Original Article CNOT7 is upregulated in hepatocellular carcinoma with hepatitis B virus related cirrhosis and regulates tumorigenesis by targeting signal transducer and activator of transcription 1

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Abstract: IFN-y/STAT1 is the major pathway involved in cancer immunosurveillance. IFN-y resistance has been proposed to be a primary cause of the poor response to IFN therapy in hepatocellular carcinoma (HCC), contributing to poor prognosis, and the transcription factor STAT1 has been proposed to be a crucial mediator of this response. CNOT7, a cytoplasmic mRNA deadenylase that shows high expression in some cancers, may negatively regulate IFN-y/STAT1 pathways. Indeed, CNOT7 can inhibit cytoplasmic STAT1 trafficking induced by IFN-y through interactions with the latent form of STAT1 in resting cells. Therefore, we hypothesized that CNOT7 protein overexpression may be responsible for the IFN-y insensitivity of hepatocellular carcinoma cells. We detected the plasma concentrations of IFN-γ in hepatocellular carcinoma with HBV related cirrhosis (HCCBC) and HBV-related cirrhosis (HBC) patients. CNOT7, STAT1 and STAT3 expression were determined in the tumor and adjacent non-tumor tissues of HCCBC patients. We treated four hepatocellular carcinoma cell lines with IFN-y and evaluated the effects on proliferation, cell cycle, and apoptosis, as well as the relative expression levels of CNOT7, STAT1, and STAT3. These effects of IFN-y were also evaluated in HepG2 cells with CNOT7 expression knocked down. In our study, the plasma concentrations of IFN-y were no significant difference between HCCBC and HBC patients. CNOT7 expression increased and STAT1 expression decreased in HCCBC tissues and HCC lines. CNOT7 depletion could significantly induce STAT1 protein expression and inhibit STAT3 protein expression in HepG2 cells. The levels of phosphorylated (P)-STAT1 and caspase3 expression were elevated and the levels of P-STAT3 and cyclinE1 expression were down-regulated after CNOT7 depletion in HepG2 cells treated with IFN-y. Overall, CNOT7 overexpression was a primary cause of IFN-y resistance which may be a reason of tumorigenesis in HCCBC. CNOT7 depletion could reverse IFN-y resistance in HCC cells. It shows potential as a new adjuvant therapy in immunological therapy for HCC.

Keywords: CNOT7, hepatocellular carcinoma, HepG2, STAT1, STAT3

Introduction

Hepatocellular carcinoma (HCC) is the most highly malignant diseases and the most prevalent cause of cancer-related deaths in the world. HCC originates on a diffuse and chronic inflammation in liver, where exists unceasing cellular injury and regeneration, finally developing to cirrhosis [1]. Such chronic inflammation in liver is considered to increase the risk of cancer development. There are many reasons to cause the liver inflammation, e.g. hepatotropic virus, medicines, and alcohol. Among them, the HBV infection is generally acknowledged the major risk factor for HCC in the world [2]. Interferon- γ (IFN- γ ; also known as type II interferon) is one type of cytokine that is critical in both innate and adaptive immunity in humans. Owing to the anti-virus and anti-tumor properties, Interferons (IFNs), including type I, II, and III IFNs, have been considered to be valuable therapeutic reagents, and widely used to be against chronic viral hepatitis and HCC. It is also deemed that IFN- γ , neither IFN- α nor IFN- β (also known as type I interferon), has the ability to control the immunogenicity of tumor cells in human [3]. Increasing the plasma concentration of IFN- γ in HCC patients has generally shown a discouraging response [4]. Several studies have demonstrated that IFN- γ exerted growth-inhibitory effects on HCC cells, but other studies shown opposite results [5, 6]. Some authors suggest that the resistance of cancer cells to IFN-y may be due to a relative deficiency of IFN signaling molecules STAT1 and overexpression of STAT1 may restore the responsiveness of cancer cells to IFN-y [7]. There are some reports indicating that the antior pro-proliferative effects of IFN-y in tumors may depend on the ratio of STAT1/STAT3 [8]. STAT1 deficiency may redirect IFN-y signaling through feedback activation of STAT3 [9]. It is shown that HCC patients, harboring tumors with higher levels of phosphorylated (P)-STAT1, possess better prognosis and live longer [10]. Actually, STAT1 negatively regulates HCC proliferation via cyclinE1 and caspase3 [11]. By contrast, approximately 60% of human HCC specimens show P-STAT3 expression, and STAT3positive tumors were found to be relatively more aggressive [12].

