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ORIGINAL ARTICLE

C1QTNF1-AS1 regulates the occurrence and development of hepatocellular carcinoma by regulating miR-221-3p/*SOCS3*

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Abstract

Background The aim of our study was to explore how C1QTNF1-AS1 regulated miR-221-3p/*SOCS3* axis in human hepatocellular carcinoma (HCC).

Methods Differentially expressed lncRNAs and genes were examined via RNA-seq. GO analysis and KEGG pathway enrichment analysis were carried out based on the function of dys-regulated mRNAs. RT-qPCR was employed to detect the relative mRNA expression level of C1QTNF1-AS1, miR-221-3p, *SOCS3* and key genes in the JAK/STAT signaling pathway in HCC tissues and cells, and western blot analysis was conducted to detect the relative protein expression levels of *SOCS3* and key proteins in the JAK/STAT signaling pathway in HCC tissues and cells. MTT assay, transwell assay and flow cytometry were utilized to assess HCC cell proliferation, invasion, migration and apoptosis. Dual luciferase reporter gene assay was used to verify the targeted relationship between C1QTNF1-AS1 and miR-221-3p, as well as between miR-221-3p and *SOCS3*. A tumorigenicity assay in nude mice was conducted to investigate the effects of C1QTNF1-AS1 on HCC tumor growth in vivo.

Results C1QTNF1-AS1 and *SOCS3* were down-regulated, while miR-221-3p was up-regulated in HCC tissues and cells. In HepG2 and Huh7 cells, overexpression of C1QTNF1-AS1 or *SOCS3*, as well as silence of miR-221-3p inhibited HCC cell proliferation, migration, and invasion and promoted HCC cell apoptosis. The results of the dual luciferase reporter gene assay indicated that miR-221-3p could directly target both C1QTNF1-AS1 and *SOCS3*. In addition, up-regulation of C1QTNF1-AS1 suppressed HCC tumor growth in vivo.

Conclusion Overexpression of C1QTNF1-AS1 down-regulated miR-221-3p and subsequently up-regulated *SOCS3*, thereby inhibiting HCC cell proliferation, migration and invasion and promoting apoptosis through the JAK/STAT signaling pathway.

Keywords C1QTNF1-AS1 · miR-221-3p · *SOCS3* · Hepatocellular carcinoma

Introduction

As one of the ten most common human malignancies and the third major cause of cancer-related death rate worldwide, hepatocellular carcinoma (HCC) results in an increasing global incidence and poor prognosis [1]. Although treatments for HCC patients are now common,

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recurrence and metastasis lead to 5-year survival rates that remain unsatisfactory. Thus, it is urgent for scientists to explore the precise mechanisms underlying liver carcinogenesis and identify new molecular targets affecting HCC tumor growth to develop new therapeutic strategies [2]. Epigenetic alteration has been shown to be a critical factor in cancer development. Dysregulation of lncRNAs and miRNAs that affect protein-coding genes and their associated signaling pathways is an important part of the epigenetic alterations in HCC and remains to be explored [3].

Long noncoding RNAs (lncRNAs) are a subtype of noncoding RNAs longer than 200 nucleotides that lack the potential to encode protein, with 50 capped and 30 polyadenylated lncRNAs currently identified. LncRNAs are involved in various biological processes by regulating gene expression in a *cis* or *trans* manner [4]. In cancer, lncRNAs are often dysregulated. Thus, elucidating lncRNA functions and molecular mechanisms will be important for developing new strategies for cancer diagnosis and treatment [5]. The functions of lncRNAs are diverse, and lncRNAs regulate gene expression at epigenetic, transcriptional, posttranscriptional, and translational levels. Previous research has shown that lncRNA-UCA1 can inhibit miR-216b to promote HCC progression through activation of the FGFR1/ERK signaling pathway. Thus, lncRNAs may act as competing endogenous RNAs (ceRNAs) that can down-regulate miRNA expression and thereby modulate their targets to affect cancer development and progression [6]. Another study in HCC indicated that lncRNA-ATB competitively binds to the miR-200 family to up-regulate ZEB1 and ZEB2, inducing epithelial–mesenchymal transition and HCC invasion [7]. However, the regulation of lncRNA C1QTNF1-AS1 in HCC is still unclear.

MicroRNAs (miRNAs) are endogenous non-coding small RNAs approximately 21 nucleotides long. MiRNAs regulate gene expression by binding to the 3′-untranslated region (3′-UTR) of target mRNAs and post-transcriptionally control target expression. Previous studies have demonstrated that by regulating tumor suppressor genes or oncogenes, miRNAs play crucial roles in many cellular biological processes, especially in tumor progression [8]. In a HCC study, scientists revealed that miR-21 was aberrantly expressed in HCC tissues and could increase migration and invasion of side population (SP) cells by directly targeting PTEN, RECK and PDCD4 [9]. As an anti-angiogenic gene-regulating miRNA, miR-221-3p is encoded by a gene cluster on the X chromosome. MiR-221-3p was found to initiate changes in proliferation, migration, invasion and apoptosis in a variety of human malignancy cells. For example, a previous study reported that miR-221-3p enhanced cell proliferation and impeded cell apoptosis in pancreatic cancer, suggesting that miR-221-3p could be a novel potential candidate for PCA treatment [10]. However, studies of miR-221-3p in HCC are absent.

SOCS3 is a member of the suppressors of cytokine signaling (SOCS) family of proteins. SOCS3 directly interacts with JAK molecules and further inhibits STAT phosphorylation by binding with a specific phosphorylated tyrosine residue in the kinase domain of JAK molecules. In addition, JAK and STAT are crucial members of the JAK/STAT signaling pathway. Cytokines and growth factors first activate JAK and then STAT to trans-activate target genes, which determine immune reaction, cell growth and differentiation. Thus, SOCS3 can terminate signal transduction through the JAK/STAT signaling pathway and thus affect tumor progression [11]. SOCS3 is often aberrantly inactivated in tumor tissues. According to a recent study, in HCC, SOCS3 was found to be silenced via methylation, which resulted in enhanced cell growth and migration through restriction of STAT activity in HCC cells [12]. SOCS3 can also be modulated by miRNA as a target gene. One study found that SOCS3 might be a target of miR-455-5p. Wang et al reported that miR-455-5p promoted non-small cell lung cancer development and metastasis by inhibiting SOCS3 [13]. Another study demonstrated that in hepatitis virus-associated HCC, miR-221 targeted SOCS1 and SOCS3 to enhance the ability of IFN to inhibit HCV replication [14]. The relationship between miR-221-3p and *SOCS3* in HCC progression has not yet been studied. In the present study, we found that C1QTNF1-AS1, miR-221-3p and *SOCS3* are dysregulated in HCC and examined their targeted regulatory relationships using bioinformatics analysis. Through biological experiments, we confirmed that C1QTNF1-AS1 regulates the miR-221-3p/*SOCS3* axis to affect the JAK/STAT signaling pathway and ultimately alters HCC cell behavior and tumor growth.

Materials and methods

Bioinformatic analysis

RNA-seq analysis was conducted to analyze differentially expressed lncRNAs and genes between normal tissues and HCC tissues. RNA-seq expression profile data were downloaded from NCBI GEO DataSets (https://www.ncbi.nlm.nih.gov/gds/), and lncRNA and gene expression data in normal and HCC tissues were obtained after quantification and background correction. Most differentially expressed genes and lncRNAs with fold change values > 2 and $p < 0.05$ were screened out using Limma package in the R environment and were presented in a heat map. Thereafter, we utilized GO plot, a R package for visually combining expression data with functional analysis, for GO enrichment analysis. We used KEGG Orthology Based Annotation System software to detect the

statistical enrichment of the candidate target genes in KEGG pathways. The targeted relationships between miRNA and mRNA were analyzed using TargetScan 7.1 (https://www.targetscan.org/vert_71/), while the binding sites between lncRNA and miRNA were predicted using miRcode (https://www.mircode.org/index.php). A co-expression network was built after the correlation coefficient between differentially expressed lncRNAs and genes was calculated.

Cell culture and tissue collection

Cells: The immortalized human liver cell line MIHA and hepatocellular carcinoma cell lines HepG2 and Huh7 were obtained from American Type Culture Collection. All three cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin in a 5% CO_2 atmosphere at 37 °C. Tissues: Samples from 11 HCC cases and 11 paired adjacent tissues were obtained from China-Japan Union Hospital, Jilin University. Specimens were stored at − 80 °C for further analysis.

