17β-hydroxysteroid dehydrogenase 4 induces liver cancer proliferation-associated genes via STAT3 activation

XIN LU[1], LINGYU KONG[2], XI WANG[3], WEIRAN LIU[4], PANPAN MA[5] and LINGLING JIANG[6]

[1]Department of Clinical Laboratory, Tangshan Maternal and Children Hospital, Tangshan, Hebei 063000; [2]College of Integrative Chinese and Western Medicine, Hebei Medical University; [3]Department of Endocrinology, The Third Affiliated Hospital of Hebei Medical University, Shijiazhuang, Hebei 050017; [4]Department of Chinese Medicine, Tangshan Maternal and Children Hospital, Tangshan, Hebei 063000; [5]Department of Blood Transfusion, Hebei Provincial People's Hospital, Shijiazhuang; [6]Department of Biochemistry and Molecular Biology, The Key Laboratory of Neurobiology and Vascular Biology, Hebei Medical University, Shijiazhuang, Hebei 050017, P.R. China

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Abstract. Accumulating evidence has implicated that the activation of signal transducer and activator of transcription 3 (STAT3) contributes to the progression of liver cancer by affecting the expression of proliferation-associated genes. A previous study reported that elevated levels of 17β-hydroxysteroid dehydrogenase 4 (HSD17B4) are observed in patients with liver cancer. The current study investigated how upregulated HSD17B4 expression promoted the expression of proliferation-associated genes in rats with liver cancer. HSD17B4 expression in rats with liver cancer was significantly increased compared with the control group as determined by reverse transcription-quantitative polymerase chain reaction and western blot assays. Immunohistochemical results revealed that STAT3 activation was positively correlated with increased HSD17B4 expression in tumor tissues from patients with liver cancer. Western blot results further suggested that HSD17B4 overexpression increased STAT3 activation via the protein kinase B and the mitogen-activated protein kinase/extracellular-signal-regulated kinase signaling pathways in HepG2 cells. The present study suggested that overexpression may promote HepG2 proliferation by enhancing expression of various downstream targets of STAT3. Targeted inhibition of HSD17B4 may describe a novel approach in the prevention and treatment of liver cancer.

Correspondence to: Professor Lingling Jiang, Department of Biochemistry and Molecular Biology, The Key Laboratory of Neurobiology and Vascular Biology, Hebei Medical University, 361 Zhongshan East Road, Shijiazhuang, Hebei 050017, P.R. China
E-mail: jianglingling1959@163.com

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Introduction

Liver cancer is the third most fatal cancer in the world, with mortality rates of ≤0.49‰ and poor prognosis within the last 5 years (1-3). Although the underlying mechanisms of liver cancer development remains to be investigated, accumulating evidence has implicated that tumor cell proliferation contributes to the process of oncogenesis (4). Signal transducer and activator of transcription 3 (STAT3) is a nuclear transcription factor that promotes tumorigenesis, mediates the occurrence of liver cancer and serves a role in liver cancer development (5-7). STAT3 is phosphorylated upon activation, translocates into the nucleus and contributes to the process of oncogenesis by modulating the expression of various genes involved in proliferation (4). Inactivation of STAT3 leads to an inhibition of proliferation in liver cancer cell lines (8-10). The identification of genes regulating STAT3 activation may provide novel approaches for the treatment of liver cancer.

17β-Hydroxysteroid dehydrogenase 4 (HSD17B4) is widely distributed in peroxisomes of mammalian cells, with the highest levels reported for the liver (11-13). Previous studies have reported an increase in HSD17B4 expression in prostate and breast cancer and further tissues and cells (14,15). A recent study reported that HSD17B4 is upregulated in patients with liver cancer and HSD17B4 overexpression promotes HepG2 proliferation by enhancing cyclin D1 expression (16). It was demonstrated that STAT3 induces the transcription of cyclin D1 and serves an important role in hepatocyte proliferation (6,7). It is suggested that HSD17B4 may enhance STAT3 activation to promote tumor cell proliferation in liver cancer. However, the mechanism by which HSD17B4 promotes STAT3 activation in liver cancer requires to be investigated.