CCR4-NOT complex is a multifunctional complex that regulates many physiological processes, including transcriptional regulation, protein modification, and mRNA deadenylation. CNOT7, a subunit of the CCR4-NOT complex, has been identified as the major cytoplasmic mRNA deadenylase in metazoans [13]. CNOT7 depletion resulted in strong inhibition of adenylate uridylate-rich elements (AREs) containing decayed mRNA in human HTGM5 cells [14, 15]. Therefore, CNOT7 could be a critical factor for ARE-bearing mRNA deadenylation and degradation. There are some reports that many IFNs, encode functional AREs that allow for tight temporal translational control of these cytokines. Deletion of certain proteins that bind to AREs targeting cytokines can have significant deleterious effects leading to immune pathologies [16, 17]. In addition, CNOT7 regulated IFN pathways by controlling STAT1 trafficking through interaction with its inactive form and by degrading STAT1-regulated mRNAs through its deadenylase activity [18].

To explore the role of CNOT7 in hepatocellular carcinoma with HBV-related cirrhosis (HCCBC), the plasma concentrations of IFN- γ were detected in HCCBC and HBC patients. The levels of CNOT7, STAT1 and STAT3 expression were determined in the tumor and their adjacent non-tumor tissues of HCCBC patients. Then, we stimulated four HCC cell lines with

IFN-γ and evaluated effects. We compared the expression levels of CNOT7, STAT1, and STAT3 in the four HCC cell lines to human liver cell line (LO2). Furthermore, we knocked down CNOT7 expression in HepG2 cells, and compared the effects between HepG2^{shCNOT7} and HepG2^{control} cells treated with IFN-γ. These results could help to elucidate the function and mechanism of CNOT7 in regulating STAT1 and its relationship to the IFN-γ/STAT1 signaling pathway in HCC cells, to gain a better understanding of the factors contributing to IFN-γ resistance and indicate new potential targets for treatment.

Materials and methods

Patients and controls

The subjects were recruited at the Shanxi Dayi Hospital from September 2012 to January 2016. It comprised 42 HCCBC patients diagnosed pathologically, 50 HBV-related cirrhosis (HBC) patients and 50 healthy controls. We excluded patients if age < 30 or > 65 years old; or hepatic function Child-Pugh = C; or any immunotherapy during the 6 month prior to sampling; or infections of HCV, HDV, HIV; or had a history of alcohol abuse or autoimmune hepatitis. We also excluded HCCBC patients with secondary tumor, or lymph node involvement, or multiple tumor: or metastasis or can not complete resection. All subjects underwent clinical, radiological or histological diagnoses that were performed in line with international diagnostic criteria. The study protocol was approved by the ethics committee of the Shanxi Medical University. Each participant signed informed consent after being explained the nature and potential risks of the study. The clinical characteristics of eligible subjects were shown in Table 1.

Cytokine assay and immunohistochemical analysis

The plasma concentrations of IFN- γ were determined by ELISA kits (Boster, Wuhan, China), according to the manufacturer's instructions. The sensitivity of ELISA kits for IFN- γ was 7 pg/ml, respectively without detecting of any cross-reactivity. The levels of CNOT7, STAT1 and STAT3 expression in the tumor and adjacent non-tumor tissues were determined by immunohistochemistry. Briefly, individual paraffinembedded tissue sections (4- μ m) were depar-

Table 1	Clinical	characteristics	of	eligihle	subjects
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	HCCBC	HBC	CON
	(n = 42)	(n = 50)	(n = 50)
Age (\geq 50/< 50 years)	29/13	35/15	35/15
Gender (male/female)	26/16	30/20	30/20
HBV-DNA median level (log_{10} copies/mL)	4.83±2.81*	5.49±6.24*	-
AFP (≥ 400/< 400 µg/L)	38/4	7/43	-
Tumor diameter (\geq 5/< 5 cm)	25/17	-	-
Differentiation (high-moderate/low)	33/9	-	-

Abbreviations: AFP, α -fetoprotein; CON, healthy controls. *Abnormal distribution, data were expressed as median \pm QR.

affinized and rehydrated. The sections were incubated with primary antibody for CNOT7 (Santa Cruz, diluted 1:200), STAT1 and STAT3 (Cell Signaling Technology, diluted 1:200). The bound antibodies were detected with biotinylated goat-anti-mouse/rabbit IgG and demonstrated with SABC method (SABC and DAB Histostain Kits; Boster). We used a thing dyed from a specimen well every time as positive control. The number of positive staining cells in five areas selected randomly from a single specimen was counted, and the intensity of staining was scored by two pathologists in a blinded manner. Where 0 = No staining is observed in cells, 1 = Weak, incomplete cytoplasm staining in any proportion of cells, or weak, complete cytoplasm staining in less than 10% of cells, 2 = Middle, complete cytoplasm staining that is non-uniform or weak but with obvious distribution in at least 10% of cells, or intense complete cytoplasm staining in 30% or less of cells, 3 = Strong, uniform intense cytoplasm staining of more than 30% of cells. Scores of 0 and 1 were considered to "low" expression and scores of 2 and 3 were indicated "high" expression. This grading was determined based on the diagnostic criteria of American Society of Clinical Oncology/College of American Pathology 2007 guidelines.