Plasmid construction and cell transfection

Negative control (NC) vector, si-C1QTNF1-AS1, pcDNA-C1QTNF1-AS1, p LUC-C1QTNF1-AS1-wt, pLUC-C1QTNF1-AS1-mut, miR-221-3p mimic, miR-221-3p inhibitor, miR-221-3p mimics+pcDNA-C1QTNF1-AS1, pLUC-*SOCS3*-wt, pLUC-*SOCS3*-mut, si-*SOCS3*, pcDNA-*SOCS3*, and pcDNA-*SOCS3*+miR-221-3p mimic were used in this study. All plasmids were designed and purchased from GenePharma (Shanghai, China). At 24 h prior to transfection, HepG2 and Huh7 cells were re-suspended in DMEM and inoculated in 6-well culture plates for 18–24 h in 5% CO_2 at 37 °C to achieve 80% confluence per well. Three hours before transfection, the cell culture medium was replaced with serum- and antibiotic-free medium. Using Lipofectamine 2000 reagent (Life Technologies, MD, USA), cells were transfected with plasmids and incubated under the same conditions. After 8 h, complete medium was added, and cells were incubated for further analysis. Selection medium (600 μg/mL G418) was applied for routine maintenance of positively transfected clones.

Real-time quantitative polymerase chain reaction (RT-qPCR) assay

Total RNA was extracted from tissue samples and cell lines using a RNAiso kit (Takara, China) according to the manufacturer's instructions. Quantification of the total RNA was performed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., MA, USA). Then, 0.2 μg of total RNA was used for reverse transcription using a TaqMan RT Kit (Toyobo, Japan) to produce cDNA. Thereafter, the product was subjected to real-time quantitative PCR analysis using Go Tag Green Master Mix (Toyobo, Japan). The PCR reaction conditions were as follows: pre-denaturation at 95 °C for 5 min; denaturation at 95 °C for 30 s; annealing at 60 °C for 10 s; extension at 72 °C for 30 s for 40 cycles, followed by 72 °C for 10 min. We used the $2^{-\Delta\Delta}$ method to quantify the relative mRNA expression levels of the examined genes and GAPDH for normalization. All of the experiments were performed in triplicate.

MTT assay

HepG2 and Huh7 cells were suspended in complete medium and then seeded in 96-well plates at 2×10^4 cells/mL. On the 1st, 2nd, 3rd, 4th, 5th, and 6th day after transfection, cells were incubated with 10 μL of 0.5% MTT reagent for 4 h at 37 °C. Then, the supernatant was discarded, and 150 μL dimethyl sulfoxide (DMSO) was added to each well. To dissolve crystals completely, the 96-well plates were shaken for 5 min. The optical density (OD) value for each sample was detected at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories, CA, USA). All of the experiments were repeated three times.

Transwell assay

According to the manufacturer's instructions, cell migration and invasion were examined with Transwell chamber assays (8 μm; Millipore, USA). The Transwell chamber was coated with 80 μL Matrigel (Takara, Japan), and then, 50 μL serum-free medium was added. HepG2 and Huh7 cells were digested with pancreatin and re-suspended after transfection. After counting, a total of 2×10^4 cells in 200 μL serum-free DMEM were incubated in the upper well of Transwell plates (BD Biosciences, San Jose, CA, USA), while 500 μL complete DMEM was placed into the lower chamber. After being cultured at 37 °C for 24 h, cells on the upper surface were washed with PBS twice and scraped with a cotton swab, while the cells that invaded and migrated to the lower surface were washed and fixed with 4% formaldehyde for 30 min, followed by staining with 1% crystal violet. Cell migration was evaluated as described above, without the Matrigel coating. Images of migrating and invading cells were obtained using an inverted fluorescence microscope (Nikon, Tokyo, Japan). Each experiment was repeated three times.

Flow cytometry

Cell apoptosis was analyzed using a PE Annexin V Kit (BD, CA, USA) according to the manufacturer's instructions. Cells were first collected, washed twice with cold phosphate-buffered saline (PBS), and then cultured in 5 μL Annexin V-FITC for 15 min without light at room temperature. A FACSCalibur flow cytometer (BD, CA, USA) was used to detect apoptosis. Analysis was carried out using FACSDiva software (BD, CA, USA). Each experiment was repeated at least three times.

Western blot analysis

Total protein was extracted from tissues and cells using RIPA lysis buffer (Beyotime, Shanghai, China). The protein concentration was quantified using a bicinchoninic acid (BCA) protein concentration assay kit (Biyuntian, Beijing, China). Then, SDS-PAGE was used to separate proteins. The isolated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA), which were then immersed in TBST containing 5% skimmed milk at room temperature for 1 h. Thereafter, the membranes were incubated at 4 °C with primary antibodies (anti-STAT3, ab119352, 1:5000; anti-STAT3 (phospho Y705), ab76315, 1:20,000; anti-c-Myc, ab32072, 1:1000; anti-MCL1, ab32087, 1:5000; anti-*SOCS3*, ab16030, 1:300; anti-β-actin, ab8227, 1:500, Abcam, MA, USA) overnight, washed with TBST three times, and incubated with secondary antibody (ab7090, 1:2000, Abcam, MA, USA) in a shaker at room temperature. The blots were then washed, and proteins were visualized using ECL-plus reagents (BD, USA). For band density measurements, ImageJ software (Version 1.48 μ, Bethesda, USA) was used, and β-actin served as the internal control. Each experiment was repeated at least three times.

Dual-luciferase reporter gene assay

HepG2 and Huh7 cells were digested by trypsin and collected. Following the manual instructions, an EasyPure Blood Genomic DNA Kit was used for genomic DNA extraction. A full-length fragment or fragment containing a mutant sequence of the 3′-UTR of the C1QTNF1-AS1 gene and *SOCS3* gene were amplified using DNA as the template. PCR products isolated from agarose gel were then ligated into pLUC plasmids, which were then used to transform DH5α competent cells. Positive colonies were amplified and sequenced. Plasmids with the correct sequence were named pLUC-C1QTNF1-AS1-wt, pLUC-C1QTNF1-AS1-mut, pLUC-*SOCS3*-wt and pLUC-*SOCS3*-mut. Then, HepG2 and Huh7 cells were co-transfected with pLUC-C1QTNF1-AS1-wt or pLUC-C1QTNF1-AS1-mut and miR-221-3p mimic or miR-NC using Lipofectamine 2000 as the transfection reagent. In addition, HepG2 and Huh7 cells were co-transfected with pLUC-*SOCS3*-wt or pLUC-*SOCS3*-mut and miR-221-3p mimic or miR-NC. After 48 h of incubation, A Dual-Glo Luciferase Assay System was used to measure the relative luciferase activity. Each experiment was repeated at least three times.

Tumor formation in nude mice

Twenty male BALB/C nude mice (4 weeks old, purchased from Shanghai SLAC Laboratory Animal Co., Ltd, China) were used for tumor formation assays. HepG2 and Huh7 cells transfected with si-C1QTNF1-AS1 and pcDNA-C1QTNF1-AS1, respectively, were injected into the right flank of mice (5×10^6 cells per inoculation point). Tumor size and weight were monitored every 7 days for the indicated time using a Vernier caliper, and the tumor volumes were calculated as length × width × width/2. Each experiment was repeated at least three times.

Statistical analysis

All statistical analyses, including construction of diagrams, were performed using GraphPad Prism 6.0 software (Version 6, CA, USA). The data are presented as the mean ± standard deviation (SD). All data were obtained from at least three repetitions of each experiment. Comparisons of multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All differences were considered significant at a p value of 0.05.

Results

RNA-seq analysis of HCC tissues defined down-regulation of C1QTNF1-AS1 and *SOCS3* in HCC tissues.

As shown in Supplementary Figure 1A, B, the green area represented the highest quality scores, and most bases were located in this area, which indicated that the results of the RNA-seq analysis were all under high quality. By performing RNA-seq analysis, a total of 40 significantly expressed genes and lncRNAs were identified, including 20 up-regulated and 20 down-regulated genes or lncRNAs, which are presented in a heatmap (p value < 0.05, fold change ≥ 2, Supplementary Figure 1C, D). In addition, variation analysis indicated that the expression profile of genes and lncRNAs in HCC tissues could easily be distinguished from that of normal controls. In short, *SOCS3* and C1QTNF1-AS1 were both down-regulated in HCC tissues.

GO function enrichment and pathway enrichment analysis validated activation of the JAK/STAT signaling pathway in HCC tissues.

GO plot was utilized to perform functional analysis based on differentially expressed lncRNAs and genes. The results showed the top-ranked biological processes (BPs), cellular component (CC) and molecular function (MF), and the most significantly related functions were xenobiotic metabolic process, integral component of plasma membrane and cell adhesion ($-\log_{10}$ adjusted p value, Supplementary Figures 2A, B, C; Supplementary Tables 1, 2, 3). KEGG pathway enrichment analysis revealed the different biological functions in HCC and normal tissue samples and that the JAK/STAT signaling pathway was up-regulated in HCC tissues (Supplementary Figure 2D, E).