The current study investigated HSD17B4 upregulation and the correlation of HSD17B4 and the expression of various proliferation-associated genes in a liver cancer rat model, which was chemically induced using diethylnitrosamine (DEN). To understand how HSD17B4 promoted liver cancer cell proliferation, HSD17B4 and STAT3 levels were evaluated

and a positive correlation between HSD17B4 and phosphorylated (p)-STAT3 in patients with liver cancer was observed. A connection to the protein kinase B (Akt) and the mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) signaling pathways was further established. The findings suggested that HSD17B4 promoted liver cancer cell proliferation via STAT3 activation and HSD17B4 inhibition may inspire liver cancer treatment in the future.

Materials and methods

Isolation of liver tissue from patients with liver cancer. Human liver tissues were obtained from 16 male patients (age, 40-70 years) with liver cancer during surgical resection in The Second and The Third Affiliated Hospital of Hebei Medical University (Shijiazhuang, China) between August 2012 and December 2013. Written informed consent was obtained from each patient prior to resection and experiments were approved by the Ethics Committee of Hebei Medical University (Shijiazhuang, China). Patients with metastatic liver cancer were excluded from the current study. Tumor and adjacent (1 cm from tumor) liver tissues were identified according to pathology results. Tissues were fixed in 10% neutral-buffered formalin overnight at 4˚C and embedded in paraffin fro 1 h at 60-62˚C.

Animals. All animal studies were approved by the Institutional Animal Care and Use Committee of Hebei Medical University (approval no. HebMU20080026; Shijiazhuang, China) and efforts were made to minimize suffering. A total of 16 male Wistar rats (age, 4-5 weeks; weight, 100-120 g) were obtained from the Central Laboratorial Animal Facility at the Faculty of Medicine of Hebei University (Shijiazhuang, China). Rats were housed in cages under controlled environmental conditions at 25˚C with 55-65% humidity, a 12-h light/dark cycle and had free access to food and water. Following acclimatization for 1 week, rats were divided into two groups (8/group) as follows: Control group (NC), not receiving DEN, injected with an equal volume of saline; and DEN group (Cancer), receiving a weekly intraperitoneal dose of DEN (70 mg/kg) for 10 weeks. All animals were sacrificed at 20 weeks (17) by exsanguination through cardiac puncture under urethane anesthesia (20%; 1.2 g/kg; intraperitoneally). Livers were isolated and fixed for histopathological analysis.

Histopathological evaluation. Human and rat specimens were processed routinely in 10% formalin buffer overnight at 4˚C and embedded in paraffin 1 h at 60-62˚C. Tissue sections (4-μm) were obtained and stained with hematoxylin and eosin 2-3 min/each at room temperature. Histopathological examinations were performed under a light microscope (magnification, x200).

Western blotting. Precut rats livers were placed in Total Protein Isolation Buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 1 mM NaF) containing protease inhibitors (leupeptin and aprotinin, 1 μg/ml each), homogenized using an ice-chilled glass homogenizer with Teflon pestle and lysed for 40 min on ice with vortex mixing every 10 min. Supernatants were collected (15,000 x g; 30 min; 4˚C), the protein concentration was determined using the Lowry method and samples were aliquoted and stored at -70˚C until further analysis.

Cells were lysed in lysis buffer (50 mM PBS pH 7.5, 200 mM NaCl, 0.5 mM EDTA pH 8.0, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 0.5% Tween-20) for protein extraction. Following incubation on ice for 30 min, cell lysates were centrifuged (12,000 x g; 20 min; 4˚C), the protein concentration was determined as indicated above and the supernatants were stored at -20˚C until use.