Cell culture

The HCC cell lines HepG2, SMMC7721, Huh7, PLC/PRF/5 and L02 (Chinese Academy of Sciences, Shanghai, China) were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell proliferation assay

The impact of IFN- γ on cell viability in the PLC/ PRF/5, HepG2, Huh7, SM-MC7721, HepG2^{control}, and HepG2^{shCNOT7} cell lines was evaluated by MTT assay (Sigma, St. Louis, MO, USA). The cells (4 × 10³ per well) were cultured on 96-well plates and incubated in medium containing 20 ng/

ml recombinant human IFN- γ (PreproTech, Rocky Hill, NJ, USA) for 24 h. The cells were washed to remove the medium containing IFN- γ and replaced with serum-free culture medium; 10 µl of the MTT reagent was added to the medium in each well and the cells were incubated for a further 4 h. All of the medium in the well was discarded, 100 µl of DMSO was added to each well, and the cells were further incubated for 1 h. The optical density was determined with the Victor X5 Multilabel Reader spectrophotometer (Perkin Elmer, Waltham, MA, USA) at 490 nm, and data were obtained from 3 replicate wells for each cell line.

Cell cycle analysis

The cells (4 × 10^5) were seeded into 6-well plates and incubated overnight. The cells were incubated in media containing 20 ng/ml of IFN- γ for 24 h. The cells were harvested and fixed with 70% cold ethanol at 4°C for 24 h. Fixed cells were resuspended with 100 µg/ml RNase, incubated with 50 µg/ml Pl (Cell Cycle Analysis kit, KeyGen BioTECH, Nanjing, China) at 37°C for 30 min in the dark, and analyzed by flow cytometry (BD Bioscience, San Jose, CA, USA).

Apoptosis assay

The cells (2×10^5) were seeded into 6-well plates and incubated overnight. The cells were incubated in medium containing 20 ng/ml of IFN- γ for 24 h. The cells were harvested and resuspended in 1 × binding buffer, to which 5 µl Annexin V-FITC/Annexin V-APC or 5 µl Pl/7-AAD (Apoptosis Detection kit, KeyGen BioTECH)were added. The cells were gently vortexed and incubated in the dark and then analyzed using flow cytometry.

Table 2. Talget Sequences of Shonor	Table 2.	Target Sequer	nces of shCNOT7
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ID	Target Sequences	Initial position	GC content (%)	
CNOT7-RNAi (#1)	5'-TACTAACAACATCTGGTAT-3'	641	31.58	
CNOT7-RNAi (#2)	5'-TGACTATCAATACCAACTA-3'	471	31.58	
CNOT7-RNAi (#3)	5'-GTTACGACTTTGGCTACTT-3'	773	42.11	
CNOT7-RNAi (#4)	5'-GTGTAATGTAGACTTGTTA-3'	495	31.58	
non-specific shRNA 5'-TTCTCCGAACGTGTCACGT-3'				

Table 3. Sequences of primers

Gene	Sequences	Amplicon Size
GADPH	Forward: 5'CTCTGCTCCTCCTGTTCGAC-3' Reverse: 5'-TTAAAAGCAGCCCTGGTGAC-3'	104 bp
CNOT7	Forward: 5'-GAGGAAGCCAACAAGCAGTC-3' Reverse: 5'-GTTCGAGGGATTCAACCAGA-3'	105 bp
STAT1	Forward: 5'-CCGTTTTCATGACCTCCTGT-3' Reverse: 5'-TGAATATTCCCCGACTGAGC-3'	228 bp
STAT3	Forward: 5'-AGTGAGTAAGGCTGGGCAGA-3' Reverse: 5'-AAGGCACCCACAGAAACAAC-3'	100 bp
cyclinE1	Forward: 5'-GGAAAATCAGACCGCCCAG-3' Reverse: 5'-CATCAGCCAGTCCAGAAGAAC-3'	176 bp
caspase3	Forward: 5'-CTGCCGGAGTCTGACTGGAA-3' Reverse: 5'-ATCAGTCCCACTGTCTGTCTCAATG-3'	97 bp

Transfection of HepG2 cells

HepG2 cells (2×10^5) were plated in a 6-well plate for 24 h before transfection. Transfection was performed with Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions using four shR-NAs specifically targeting CNOT7, non-specific shRNA as a control (**Table 2**), and recombinant plasmids encoding CNOT7 (GV230-CNOT7) and the empty vector (GV230-EV) (GeneChem, Shanghai, China). The depletion and overexpression efficiency was evaluated by RT-qPCR and western blot analysis. Selection of the stably transfected cells was initiated 48 h after transfection in medium containing 450 ng/ml of puromycin.

