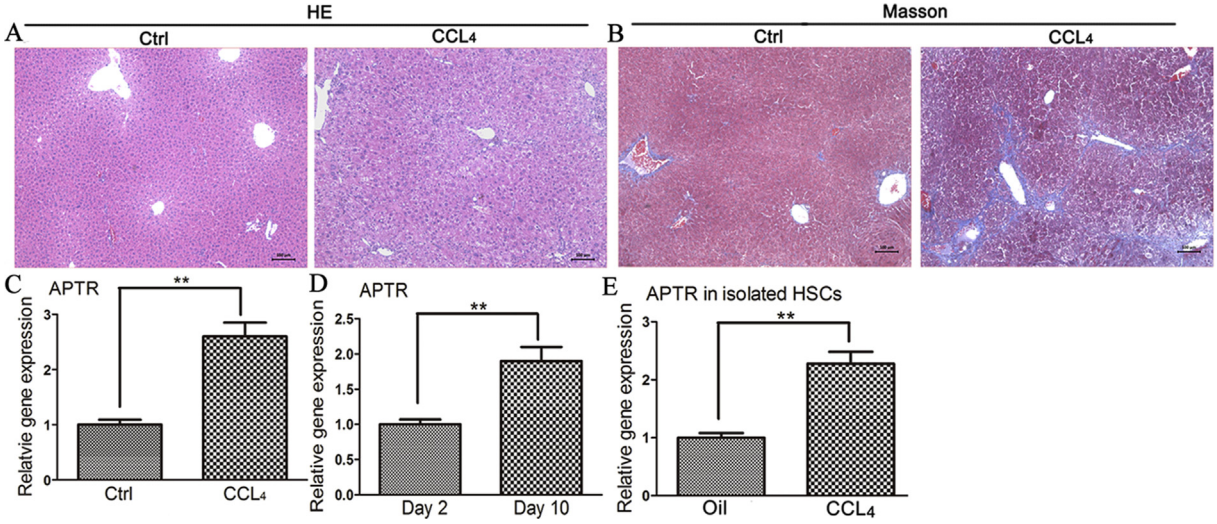

Fig. 1. Expression of APTR in fibrotic liver tissues and activated HSCs. (A and B) Normal liver tissues and fibrotic liver tissues were assessed by HE staining and Masson staining. Scale bars, 100 μm. (C) Expression of APTR in normal liver tissues (n = 9) and CCL_4-induced fibrotic liver tissues (n = 9). (D) Expression of APTR in primary HSCs at day 2 and day 10. (E) Expression of APTR in HSCs isolated from mice treated with CCL_4 or oil for 8 weeks. $^{**}P < 0.01$.