MiR-221-3p was predicted to target both C1QTNF1-AS1 and *SOCS3* in HCC.

miRNAs that might directly target C1QTNF1-AS1 or *SOCS3* were predicted using miRcode and TargetScan 7.1, respectively. 14 miRNAs were predicted to be able to directly target both C1QTNF1-AS1 and *SOCS3*. As shown in Fig. 1a, miR-221-3p was predicted to target both C1QTNF1-AS1 and *SOCS3*. Furthermore, the binding sites between C1QTNF1-AS1 and miR-221-3p, as well as *SOCS3* and miR-221-3p, are shown in Fig. 1b. In addition, according to the co-expression network, *SOCS3* was demonstrated to be co-expressed with C1QTNF1-AS1, as shown in Fig. 1c. Taken together, miR-221-3p was predicted to target both C1QTNF1-AS1 and *SOCS3* in HCC.

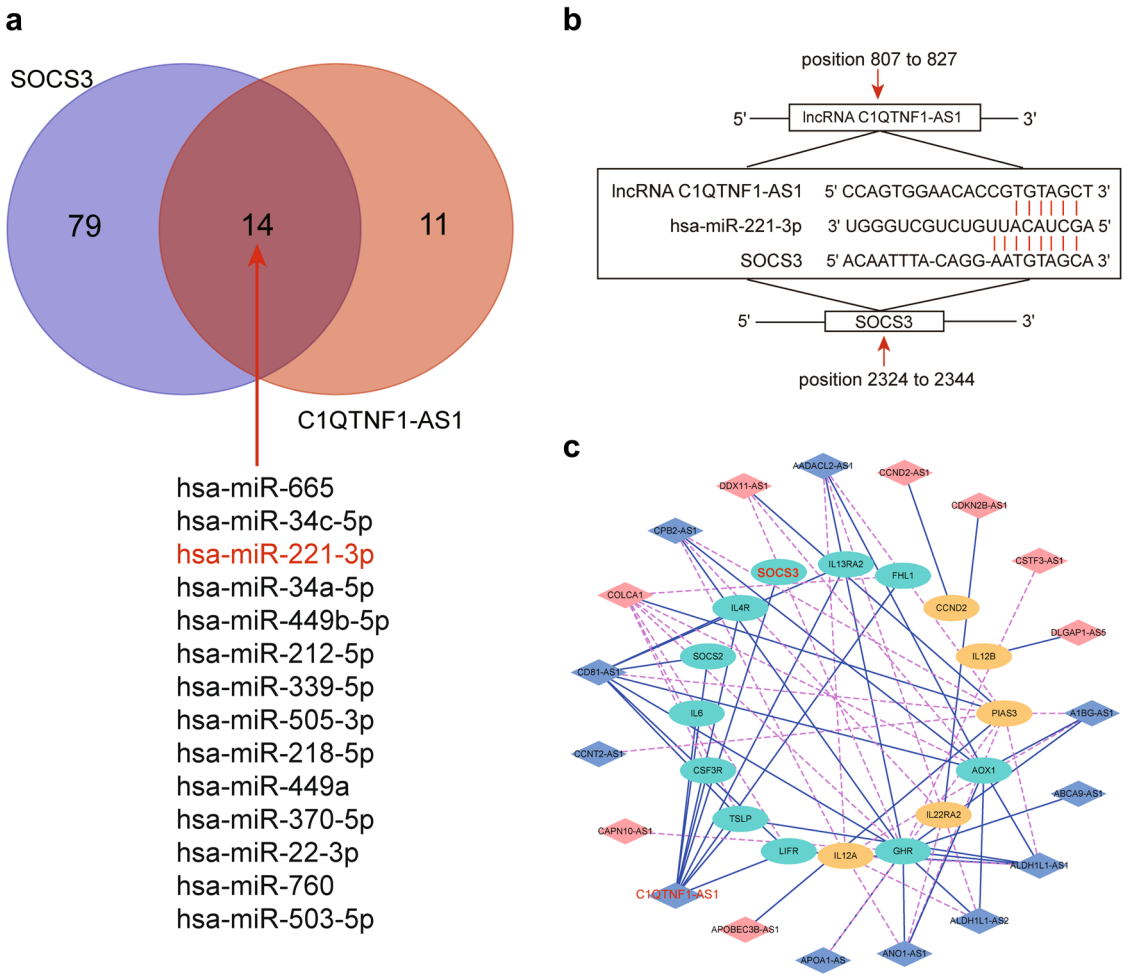
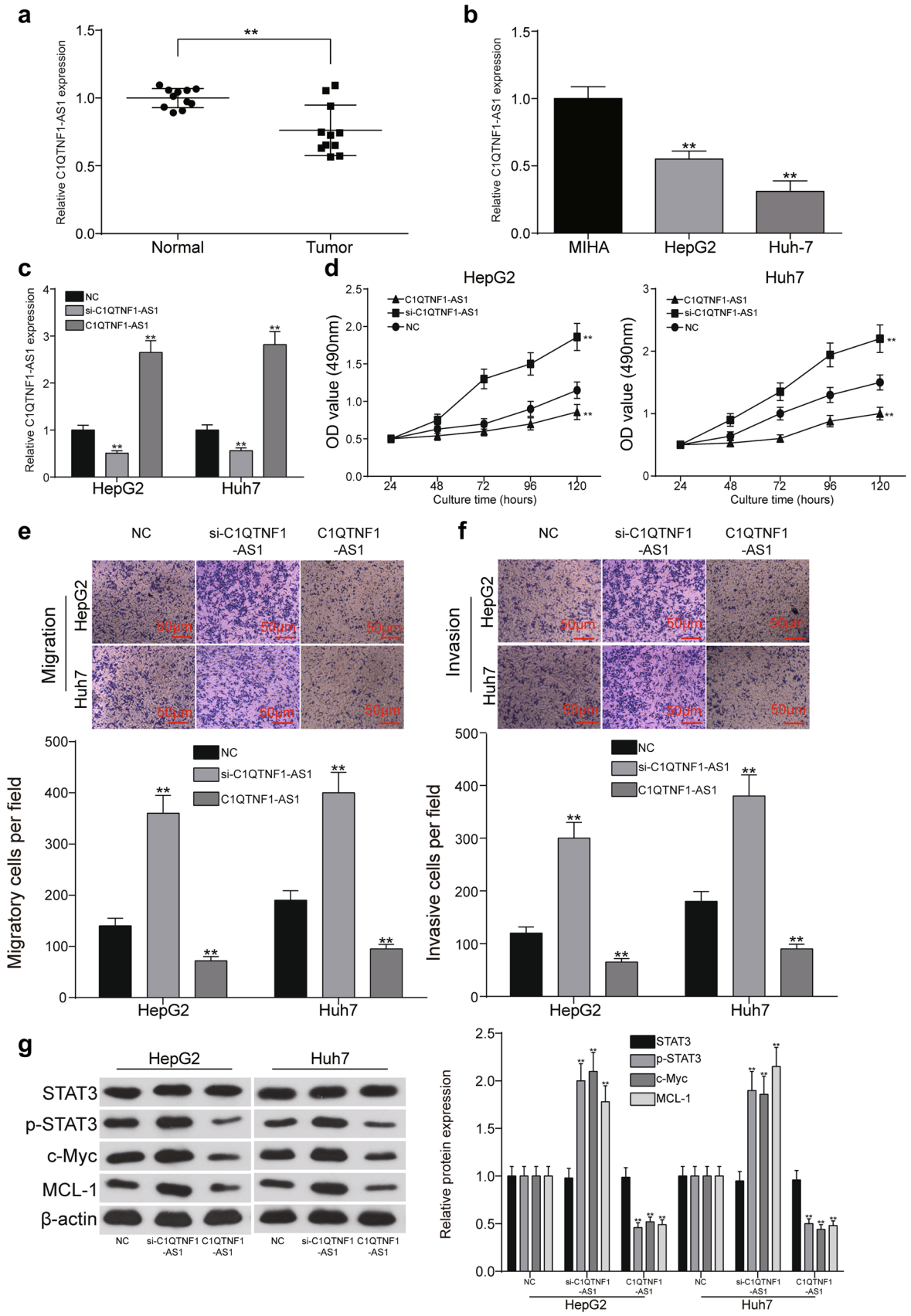