RT-qPCR analysis

The cells were washed with PBS, and total RNA was isolated using SuperEnhanced TRIzol reagent (Sangon Biotech, Shanghai, China) and reverse-transcribed into cDNA using Fermentas K1622 RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). The PCRs were run on a FastStart Universal SYBR Green Master (ROX) (Roche, Mannheim, Germany) and quantified on the ABI StepOnePlus RT-gPCR system (Applied Biosystems, Foster, CA, USA). The relative gene expression was normalized to the level of GAPDH transcript and quantified by the $2^{-\Delta\Delta CT}$ method. The amplification were performed at 94°C for 10 min for Tag enzyme activation and subjected to 40 cycles of 94°C for 15 sec and 60°C for 60 sec. The sequences of primers were shown in Table 3.

Western blot analysis and coimmunoprecipitation

Cell proteins were extracted and the concentrations were measured using a BCA protein assay (Boster). Proteins were subjected to Western blot analysis using primary antibody for CNOT7 (diluted 1:500), STAT1, STAT3, P-STAT1 (Tyr701), P-STAT3 (Tyr705) (Cell Signaling Technology, diluted 1:

1000), cyclinE1, caspase-3, cleaved caspase-3 (Abcam, diluted 1:1000), and GAPDH (Abcam, diluted 1:5000). The relative levels of each target protein to the control GAPDH were determined by the AlphaView-FluorChemQ Gel Imaging System (ProteinSimple, Silicon Valley, CA, USA). For co-immunoprecipitation (Co-IP), 800 mg of the lysates were incubated with the appropriate antibody (1-2 mg) for 3-4 h at 4°C followed by 1 h incubation with Protein A/G PLUS-Agarose beads (Santa Cruz). The resulting immunoprecipitates were washed at least three times in RIPA lysis buffer before being resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Co-IP assays were performed with rabbit IgG (Santa Cruz) as a negative control.

Statistical analysis

Clinicopathological variables of CNOT7, STAT1 and STAT3 were assessed by the x^2 test. The rest of data were evaluated using the Student *t*-test or one-way ANOVA with SPSS 22.0, and were expressed as the mean \pm SD. The significance level was set to 0.05.



Figure 1. The role of CNOT7, STAT1, and STAT3 in the IFN- γ resistance of HCCBC patients. A. ELISA: Data are representative images or expressed as the mean \pm SD. Multiple comparisons were evaluated with the SNK-q method. Values with different letters differ significantly (P < 0.05). B. Immunohistochemistry: CNOT7, STAT1 and STAT3 expressions were analyzed in HCCBC tissues and adjacent non-tumor tissues (magnification, × 400). The cells with yellow-brown staining are immunopositive (Scale bar: 50 µm).

 Table 4. CNOT7, STAT1 and STAT3 expressions in tumor and adjacent non-tumor tissues

Ν		CNC	DT7	*P-	STAT1		*P-	STAT3		P-
		High	Low	values	High	Low	values	High	Low	values
Tumor	42	25	17	0.016	13	29	0.004	11	31	0.263
Adjacent	42	14	28		26	16		9	33	

Abbreviations: Tumor, tumor tissues; Adjacent, adjacent non-tumor tissues; High, high expression; Low, low expression. *Based on x² test.

Results

Plasma concentrations of IFN-y were no significant difference between HCCBC and HBC patients and CNOT7 expression increased and STAT1 expression decreased in liver tumor tissues

Plasma concentrations of IFN-y were determined by ELISA kits in 50 healthy controls, 50 HBC and 42 HCCBC patients. There was no significant difference between HBC and HCCBC patients. Both of them were higher than healthy controls (Figure 1A; P < 0.05). Immunohistochemistry was performed to evaluate CNOT7, STAT1 and STAT3 protein expression in 42 paired HCCBC and their adjacent non-tumor tissues (Figure 1B). Target protein was expressed predominantly in the cytoplasm in tumor and adjacent non-tumor tissues. CNOT7 were significantly higher in tumor compared to their adjacent non-tumor tissues (P < 0.05; Table 4). STAT1 was significantly downregulated in tumor tissues (P < 0.01; Table 4). STAT3 was no significant difference between them.