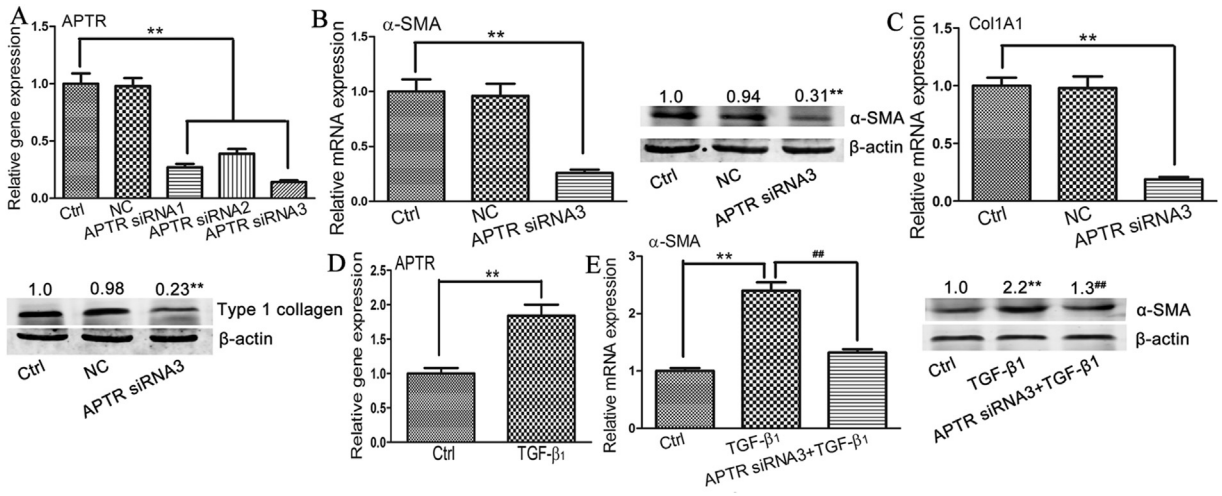

Fig. 2. Knockdown of APTR suppresses HSC activation *in vitro*. (A) Relative mRNA expression of APTR was evaluated by qRT-PCR in primary HSCs transfected with APTR siRNAs. (B) The mRNA and protein levels of α-SMA were analyzed by qRT-PCR and western blotting in primary HSCs transfected with APTR siRNA3. (C) The mRNA and protein levels of Col1A1 were analyzed by qRT-PCR and western blotting in primary HSCs transfected with APTR siRNA3. (D) The level of APTR was analyzed by qRT-PCR in primary HSCs treated with TGF-β_1. (E) APTR siRNA3 prevents TGF-β_1-induced upregulation of α-SMA. $^{**}P < 0.01$ compared with the control. $^{\#\#}P < 0.01$ compared with TGF-β_1.

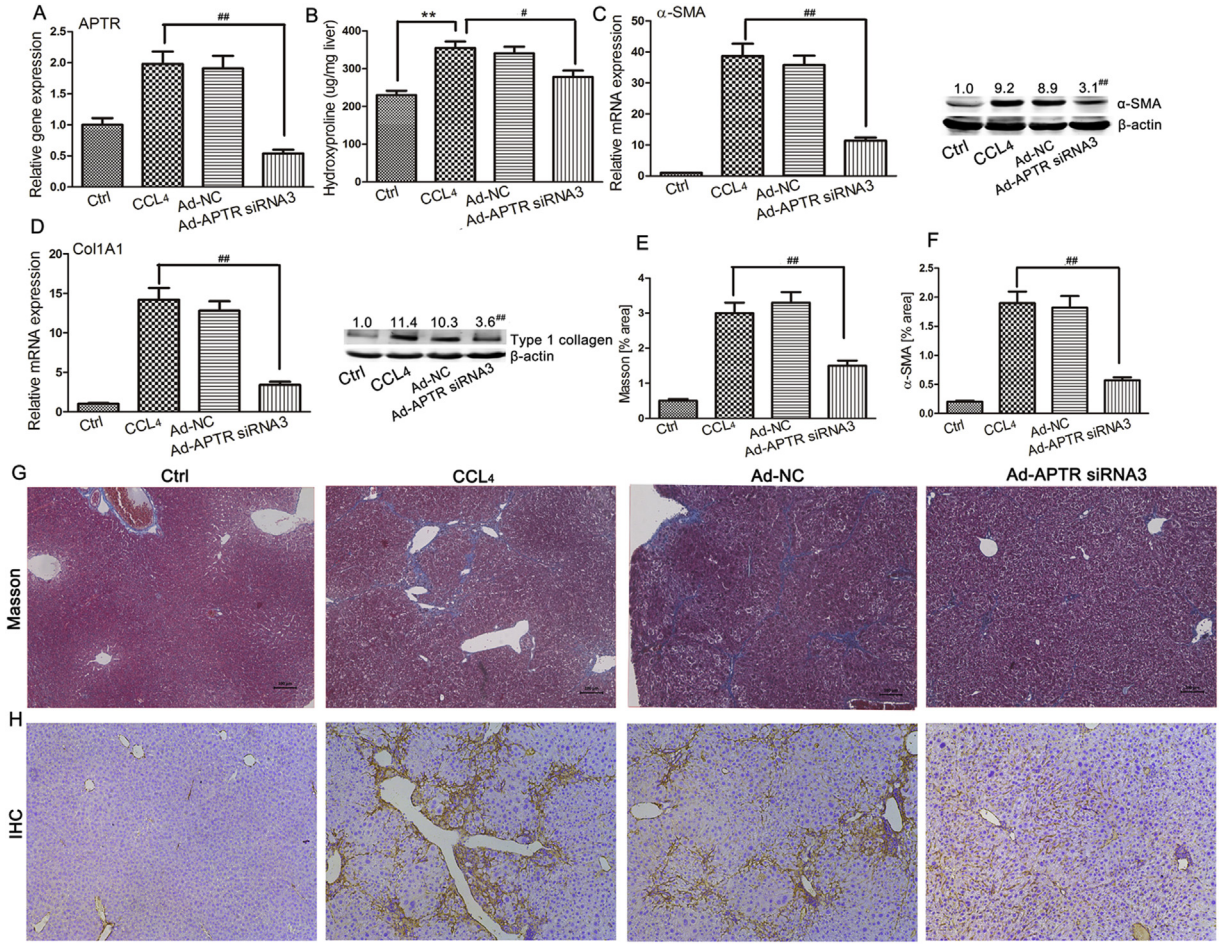

Fig. 3. Inhibitory role of APTR siRNA3 in the progression of liver fibrosis *in vivo*. (A) Hepatic APTR expression was analyzed by qRT-PCR. (B) Quantification of hepatic hydroxyproline content; the data are expressed as hydroxyproline (μg)/liver wet weight (mg). (C) The mRNA and protein expression of α-SMA was analyzed by qRT-PCR and western blotting. (D) The mRNA and protein expression of Col1A1 was analyzed by qRT-PCR and western blotting. (E and G) Accumulation of collagen was assessed by Masson staining. Scale bars, 100 μm. (F and H) Hepatic α-SMA protein expression was determined by an IHC assay. $^{**}P < 0.01$ compared with the control. $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ compared with CCl_4.