Fig. 1 miR-221-3p was the target of C1QTNF1-AS1 and SOCS3. **a** Prediction and screening of miRNA co-targeted by SOCS3 and C1QTNF1-AS1. miR-221-3p was one of the targets; **b** targeting binding site between C1QTNF1-AS1/miR-221-3p and between SOCS3/miR-221-3p; **c** co-expression network of mRNA and lncRNAs. C1QTNF1-AS1 and *SOCS3* were included


a
Relative C1QTNF1-AS1 expression
Normal
Tumor
b
Relative C1QTNF1-AS1 expression
MIHA
HepG2
Huh-7
c
Relative C1QTNF1-AS1 expression
NC
si-C1QTNF1-AS1
C1QTNF1-AS1
HepG2
Huh7
d
HepG2
Huh7
C1QTNF1-AS1
si-C1QTNF1-AS1
NC
OD value (490nm)
Culture time (hours)
e
NC
si-C1QTNF1 -AS1
C1QTNF1 -AS1
Migration
HepG2
Huh7
50μm
Migratory cells per field
f
Invasion
Invasive cells per field
g
HepG2
Huh7
STAT3
p-STAT3
c-Myc
MCL-1
β-actin
Relative protein expression

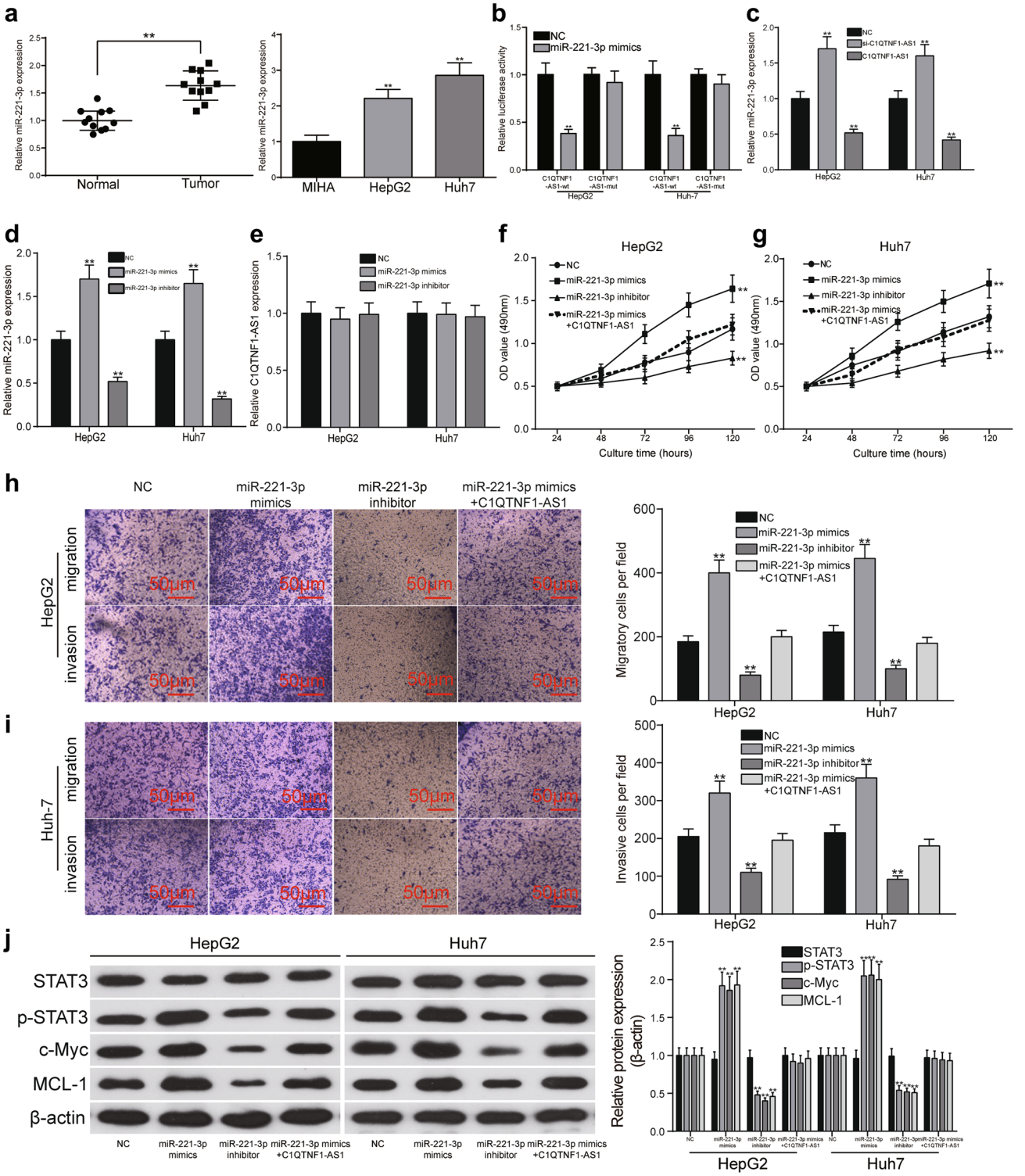
◂**Fig. 2** C1QTNF1-AS1 affected HCC progression through the JAK/STAT signaling pathway. **a** qPCR results showed low C1QTNF1-AS1 expression in HCC tissues. $^{**}p<0.01$ compared with the normal group; **b** The expression of C1QTNF1-AS1 in HepG2 and Huh7 cell lines was lower than that in the normal human liver cell line MIHA. $^{**}p<0.01$ compared with the MIHA group; **c** assessment of transfection efficiency and screening of interference RNA. $^{**}p<0.01$ compared with the NC group; **d** MTT assay showing that C1QTNF1-AS1 slowed down the growth of the HCC cell line Huh7, while si-C1QTNF1-AS1 enhanced cell growth. $^{**}p<0.01$ compared with the NC group; **e, f** transwell assay to determine the migratory and invasive capacity of HepG2 and Huh7 cells transfected with either si-C1QTNF1-AS1 or C1QTNF1-AS1. C1QTNF1-AS1 inhibited the migration and invasion of HCC cells. $^{**}p<0.01$ compared with the NC group; **g** C1QTNF1-AS1 inhibited the JAK/STAT signaling pathway, which was manifested by the decline in phosphorylated STAT and the decrease in the expression of the downstream proteins c-Myc and MCL-1. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the NC group

C1QTNF1-AS1 affected HCC progression through the JAK/STAT signaling pathway.

Relative mRNA expression level of C1QTNF1-AS1 in both HCC tissues and cell lines was detected by RT-qPCR. Compared with the 11 normal tissues, C1QTNF1-AS1 was significantly down-regulated in the 11 HCC tissues ($p<0.001$, Fig. 2a). In addition, the relative mRNA expression level of C1QTNF1-AS1 was detected in the HCC cell lines, including HepG2 and Huh7, and in the normal human liver cell line MIHA, and the results verified down-regulation of C1QTNF1-AS1 in HCC cells ($p<0.01$, Fig. 2b). To assess transfection efficiency, relative mRNA expression level of C1QTNF1-AS1 in HepG2 and Huh7 cells transfected with NC, si-C1QTNF1-AS1 and C1QTNF1-AS1 was detected and the results are shown in Fig. 2c. In addition, OD growth curve constructed by MTT assay data indicated that down-regulation of C1QTNF1-AS1 greatly increased the cell proliferation rate, while up-regulation of C1QTNF1-AS1 greatly decreased the cell proliferation rate in HepG2 and Huh7 cells compared with NC group ($p<0.01$, Fig. 2d). Transwell assays indicated that the invasive and migratory ability of HepG2 and Huh7 cells in the si-C1QTNF1-AS1 group was significantly higher than in the NC group ($p<0.01$), while HepG2 and Huh7 cells with overexpression of C1QTNF1-AS1 showed lower invasion and migration ability than in the NC group ($p<0.01$, Fig. 2e, f). Flow cytometry analysis revealed that up-regulation of C1QTNF1-AS1 increased the cell apoptosis rate while suppression of C1QTNF1-AS1 showed the opposite trend in both HepG2 and Huh7 cells ($p<0.01$, Supplementary Figure 3A). As indicated in Fig. 2g, western blot analysis showed that overexpression of C1QTNF1-AS1 inactivated the JAK/STAT signaling pathway by down-regulating related proteins. Down-regulation of C1QTNF1-AS1 in HepG2 and Huh7 cells greatly increased STAT phosphorylation and the expression of the downstream proteins c-Myc and MCL-1, while overexpression of C1QTNF1-AS1 resulted in the opposite tendency compared with the NC group ($p<0.01$). Above all, C1QTNF1-AS1 affected HCC progression through the JAK/STAT signaling pathway.

Down-regulation of C1QTNF1-AS1 promoted cell proliferation yet suppressed cell apoptosis by targeting miR-221-3p in HCC.