IFN- γ resistance of HepG2 cells is greatest among HCC cells

The viability of the four HCC cell lines was examined with an MTT assay following treatment with IFN- γ . As shown in **Figure 2A**, cell proliferation was significantly inhibited by IFN- γ in the PLC/PRF/5, Huh7, and SMMC7721 cell lines compared to untreated control cells (P < 0.05); however, HepG2 cells were not inhibited following treatment. Cell cycle analysis with

flow cytometry based on PI staining showed that after IFN-y treatment, the percentage of PLC/PRF/5 and SMMC7721 cells in GO/G1phase increased significantly, suggesting that IFN-y induced GO/G1-phase cell cycle arrest (Figure 2B; P < 0.05). However, IFN- γ did not induce GO/G1-phase cell cycle arrest in Hep-G2 and Huh7 cells (data not shown). Furthermore, evaluation of apoptosis with flow cytometry and AnnexinV-FITC/PI staining showed that after treatment with IFN-y, the early apoptosis rates of PLC/PRF/5, Huh7, and SMMC7721 cells increased significantly, suggesting that IFN-y induced apoptosis (Figure **2C**: P < 0.05). However, the early apoptosis rates of HepG2 cells only increased slightly (data not shown).

CNOT7 and STAT3 expression increased and STAT1 decreased in HepG2 cells

The mRNA and protein expression levels of CNOT7, STAT1 and STAT3 expression in the four HCC cell lines and LO2 cells were analyzed by RT-qPCR and western blot analysis, respectively. As shown in **Figure 3A**, the mRNA expression levels of *CNOT7* and *STAT3* were significantly higher in the four HCC cell lines compared to LO2 cells (P < 0.05), and were highest in HepG2

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Figure 2. Resistance of HCC cell lines to IFN- γ . Four HCC cell lines were treated with or without 20 ng/ml of IFN- γ for 24 h. The viability, cell cycle, and apoptosis were determined by MTT and flow cytometric analysis. Data are representative images or expressed as the mean \pm SD of individual groups of cells from 3 separate experiments. A. MTT assay: the asterisk indicates a significant difference between treatment groups and control groups (*P < 0.05). B. Cell cycle: cells were fixed and stained with PI. Cellular DNA content was analyzed by flow cytometric analysis and the cell distribution in various cell cycle phases was analyzed. C. Cell apoptosis: apoptosis was detected using the Annexin-V-FITC/PI assay. The lower right quadrant showed that cells were undergoing apoptosis.

cells among the HCC cells (P < 0.05). STAT1 mRNA expression in HepG2 cells was lower than that in PLC/PRF/5 and SMMC7721 cells (P < 0.05). As shown in **Figure 3B**, the patterns of CNOT7, STAT1 and STAT3 protein expression in the four HCC cell lines and LO2 were identical to those observed for mRNA expression, although with slightly smaller differences. The analysis of CNOT7 and STAT1 protein level by western blot confirmed the immunohistochemistry results. These data suggested that CNOT7 and STAT1 may be relevant to the IFN- γ resistance in HCC.

Modulation of CNOT7 expression alters STAT1 protein expression

Considering the results described above, HepG2 cells appeared to be the most suitable cell line for further evaluating the mechanism of IFN-v resistance in HCC. HepG2 cells were transfected with CNOT7-specific shRNA and non-specific shRNA as a control, followed by transfection with the recombinant plasmids encoding CNOT7 (GV230-CNOT7) and an empty vector (GV230-EV). As shown in Figure 4A, the depletion effect of shCNOT7 #2 was stronger than that of the others tested (P < 0.05), and was therefore used in the following experiments. CNOT7 protein expression of HepG2^{CNOT7} cells was significantly up-regulated compared to that of HepG2^{EV} cells (P < 0.05), with similar results obtained for CNOT7 mRNA expression. As shown in Figure 4B, the level of STAT1 protein was greater in HepG2^{shCNOT7} cells than in HepG2^{control} cells (P < 0.05). In parallel, the level of STAT1 protein was down-regulated in HepG2^{CNOT7} cells compared to that in HepG2^{EV} cells (P < 0.05). However, there was no signifi-



Figure 3. The role of CNOT7, STAT1, and STAT3 in the IFN-y resistance of HCC cell lines. A. SYBR green RTqPCR analysis was performed for detection of transcript levels of CNOT7, STAT1, and STAT3 in 4 HCC cell lines and LO2 cells. The results were normalized using the GAPDH mRNA level as an internal control. Transcript levels in L02 cell were set to 1 and other values are expressed relative to this. Data are representative images or expressed as the mean ± SD of individual groups of cells from 3 separate experiments. Multiple comparisons were evaluated with the SNK-q method. Values with different letters differ significantly (P < 0.05). B. CNOT7, STAT1, and STAT3 protein levels were analyzed by western blot. GAPDH was used as the internal control. The experiments were performed in triplicate.