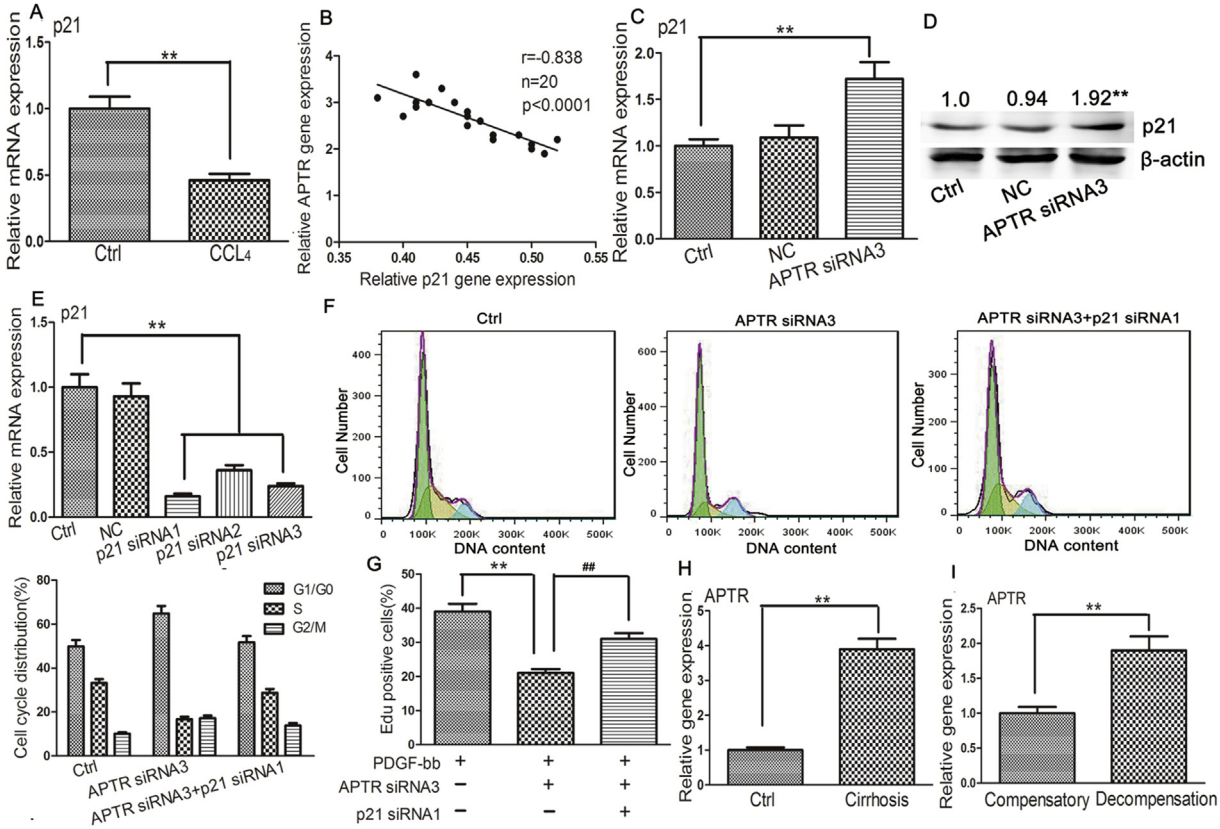

Fig. 4. p21 silencing attenuates the inhibitory effect of APTR knockdown on cell cycle and cell proliferation as well as serum APTR levels were increased in patients with liver cirrhosis. (A) Relative mRNA expression of p21 in normal liver tissues (n = 9) and fibrotic liver tissues (n = 9) was analyzed by qRT-PCR. (B) Correlation between the gene expression of p21 and APTR in 20 liver fibrosis tissue specimens ($r = -0.838$, $p < 0.0001$). (C and D) The mRNA and protein levels of p21 were measured by qRT-PCR and western blotting in primary HSCs transfected with APTR siRNA3. (E) Relative mRNA expression of p21 was analyzed by qRT-PCR in primary HSCs transfected with p21 siRNAs. (F) Inhibitory effect of APTR silencing on cell cycle was rescued by p21 siRNA1. (G) Inhibitory effect of APTR silencing on cell proliferation was attenuated by p21 siRNA1. (H) Serum APTR levels were analyzed by qRT-PCR in control patients (n = 24) and liver cirrhotic patients (n = 34). (I) Serum APTR levels were analyzed by qRT-PCR in patients with compensated cirrhosis (n = 16) and patients with decompensated cirrhosis (n = 18). $^{**}P < 0.01$ compared with the control or compensatory. $^{\#\#}P < 0.01$ compared with APTR siRNA3.

phase population was increased to 28.79%, while the G1 population was reduced to 51.71% (Fig. 4F).