Relative mRNA expression of miR-221-3p in both HCC tissues and cell lines was detected by RT-qPCR and is shown in Fig. 3a. In the 11 paired tissues, miR-221-3p was greatly up-regulated in HCC tissues ($p<0.001$). In addition, a similar trend was observed in the HepG2 and Huh7 HCC cell lines compared with the normal human liver cell line MIHA ($p<0.01$). Based on the bioinformatic analysis, miR-221-3p may be a target of C1QTNF1-AS1. To verify this, HepG2 cells and Huh7 cells were co-transfected with miR-221-3p mimic together with luciferase reporter vectors encoding the wild-type or mutated 3′ UTR of C1QTNF1-AS1. Dual-luciferase reporter gene assay showed that the addition of miR-221-3p mimic inhibited luciferase activity in the wild-type group, indicating that miR-221-3p could bind to the 3′-UTR seed sequence of C1QTNF1-AS1 ($p<0.01$, Fig. 3b). Inhibition of C1QTNF1-AS1 in HepG2 and Huh7 cells enhanced the expression of miR-221-3p compared with NC group cells, which could be reversed with up-regulation of C1QTNF1-AS1 ($p<0.01$, Fig. 3c). Transfection of HCC cells with miR-221-3p mimic and miR-221-3p inhibitor led to up-regulation and down-regulation, respectively, of miR-221-3p expression ($p<0.01$, Fig. 3d), indicated successful transfection However, either up-regulation or down-regulation of miR-221-3p did not alter the expression of the up-stream factor C1QTNF1-AS1 ($p<0.01$, Fig. 3e). OD values measured in MTT assays showed that overexpression of miR-221-3p promoted the proliferation rate of HCC cells, which could be retrieved with up-regulation of C1QTNF1-AS1 ($p<0.01$, Fig. 3f, g). Transwell assays indicated that up-regulation of miR-221-3p enhanced the invasive and migratory ability of HepG2 and Huh7 cells, which could be counteracted with suppression of miR-221-3p. Besides, up-regulation of miR-221-3p enhanced the invasive and migratory ability of HepG2 and Huh7 cells compared with that in the NC group ($p<0.01$), which could be rescued with overexpression of C1QTNF1-AS1 ($p<0.01$, Fig. 3h, i). Flow cytometry analysis revealed that the apoptosis rate was significantly lower than that in the NC group when the expression of miR-221-3p was increased in HepG2 and Huh7 cells, while HepG2 and Huh7 cells transfected with miR-221-3p inhibitor exhibited the opposite results. In addition, up-regulation of miR-221-3p down-regulated cell apoptotic rate, which could be retrieved with co-transfection of C1QTNF1-AS1 ($p<0.01$, Supplementary Figure 3B).



Western blot analysis results shown in Fig. 3j indicated that up-regulation of miR-221-3p activated the JAK/STAT signaling pathway, which could be observed by the increase in STAT phosphorylation and the up-regulation of downstream proteins, including c-Myc and MCL-1 ($p < 0.01$), while down-regulation of miR-221-3p triggered opposite effect.

SOCS3 was the target of miR-221-3p regulated by C1QTNF1-AS1

RT-qPCR results showed the relative expression of *SOCS3*. *SOCS3* was down-regulated in HCC tissues compared with

◂**Fig. 3** MiR-221-3p affected HCC progression and the JAK/STAT signaling pathway regulated by C1QTNF1-AS1. **a** qPCR results showed that high miR-221-3p expression in HCC tissues. $^{**}p < 0.01$ compared with the normal group; **b** dual luciferase reporter gene assay confirmed targeted binding between C1QTNF1-AS1 and miR-221-3p. $^{**}p < 0.01$ compared with the NC group; **c** RT-qPCR verified that the expression of miR-221-3p was negatively regulated by C1QTNF1-AS1. $^{**}p < 0.01$ compared with the NC group; **d**, **e** miR-221-3p transfection efficiency and validation showing that the expression of miR-221-3p had no significant effect on the expression of C1QTNF1-AS1. $^{**}p < 0.01$ compared with the NC group; **f**, **g** MTT assay showing that miR-221-3p promoted the growth of the HCC cell lines, while the addition of C1QTNF1-AS1 counteracted this effect. Inhibition of miR-221-3p inhibited the proliferation of cancer cells, but inhibition of upstream C1QTNF1-AS1 restored the proliferation ability of cancer cells. $^{**}p < 0.01$ compared with the NC group; **h**, **i** transwell assay to determine the migratory and invasive capacity of HepG2 and Huh7 cells. miR-221-3p promoted the migration and invasion of cancer cells, which was offset with the addition of C1QTNF1-AS1, and inhibition of miR-221-3p inhibited migration and invasion of cancer cells, which was recovered with inhibition of the upstream C1QTNF1-AS1. $^{**}p < 0.01$ compared with the NC group; **j** miR-221-3p activated the JAK/STAT signaling pathway, which was manifested by the increase in STAT phosphorylation and increase in c-Myc and MCL-1 expression, and inhibition of miR-221-3p led to the opposite results. The above situation was regulated by upstream C1QTNF1-AS1. $^{**}p < 0.01$ compared with the NC group

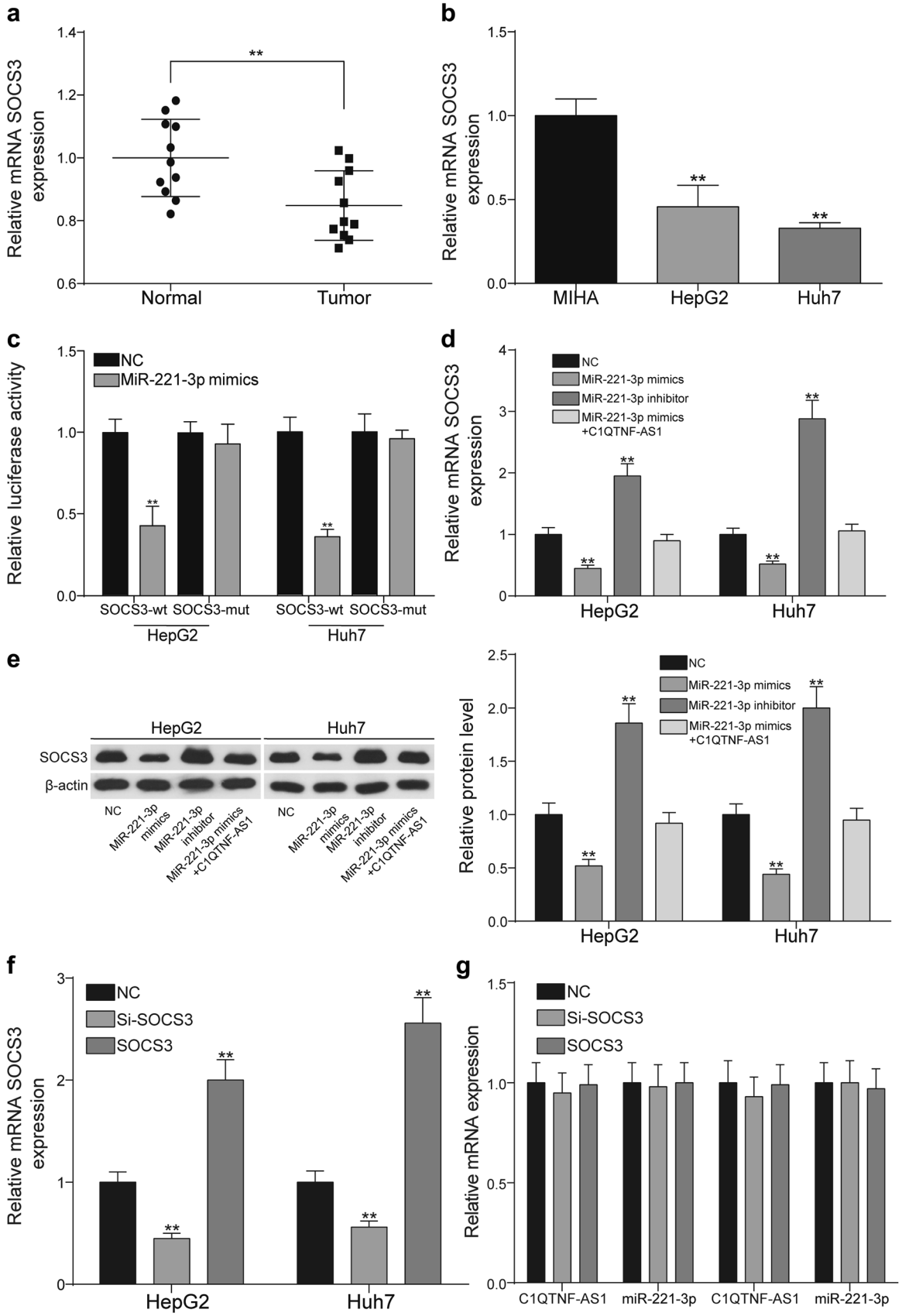
normal tissues ($p < 0.01$, Fig. 4a). Besides, *SOCS3* was also more highly expressed in HCC cell lines than in the normal human liver cell line MIHA ($p < 0.01$, Fig. 4b). To verify whether *SOCS3* was a target of miR-221-3p, HepG2 cells and Huh7 cells were co-transfected with miR-221-3p mimic together with luciferase reporter vectors encoding the wild-type or mutated 3′ UTR of *SOCS3*. Figure 4c showed that miR-221-3p mimic inhibited luciferase activity in the wild-type group, indicating that miR-221-3p could bind to the 3′-UTR seed sequence of *SOCS3* ($p < 0.01$). RT-qPCR and western blot analysis results revealed the regulatory relationships among *SOCS3*, miR-221-3p and C1QTNF1-AS1 in HepG2 and Huh7 cells. Up-regulation of miR-221-3p inhibited the expression level of *SOCS3*, but the addition of C1QTNF1-AS1 dragged the low level expression back. In addition, inhibition of miR-221-3p or overexpression of C1QTNF1-AS1 led to the completely opposite results, indicating that *SOCS3* was a target gene of miR-221-3p regulated by C1QTNF1-AS1 ($p < 0.01$, Fig. 4d, e). Transfection efficiency was tested by evaluating the expression of *SOCS3* after transfection of HCC cells with si-*SOCS3* and pcDNA-*SOCS3* ($p < 0.01$, Fig. 4f). However, either down-regulation or overexpression of *SOCS3* had no influence on C1QTNF1-AS1 and miR-221-3p according to the results shown in Fig. 4g because there was no significant change in C1QTNF1-AS1 and miR-221-3p levels after transfection of HepG2 and Huh7 cells with si-*SOCS3* or pcDNA-*SOCS3*.