cant difference in *STAT1* mRNA expression between HepG2^{shCNOT7} and HepG2^{control} cells, and between HepG2^{CNOT7} and HepG2^{EV} cells (date not show). There was also no significant difference in the mRNA and protein levels of STAT3 between these groups (date not show). These results prompted us to investigate a possible physical interaction between CNOT7 and STAT1 proteins. As shown in **Figure 4C**, the Co-IP assay revealed a clear interaction between STAT1 and CNOT7 in HepG2 cells.

CNOT7 protein depletion reversed IFN-γ resistance in HepG2 cells

As shown in Figure 5A, IFN-y significantly inhibited the proliferation of HepG2^{shCNOT7} cells (P <0.05) but not HepG2^{control} cells. Flow cytometry with PI staining showed that following IFN-y treatment, the percentage of HepG2^{shCNOT7} cells in the GO/G1-phase increased significantly, suggesting that IFN-y induced GO/G1-phase cell cycle arrest (P < 0.05); this increase was not observed in the untreated HepG2^{shCNOT7} cells compared to HepG2^{control} levels (Figure 5B). Evaluation of apoptosis with flow cytometry and AnnexinV-APC/7-AAD staining showed that following IFN-y treatment, the early apoptosis rates of HepG2^{shCNOT7} cells increased significantly, suggesting that IFN-y significantly induced HepG2^{shCNOT7} apoptosis (P < 0.05). However, compared with the HepG2^{control} cells, the early apoptosis rates of HepG2^{shCNOT7} cells increased only slightly. These results suggest that knocking down CNOT7 protein expression could reverse IFN-y resistance in HepG2 cells.

IFN-y up-regulated P-STAT1 and caspase3 and down-regulated P-STAT3 and cyclinE1 in CNOT7-deficient HepG2 cells

As shown in Figure 6A, STAT1 protein expression increased in both $HepG2^{shCNOT7}$ and HepG2^{control} cells after IFN-y treatment in a time-dependent manner, although the expression level of STAT1 protein was consistently greater in HepG2^{shCNOT7} cells. There was no significant difference in STAT1 mRNA expression between untreated HepG2^{shCNOT7} and HepG2^{control} cells, but STAT1 mRNA expression showed the same pattern as the protein expression after treatment with IFN- γ (P < 0.05). In the absence of IFN-y stimulation, P-STAT1 protein was not observed in either untreated cell line. However, P-STAT1 protein was detected 2 h after IFN-y treatment and then consistently expressed over 24 h in HepG2^{shCNOT7} cells. P-STAT1 protein was also detected 2 h after treatment in HepG2^{control} cells, although the effect only lasted for 8 h. This result suggests



Figure 4. Modulation of CNOT7 expression alters STAT1 protein expression. A. RT-qPCR and western blot analysis: HepG2 cells were transfected with four different shCNOT7 and non-specific shRNA as control and the recombinant plasmids of GV230-CNOT7 and GV230-empty vector (EV). Multiple comparisons were evaluated with the SNK-*q* method. Values with different letters differ significantly (P < 0.05). B. STAT1 expression after transfection of HepG2 cells were determined by western blot. C. CNOT7 and STAT1 interact. Extracts from HepG2 cells transfected with shCNOT7 and control shRNA were immunoprecipitated with either normal rabbit IgG or anti-CNOT7 antibody. Immunoprecipitates were then analyzed by immunoblotting with anti-STAT1 antibody. The experiments were performed in triplicate.

that induction of STAT1 protein over-expression could increase the level of P-STAT1 protein and even prolong its expression significantly. Furthermore, IFN-y treatment increased the level of STAT3 protein expression in HepG2^{shCNOT7} and HepG2^{control} cells in a time-dependent manner, with similar results for STAT3 mRNA. P-STAT3 expression was detected in both the treated and untreated cell lines. P-STAT3 increased 2 h after IFN-y treatment and then decreased after 8 h in both HepG2^{shCNOT7} and HepG2^{control} cells. However, both P-STAT3 and STAT3 expression was significantly down-regulated in HepG2^{shCNOT7} compared to HepG2^{control} cells after treatment with IFN-y. The similar effect was observed for STAT3 mRNA expression (P < 0.05). These results indicated that the expression of STAT3 and P-STAT3 may be competitively inhibited by induction of STAT1 protein overexpression.