We further investigated whether p21 was required for the APTR-induced cell proliferation by Edu incorporation assay. In the presence of PDGF-bb, APTR knockdown resulted in a decrease of HSC proliferation, while p21 siRNA1 could increase the down-regulated cell proliferation by APTR knockdown (Fig. 4G).

To sum up, our findings suggested that p21 is involved in the profibrogenic effects of APTR on HSCs.

3.6. Serum APTR levels are increased in patients with liver cirrhosis

We studied whether these results in mice could be validated in the serum of patients with liver cirrhosis. Serum levels of APTR in patients with liver cirrhosis were higher than those in control patients (Fig. 4H). We further investigated whether serum APTR levels were related with the severity of liver cirrhosis. Serum levels of APTR in patients with decompensated cirrhosis were higher than those in patients with compensated cirrhosis, demonstrating that the severity of liver cirrhosis could be reflected by serum APTR levels (Fig. 4I).

4. Discussion

In this study, we provided evidence for a role of APTR in liver fibrosis. In addition, we elucidated the mechanism of APTR in liver fibrogenesis. Finally, the serum levels of APTR were related with the severity of liver cirrhosis. Our findings provided substantial basis for developing a gene therapy to prevent the progression of liver fibrosis.

The expression of APTR was increased in mouse liver fibrosis and activated HSCs. Increased APTR expression was also found in human liver cirrhosis. Although there is little homology between different species regarding lncRNAs, our results implied that the functions of APTR appear to be conserved and knockdown of APTR has antifibrotic activity.

lncRNAs have emerged as pivotal mediators of gene expression and their dysregulation is a usual feature of human diseases [20–22]. Accordingly, it is possible that correction of deregulated lncRNA expression can return a cell from a pathological state to its normal phenotype. Our data indicated that knockdown of APTR reduces the expression of α-SMA and Col1A1 *in vitro*. Adenovirus-mediated gene transfer provided an efficient reduction of APTR expression in liver tissues, resulting in the decline of collagen synthesis and hydroxyproline contents. Our findings has clearly demonstrated that APTR plays a critical role in liver fibrogenesis, and we identify APTR as a profibrotic mediator in live fibrosis.

APTR is necessary for cell proliferation. APTR silencing can induce p21 transcription, thereby inhibiting cell growth in the U87 cells [15]. Therefore, we investigated whether APTR functions through this mechanism in HSCs. Our results demonstrated

that there is a negative correlation between the expression of APTR and p21 in mice liver fibrosis samples. Decreases in APTR could increase the expression of p21. Importantly, the decreased S phase and cell proliferation by APTR knockdown was partially rescued by p21 siRNA1 in primary HSCs. In summary, our data clarified that APTR can accelerate cell cycle and increase the proliferation of HSCs via negatively regulating p21 in mouse liver fibrosis.

APTR is encoded elsewhere in the p21 gene locus. It is not regulated by the tumor suppressor p53 [15]. The mechanism involved in the regulation of APTR expression in HSCs has yet to be elucidated. Progression of liver fibrosis is accompanied by dysregulation of cytokines, among which TGF-β_1 is the most dominant stimulus to ECM production [23]. Here we showed that the expression of APTR is increased in HSCs upon stimulation of TGF-β_1. Silencing of APTR reduced the upregulation of α-SMA induced by TGF-β_1. Thus, we identified APTR as a new member of TGF-β_1-dependent ncRNAs that are involved in liver fibrosis.

Serum lncRNAs have been extensively studied as biomarkers of human diseases [24,25]. Circulating lncRNA TrAnscript Predicting Survival in AKI is a predictor of mortality in critically ill patients with acute kidney injury [26]. Plasma H19 levels may become a new complementary tumor marker for gastric cancer [27]. Our data indicated that APTR is elevated in the serum of cirrhotic patients, especially in patients with decompensated cirrhosis. To sum up, we provided evidence of APTR as a novel biomarker for liver cirrhosis.

To date, there are no efficient antifibrotic drugs available for chronic liver disease. Gene therapy is a promising approach. Thus, APTR may become a therapeutic target for liver fibrosis in the future.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.124.

Transparency document

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