SOCS3 affected HCC progression and the JAK/STAT signaling pathway

OD values measured in MTT assays showed that up-regulation of *SOCS3* inhibited the proliferation rate of HCC cells. However, suppression of miR-221-3p decreased the proliferation ability of HCC cells. Besides, up-regulation of *SOCS3* inhibited proliferation rate, which could be rescued to normal level with co-transfection of miR-221-3p mimics ($p < 0.01$, Fig. 5a, b). Transwell assays showed that up-regulation of *SOCS3* inhibited the invasive and migratory rate of HepG2 cells and Huh7 cells compared with NC group cells ($p < 0.01$), and up-regulation of miR-221-3p at the same time counteracted the increase. ($p < 0.01$, Fig. 5c, d). Flow cytometry results showed that cell apoptosis rate was significantly lower than in NC group cells when the expression level of *SOCS3* was decreased in HepG2 and Huh7 cells, while HepG2 and Huh7 cells transfected with pcDNA-*SOCS3* presented the opposite results. In addition, overexpression of *SOCS3* led to enhancement of cell apoptosis, which could be reversed with up-regulation of miR-221-3p moderated the above results ($p < 0.01$, Supplementary Figure 3C). The results of western blot analysis shown in Fig. 5e showed that relative protein expression level of SOCS3 could be increased by overexpression of SOCS3, which could be instead decreased by the silence of SOCS3. In addition, up-regulation of *SOCS3* inactivated the JAK/STAT signaling pathway as indicated by decreased protein expression levels of genes in the JAK-STAT signaling pathway, including phosphorylated form of STAT (p-STAT), c-Myc and MCL ($p < 0.01$), which could be instead reversed with suppression of *SOCS3* in HCC. In brief, *SOCS3* affected HCC progression and JAK/STAT signaling pathway.

Overexpression of C1QTNF1-AS1 inhibited HCC tumor growth in vivo.

Overexpression of C1QTNF1-AS1 dramatically inhibited HCC tumor growth, which was reversed by down-regulation of C1QTNF1-AS1 in vivo. With respect to tumor volume, tumors in the C1QTNF1-AS1 group were dramatically smaller than those in the NC group. On the 35th day, tumors were collected from the nude mice and weighed with an electronic balance. The results showed that up-regulation of C1QTNF1-AS1 also led to lighter tumors than those in the NC group (Fig. 6a–c, $p < 0.01$). After tumor formation in nude mice, the relative expression levels of miR-221-3p, *SOCS3* and proteins in the JAK/STAT signaling pathway were detected by RT-qPCR and western blot analysis. As shown in Fig. 6d, suppression of C1QTNF1-AS1 increased expression of miR-221-3p, which was decreased by down-regulation of C1QTNF1-AS1. In addition, down-regulation of C1QTNF1-AS1 decreased expression of *SOCS3*, which


a
Relative mRNA SOCS3 expression
1.4
1.2
1.0
0.8
0.6
**
Normal
Tumor
b
Relative mRNA SOCS3 expression
1.5
1.0
0.5
0.0
**
**
MIHA
HepG2
Huh7
c
Relative luciferase activity
1.5
1.0
0.5
0.0
NC
MiR-221-3p mimics
**
**
SOCS3-wt
SOCS3-mut
SOCS3-wt
SOCS3-mut
HepG2
Huh7
d
Relative mRNA SOCS3 expression
4
3
2
1
0
NC
MiR-221-3p mimics
MiR-221-3p inhibitor
MiR-221-3p mimics +C1QTNF-AS1
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HepG2
Huh7
e
HepG2
Huh7
SOCS3
β-actin
NC
MiR-221-3p mimics
MiR-221-3p inhibitor
MiR-221-3p mimics +C1QTNF-AS1
NC
MiR-221-3p mimics
MiR-221-3p inhibitor
MiR-221-3p mimics +C1QTNF-AS1
Relative protein level
2.5
2.0
1.5
1.0
0.5
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NC
MiR-221-3p mimics
MiR-221-3p inhibitor
MiR-221-3p mimics +C1QTNF-AS1
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HepG2
Huh7
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Relative mRNA SOCS3 expression
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2
1
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NC
Si-SOCS3
SOCS3
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HepG2
Huh7
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Relative mRNA expression
1.5
1.0
0.5
0.0
NC
Si-SOCS3
SOCS3
C1QTNF-AS1
miR-221-3p
C1QTNF-AS1
miR-221-3p

◂**Fig. 4** SOCS3 was the target gene of miR-221-3p regulated by C1QTNF1-AS1. **a** RT-qPCR results showed that SOCS3 expression was lower in HCC tissues. $^{**}p < 0.01$ compared with the normal group; **b** RT-qPCR results showed that *SOCS3* expression was lower in HCC cells. $^{**}p < 0.01$ compared with the MIHA group; **c** dual luciferase reporter gene assays confirmed targeted binding of *SOCS3* and miR-221-3p. $^{**}p < 0.01$ compared with the NC group; **d**, **e** RT-qPCR and western blot results showed that the expression of SOCS3 was negatively regulated by miR-221-3p and changed with the expression of upstream C1QTNF1-AS1. $^{**}p < 0.01$ compared with the NC group; **f** Transfection efficiency of *SOCS3*. $^{**}p < 0.01$ compared with the NC group; **g** the expression of SOCS3 had no significant effect on upstream C1QTNF1-AS1 and miR-221-3p

was increased by up-regulation of C1QTNF1-AS1, as shown in Fig. 6d. Furthermore, silence of C1QTNF1-AS1 up-regulated the relative protein expression levels of genes in the JAK-STAT signaling pathway. Overall, overexpression of C1QTNF1-AS1 inhibited tumor growth in vivo (Fig. 6f, $p < 0.01$).

Discussion

Over the past 20 years, mortality due to HCC has consistently increased. Despite advancements in diagnosis and treatments, such as liver resection, liver transplantation and radiofrequency ablation, the prognosis of HCC patients is still very poor [15]. Since the process of hepatocarcinogenesis has always been described to involve a range of genetic alterations, the key roles of recently identified lncRNAs, miRNAs and related protein-coding genes in hepatocarcinogenesis could now inspire the development of new diagnostic and treatment strategies and improve the overall prognosis of HCC patients [16]. In our present study, we investigated differentially expressed lncRNAs and genes between HCC tissues and normal tissues and predicted their target miRNAs using bioinformatics analysis. C1QTNF1-AS1 and *SOCS3* were both found to be down-regulated in HCC tissues, while their target miR-221-3p was up-regulated. We found that C1QTNF1-AS1 inhibited cell proliferation, migration, and invasion and the JAK/STAT signaling pathway and promoted apoptosis in HCC cells by suppressing miR-221-3p and further promoting *SOCS3* expression. This novel C1QTNF1-AS1/miR-221-3p/*SOCS3* regulatory axis was elucidated in HCC tissues and cells.