To elucidate the mechanisms underlying the action of STAT1, cyclinE1 and caspase3 expres-

sion levels were also analyzed by RT-qPCR and western blot. As shown in **Figure 6B**, there was no significant difference in the levels of *cyclinE1* and *caspase3* mRNA between HepG2^{control} and HepG2^{shCNOT7} cells. However, after IFN- γ stimulation for 24 h, the expression level of *cyclinE1* mRNA in HepG2^{shCNOT7} cells was significantly inhibited, whereas *caspase3* mRNA expression was significantly enhanced (P < 0.05), with no changes observed in HepG2^{control} cells. The same patterns were observed for changes in protein levels of cyclinE1 and caspase3.

Discussion

HCC is one of the most common cancers worldwide. Epidemiological studies have showed that cirrhosis with hepatitis virus infection is the most predominant risk factor for HCC development. Most of the hepatitis following cirrhosis is HBV infection in China [19]. IFN- γ may function as a tumor suppressor by enhancing



Figure 5. IFN-y resistance was reversed by CNOT7 protein depletion. HepG2^{shCNOT7} and HepG2^{control} cells were treated with or without 20 ng/ml of IFN-γ for 24 h. The viability, cell cycle, and apoptosis were determined by MTT and flow cytometric analysis. Data are representative images or expressed as the mean ± SD of individual groups of cells from 3 separate experiments. A. MTT assay: the asterisk indicates a significant difference between treatment groups and control groups (*P < 0.05). B. Cell cycle: cells were fixed and stained with PI. Cellular DNA content was analyzed by flow cytometric analysis and cell distribution in various cell cycle phases was analyzed. C. Cell apoptosis: apoptosis was determined using the Annexin-V-APC/7-AAD assay. The lower right quadrant shows that cells were undergoing apoptosis.

tumor cell immunogenicity and cancer immunosurveillance [4]. In our study, we detected the levels of plasma IFN-y in HBC and HCCBC patients. There was no significant difference between these two groups. We found that the levels of plasma IFN-y in healthy controls were significantly lower than these two patients groups. Therefore, we assumed that a major factor resulting in the poor outcome may be IFN-y resistance of HCC cells, rather than the levels of plasma IFN-y concentrations decreasing in HCCBC patients. Then, we analyzed the levels of CNOT7, STAT1 and STAT3 expression in HCCBC patients. STAT1 were significantly lower in HCCBC compared to adjacent nontumor tissues. STAT1 is the crucial signaling molecules activated by IFN-y. STAT1 deficient may be responsible to the IFN-y insensitivity. This result supports a previous



Figure 6. IFN- γ signaling is redirected through feedback by CNOT7 protein depletion in HepG2^{shCNOT7} cells. A. After IFN- γ stimulation for the indicated times, the levels of *STAT1* and *STAT3* mRNA were measured in HepG2^{shCNOT7} and HepG2^{control} cells by RT-qPCR (*P < 0.05); the levels of tyrosine 701 phosphorylation of STAT1, STAT1, tyrosine 705 phosphorylation of STAT3 and STAT3 were measured in HepG2^{shCNOT7} and HepG2^{control} cells by western blot. B. HepG2^{shCNOT7} and HepG2^{control} cells by western blot. B. HepG2^{shCNOT7} and HepG2^{control} cells were treated with or without 20 ng/ml of IFN- γ for 24 h. The levels of cyclinE1 and caspase3 were measured by western blot and RT-qPCR. Data are representative images or expressed as the mean \pm SD of individual groups of cells from 3 separate experiments (*P < 0.05).

study [11]. CNOT7 regulated IFN pathways by controlling STAT1. It was much higher expression in the HCCBC than adjacent non-tumor tissues. This result was consistent with our hypothesis. However, the expressions of STAT3 were no significant differences in HCCBC and adjacent non-tumor tissues. This result was different from other reports [12]. One possible reason is that high IFN- γ concentration may stimulate HCC cells constantly, and then inhibit the STAT3 expression of HCCBC patients relatively. In order to further validate our hypothesis, we screened four HCC cell lines to detect the susceptibility to IFN- γ . We finally found that the levels of CNOT7 and STAT1 expression were correlated with IFN- γ resistance, and that CNOT7 depletion could reverse the IFN- γ resistance of HCC cells via adjustment of the STAT1/STAT3 ratio, and consequent promotion of STAT1 activation.