LncRNAs have received much attention since they were first discovered. They are involved in gene expression at the epigenetic level, as well as in tumorigenesis. Previous studies have shown that lncRNAs are important regulators of tumorigenesis and subsequent prognosis and metastasis of malignancies [17]. For example, Han et al. reported that down-regulation of lncRNA GAS6-AS1 affects the development and progression of non-small cell lung cancer, and thus, GAS6-AS1 has become a potential candidate diagnostic target for non-small cell lung cancer [18]. C1QTNF1-AS1 was first identified by RNA-seq carried out by Fagerberg et al. [19]. However, research on C1QTNF1-AS1 was still absent until now. In our study, we identified down-regulation of C1QTNF1-AS1 in HCC tissues for the first time. By applying a gain-of-function approach, we demonstrated that up-regulation of C1QTNF1-AS1 restrained proliferation, migration and invasion and enhanced the apoptosis of HCC cells by suppressing miR-221-3p, which indicates that dys-regulation of C1QTNF1-AS1 may affect HCC progression by acting as a sponge for miR-221-3p. Through our novel discovery of the low C1QTNF1-AS1 expression in HCC tissues and elucidation of its regulatory axis in HCC, new potential therapeutic targets for HCC may have been found. Further study may be urgently necessary to apply C1QTNF1-AS1 to the development of new clinical diagnosis and treatment methods.

One of the most important lncRNA regulation mechanisms is that lncRNA can act as ceRNA to suppress miRNA and further affect miRNA target genes. Then, miRNA modulates gene expression and participates in the development and progression of different tumors [20]. In our investigation, the target miRNA of C1QTNF1-AS1 identified by bioinformatic analysis and dual-luciferase reporter gene assay was miR-221-3p. Previous studies have reported that miR-221-3p might be involved in many cancer cell malignant behaviors, such as proliferation, apoptosis, invasion, metastasis, and chemoresistance. One example demonstrates the key role of miR-221-3p in chemoresistance. In 5-FU-resistant pancreatic cancer cells, overexpression of miR-221-3p promoted cell proliferation, migration, invasion, and epithelial–mesenchymal transition (EMT) by inhibiting RB1 expression [21]. In our study, we detected the expression of miR-221-3p in HCC tissues and adjacent normal tissues. The results showed that the expression of miR-221-3p was relatively higher in HCC tissues, which suggested that miR-221-3p may be involved in HCC pathogenesis or development. Research regarding the role miR-221-3p plays in HCC progression and metabolism has not been performed prior to our study, but there are some studies on miR-221-3p in other cancer cells. For instance, a breast cancer study reported by Deng et al. found that down-regulation of miR-221-3p may contribute to the poor prognosis of TNBC patients by regulating PARP1. MiR-221-3p acts as an inhibitor of PARP1 by binding its 3′-untranslated region [8]. In colon cancer, by evaluating the relationship between miR-221-3p expression and clinical features and prognosis of patients, Tao et al. demonstrated that overexpression of miR-221-3p was associated with a lower survival rate. Thus, miR-221-3p may become a new signature for accurate prognostic evaluation [22]. Our discovery of high miR-221-3p expression in HCC suggests it might be a new therapeutic target and novel prognostic biomarker. Thus, study of miR-221-3p may be

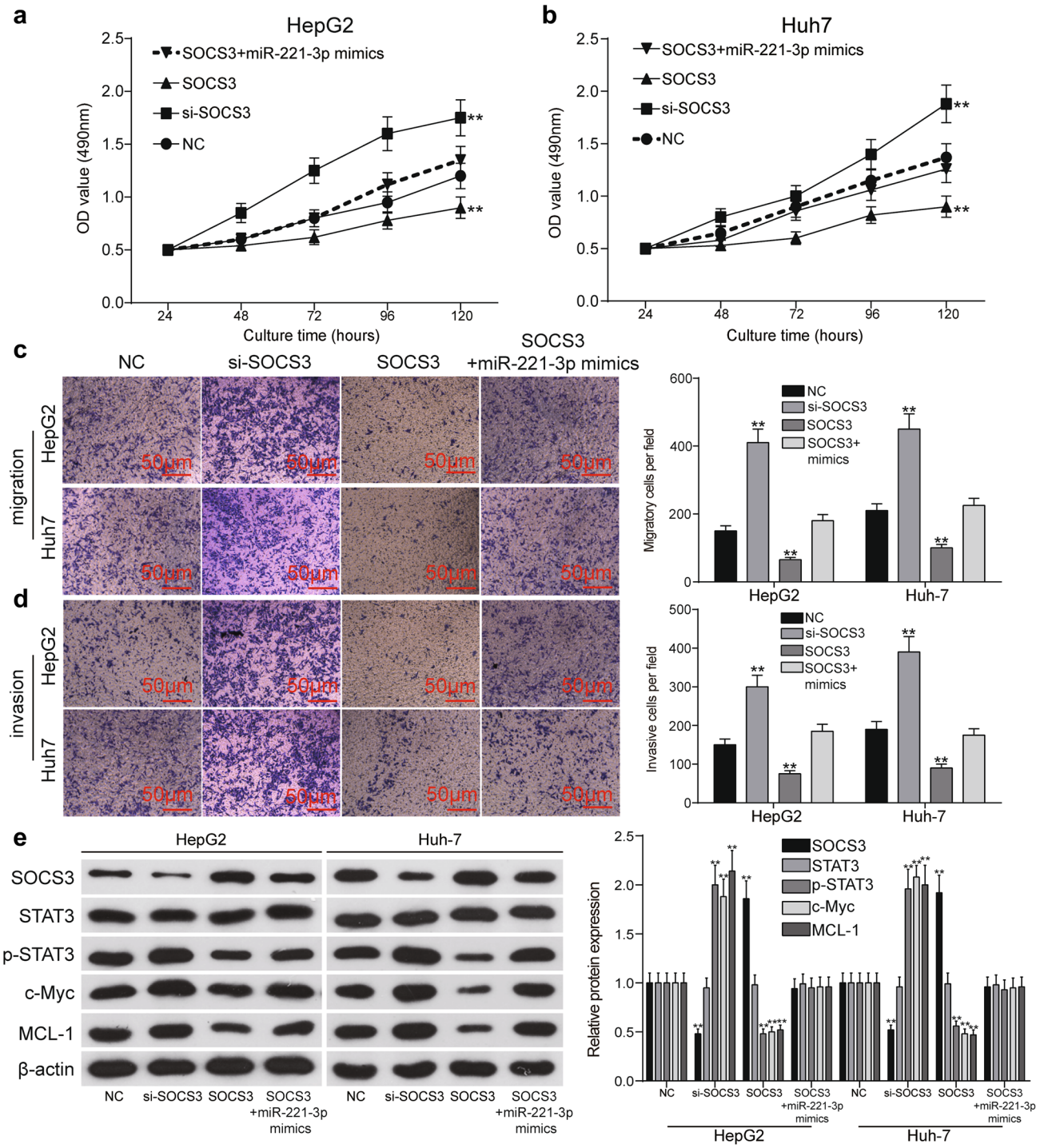
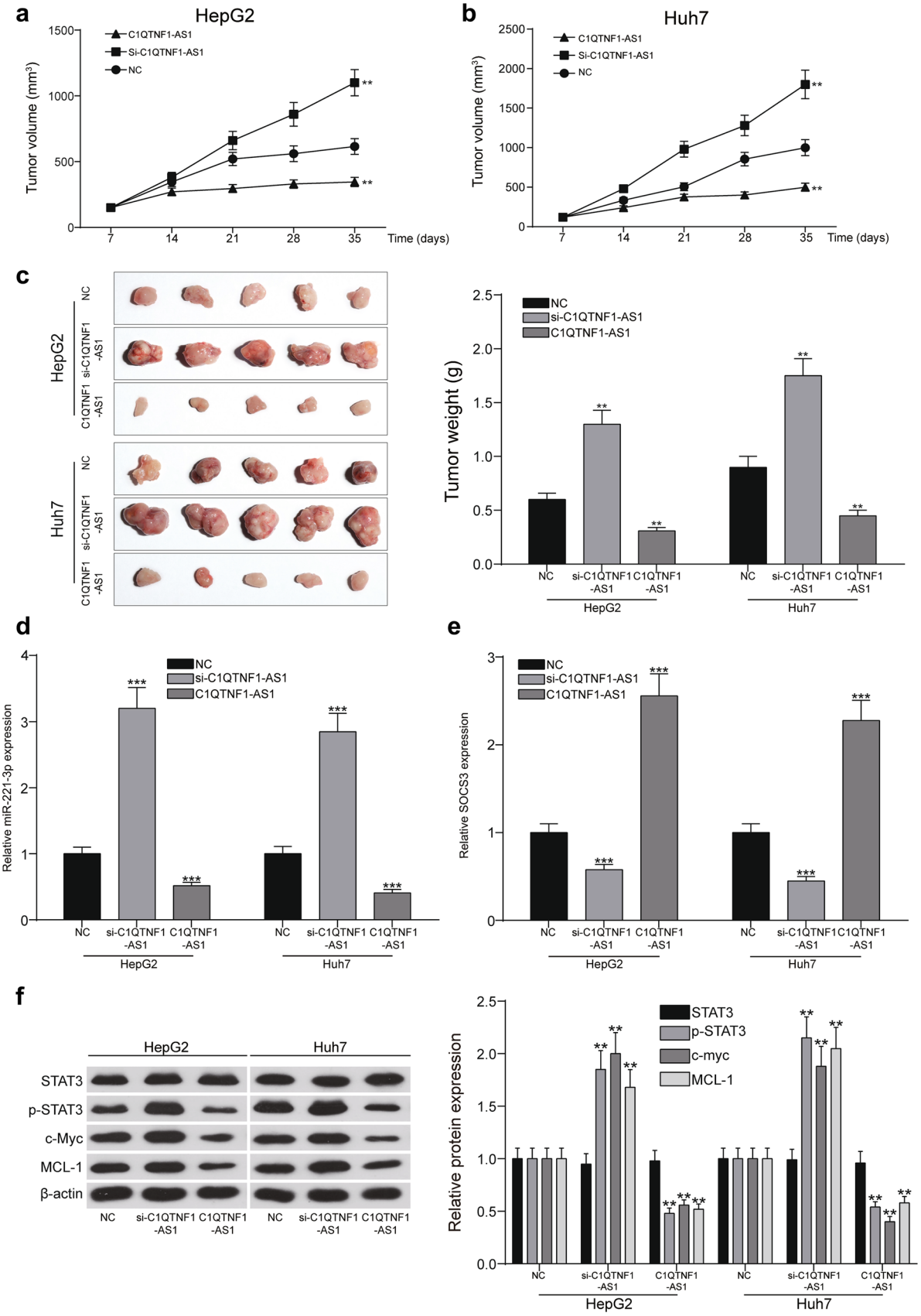


Fig. 5 *SOCS3* affected HCC cell behavior and the JAK/STAT signaling pathway regulated by miR-221-3p. **a**, **b** MTT assay showed that SOCS3 can inhibit the proliferation of cancer cells. With the addition of miR-221-3p, the inhibitory effect was offset, and inhibition of SOCS3 can promote the proliferation of cancer cells, but the proliferation ability of the cancer cells decreased with inhibition of upstream miR-221-3p. $^{**}p < 0.01$ compared with the NC group; **c**, **d** transwell assay to determine the migratory and invasive capacity of HepG2 and Huh7 cells. *SOCS3* inhibited the migration and invasion of cancer cells. With the addition of miR-221-3p, the inhibitory effect was offset, and inhibition of SOCS3 promoted the migration and invasion of cancer cells, but the migration and invasion ability of cancer cells decreased with inhibition of upstream miR-221-3p. $^{**}p < 0.01$ compared with the NC group; **e***SOCS3* inhibited the JAK/STAT signaling pathway, which was manifested by decreased protein expression levels of genes in the JAK-STAT signaling pathway, including phosphorylated form of STAT (p-STAT), c-Myc and MCL, which could be instead reversed with suppression of *SOCS3* in HCC. $^{**}p < 0.01$ compared with the NC group

utilized to improve treatment decisions and advance prognostic predictions.

The effects of miRNAs are exerted by producing an RNA-induced silencing complex (RISC) to restrain the translation of target genes or by directly degrading target genes. TargetScan analysis was applied to predict *SOCS3* as the potential target gene of miR-221-3p. In addition, down-regulation of SOCS3 was tested in HCC tissues and cells when miR-221-3p was up-regulated. Furthermore, dual-luciferase reporter gene assays demonstrated that *SOCS3* could physically interact with miR-221-3p. All of the above results suggested that *SOCS3* is the terminus of the lncRNA–miRNA–mRNA axis in HCC. In fact, accumulating studies have focused on the regulation of SOCS3 in cancer. In line with our report, a study by Guo et al. reported the same low expression of *SOCS3* in HCC. Furthermore, they discovered that inhibition of *SOCS3* could enhance STAT3 activation to increase HCC cell proliferation, which reminded us of the application of increasing *SOCS3* levels in HCC prevention [23]. A report on regulation of *SOCS3* in pancreatic cancer cells determined that restoration of miRNA let-7 promoted the expression of SOCS3 and then impeded the activation of STAT3 [24]. Both of the above studies indicated a negative correlation between SOCS3 and STAT. As a member of the protein suppressor of cytokine signaling family, the expression of SOCS3 is tightly related to many signaling pathways and ultimately affects protein encoding in cells. We detected certain key proteins in the JAK/STAT signaling pathway, and the results showed that overexpression of *SOCS3* inhibited STAT phosphorylation, which was consistent with previous studies. The mechanism of this inhibition was reported to be that *SOCS3* acts as a blocker of JAK kinase and inhibits its interaction with STAT [25]. In our report, overexpression of *SOCS3* eventually led to an increase in proliferation, invasion, and migration and a decrease in apoptosis in HCC cells, possibly resulting from suppression of the JAK/STAT signaling pathway. Zhang et al. reported that in human melanoma A375 cells blocking of the JAK/STAT signaling pathway enhanced apoptosis and inhibited proliferation, consistent with the conclusion of our study [26]. The influence of SOCS3 on tumor progression may also be mediated through other signaling pathways. A study on the effect of SOCS3 on cell migration reported by Stevenson et al. indicated that in murine embryonic fibroblasts and human 293T cells expressing SOCS3, migration ability towards the chemokine CCL11 was reduced by enhancement of both FAK and RhoA activity by SOCS3 [27]. Our identification of the target gene SOCS3 and its regulation of the JAK/STAT signaling pathway may provide a new idea for therapeutic strategies for HCC.

To sum up, we found a novel C1QTNF1-AS1/miR-221-3p/*SOCS3* regulatory axis in HCC. Through prediction based on a ChIP assay and identification in a series of biological experiments, we demonstrated dysregulation of C1QTNF1-AS1, miR-221-3p and *SOCS3* in HCC tissues and revealed physical binding between C1QTNF1-AS1/miR-221-3p and miR-221-3p/*SOCS3*. We also confirmed the effect of the regulatory axis on HCC cell proliferation, invasion, migration and apoptosis through the JAK/STAT signaling pathway. Our findings could lead to the development of better diagnostic and prognostic approaches for HCC patients. However, our study has limitations; we only took two cell lines into consideration, and the mechanism underlying the effects of SOCS3 on HCC progression is still not clear because we only tested one signaling pathway. Besides, more other experiments, such as RNA-seq and GSEA analysis under overexpression of C1QTNF-AS, investigation of relationships between dysregulation of genes in JAK/STAT signaling pathway and aggressiveness of HCC will be carried out for further validation of roles of C1QTNF-AS/miR221-3p/*SOCS3* in HCC progression and regulation of the aggressiveness of HCC.


a
HepG2
C1QTNF1-AS1
Si-C1QTNF1-AS1
NC
Tumor volume (mm3)
Time (days)
b
Huh7
c
HepG2
Huh7
NC
si-C1QTNF1-AS1
C1QTNF1-AS1
Tumor weight (g)
d
Relative miR-221-3p expression
e
Relative SOCS3 expression
f
STAT3
p-STAT3
c-Myc
MCL-1
β-actin
Relative protein expression

◂**Fig. 6** Overexpression of C1QTNF1-AS1 inhibits tumor growth. **a–c** Tumors were collected from nude mice injected with HCC cells transfected with si-C1QTNF1-AS1, C1QTNF1-AS1 or NC. Overexpression of C1QTNF1-AS1 inhibited the growth of tumors, and inhibition of C1QTNF1-AS1 expression promoted the formation of tumors. $^{**}p < 0.01$ compared with the NC group; **d–f** expression of miR-221-3p, SOCS3 and JAK/STAT signaling pathway proteins in nude mice tumorigenicity assay. $^{**}p < 0.01$ compared with the NC group

Funding None.

Compliance with ethical standards

Conflict of interest Hang Li, Bo Zhang, Meng Ding, Shang Lu, Hui Zhou, Dajun Sun, Gang Wu and Xianfeng Gan declare that they have no competing interests.

Ethics approval All procedures performed in studies involving animal participants were in accordance with the ethical standards of China-Japan Union Hospital, Jilin University. The entire study was approved by the China-Japan Union Hospital, Jilin University.

Informed consent Informed consent was collected from patients to approve utilization of their tissues for research purposes.

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