CNOT7 is the core deadenylase subunit of the CCR4-NOT complex. CNOT7 plays a key role in

regulating MHC-I mRNA deadenylation and drives tumor cell autonomous metastatic [20, 21]. CNOT7 overexpression can repress the anti-proliferative gene MSMB and PMP22 in human breast cancer, and knockdown of CNOT7 protein expression resulted in STAT1 overexpression and a hyper-activated subset of STAT1-regulated genes leading to retarded MCF7 cell growth [18, 22]. In the present study, CNOT7 was up-regulated in HCC cells and was positively correlated with IFN-y resistance; furthermore, CNOT7 depletion promoted STAT1 protein but not mRNA expression, with further Co-IP analysis suggesting a direct interaction between STAT1 and CNOT7 proteins. Therefore, our results indicate that CNOT7 may be a negative regulator of the IFN-y pathways by controlling STAT1 trafficking through interaction with its latent form. Specifically, the IFN-y-induced migration of P-STAT1 to the nucleus may be inhibited by the suppressive effects of CNOT7 on STAT1 and P-STAT1 expression through interaction with STAT1. Indeed, knockdown of CNOT7 protein expression promoted STAT1 and P-STAT1 protein expression in IFN-y-treated cells. The finding of a direct interaction between STAT1 and CNOT7 is similar to a previous report [18], although the mechanism proposed is slightly different.

The STAT family members play an important role in cytokine-related signaling processes. The major STAT protein activated by IFN-y is STAT1, and many IFN-y functions are mediated by direct activation of immune effector genes via STAT1 regulation. STAT1 is mainly recognized for its tumor-suppressing role, and is frequently silenced in many cancers [23, 24]. In our study, downregulation of STAT1 expression was correlated with the degree of IFN-y resistance of HCC cell lines, and the promotion of STAT1 protein expression by depletion of CNOT7 could markedly enhance the anti-HCC activities of IFN-y. STAT1 mediates antitumor functions after its activation via tyrosine phosphorylation (P-STAT1). Accordingly, it is generally considered that high STAT1 levels might result in better antitumor effects by enhancing P-STAT1 expression in response to IFN-y. Some reports have suggested that P-STAT1 could inhibit the expression level of cyclinE1 and induce the expression of caspase3 proteins [25, 26]. CyclinE1 is associated with CDK2 and is crucial for regulating the G1/S phase transition. Furthermore, IFN- γ can induce GO/G1 phase arrest by down-regulating cyclinE1 [27], and can also induce the cleavage of caspase3, which is the key driver of apoptosis [28]. Breast cancer patients with higher levels of phosphorylated and DNA-bound STAT1 show a better prognosis and live longer [29]. Consistent with this idea, in the present study, knockdown of CNOT7 protein expression (HepG2^{shCNOT7}) significantly promoted STAT1 and P-STAT1 protein expression following IFN- γ treatment, and down-regulated cyclinE1 and elevated caspase3 expression, reversing IFN- γ resistance.

STAT3 is another member of the STAT family. and has an opposing role to STAT1 in many biological processes [30]. STAT3 contributes to the development and progression of many types of cancers [12]. Inhibition of STAT3 activation reduced liver tumor cell growth in vitro and in vivo [31, 32], whereas activation of STAT3 promoted HCC development [24, 33]. Similar to previous reports, IFN-y significantly prevented the proliferation of HepG2^{shCNOT7} cells via inhibiting STAT3 and P-STAT3 protein expression relativity. On the basis of our results, we tried to prove the possible mechanism by which STAT1 and STAT3 may show mutual competitive inhibition after IFN-y treatment. The effects of IFN-y on tumors appear to be determined by the ratio of STAT1 to STAT3. STAT1 deficiency redirects IFN signaling through feedback activation of STAT3; if STAT1 is overexpressed, the opposite occurs. Indeed, in STAT3-deficient cells, STAT3-activators such as interleukin-6 trigger an IFN-y-like response through STAT1 activation, whereas in STAT1-deficient cells, IFN-y can trigger STAT3-dependent proliferative responses [34].

In summary, our results demonstrate that the occurrence of HCCBC partially attribute to IFN- γ resistance. IFN- γ resistance is correlated with CNOT7 overexpression and STAT1 deficiency. Given that CNOT7 is a key factor in IFN-negative regulation, the combination of CNOT7 depletion and IFN- γ appears to be highly effective for the inhibition of HCC cell proliferation *in vitro*, which may contribute to the development of new therapeutic approaches against liver cancer. CNOT7 knockdown had little or no effect on primary tumor cell, indicating that pharmaceutical suppression CNOT7 may not be unacceptably toxic in the clinic [20]. Furthermore, due to

its enhancing immunologic function, CNOT7 depletion is considered to be a superior adjuvant therapy in immunological therapy for HCC.

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Disclosure of conflict of interest

None.

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