A total of 20 or 40 μg protein was used in the analysis of HSD17B4 overexpressing or knockdown samples, respectively. Proteins were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% skim milk powder for 1 h at room temperature and incubated overnight at 4˚C with the following primary antibodies: Mouse anti-HSD17B4 (cat. no. sc-365167; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-cyclin D1 (cat. no. 2922S; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit antiproliferating cell nuclear antigen (PCNA; cat. no. 13110; 1:1,000; Cell Signaling Technology, Inc.), mouse anti-β-actin (cat. no. sc-47778; 1:1,000; Santa Cruz Biotechnology, Inc.), rabbit anti-p-STAT3 (cat. no. sc-135649; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-STAT3 (cat. no. sc-482; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-p-Akt (cat. no. sc-7985; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-Akt (cat. no. sc-8312; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-p-MEK (cat. no. sc-101733; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-MEK (cat. no. sc-9259; 1:50; Santa Cruz Biotechnology, Inc.), rabbit anti-p-ERK (cat. no. sc-23759; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-ERK (cat. no. sc-292838; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-p-c-Jun N-terminal kinase (JNK; cat. no. sc-135642; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-JNK (cat. no. sc-571; 1:500; Santa Cruz Biotechnology, Inc.), rabbit anti-p-p38 (cat. no. sc-101759; 1:500; Santa Cruz Biotechnology, Inc.), and rabbit anti-p38 (cat. no. sc-156091; 1:500; Santa Cruz Biotechnology, Inc.). Following incubation with goat anti-mouse or anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (cat. nos. 610-703-002 and 611-142-002, respectively; 1:5,000; Rockland Immunochemicals Inc., Limerick, Pennsylvania, USA) for 1 h at room temperature, bands were visualized using an enhanced chemiluminescence kit (Fuazon Fx; Vilber Lourmat, Marne-la-Vallée, France). Images were captured and processed by FusionCapt Advance Fx5 (Vilber Lourmat). The relative gray scale indicated the expression of various genes, with β-actin as the protein loading control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from rat livers using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. cDNA was synthesized using a SuperScript Reverse Transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.) with the following protocol: 5 min at 65˚C, 2 min at 37˚C, 10 min at 25˚C, 50 min at 37˚C and 15 min at 70˚C. cDNA was

Table I. Polymerase chain reaction primers.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
18S rRNA (M11188)	CGCCGCTAGAGGTGAAATTC	CCAGTCGGCATCGTTTATGG
HSD17B4 (NM_024392)	GGTGGTAAAGAAAGTAAATG	AATTGTGATGGTCGTGTC
cyclin D1 (NM_171992.4)	GGAGCAGAAGTGCGAAGA	GGGTGGGTTGGAAATGAA
PCNA (NM_022381.3)	ACAGAGCATGGATTCGTCTCAC	AGAAAACTTCACCCCGTCCTTT

HSD17B4, 17β-hydroxysteroid dehydrogenase 4 ; PCNA, proliferating cell nuclear antigen; r, ribosomal.

used as a template for qPCR with a SYBR Green PCR Master Mix kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed using a Rotor-Gene 3000 Detection System (GeneBio Systems, Inc., Oakville, ON, Canada) using the following protocol: Initial denaturation step at 95˚C for 30 sec, followed by 40 cycles of denaturation for 5 sec at 95˚C, annealing for 30 sec at 55˚C and elongation for 20 sec at 72˚C. 18S ribosomal RNA was used as internal control and for normalization. All reactions were performed in triplicate. Relative mRNA expression was determined using the $2^{-\Delta\Delta Cq}$ method (18). Primer details are presented in Table I.

Immunohistochemistry (IHC) assays. Immunohistochemistry was performed with paraffin-embedded human or rat tissue sections (4-μm) as previously described (19). Briefly, liver sections were blocked with goat serum (1:1; OriGene Technologies, Inc., Rockville, MD, USA) at 37˚C in a wet box for 30 min and incubated with a mouse anti-HSD17B4 antibody (cat. no. PA1727; 1:1,000; Santa Cruz Biotechnology, Inc.) and rabbit anti-STAT3 antibody (cat. no. sc-135649; 1:500; Santa Cruz Biotechnology, Inc.) overnight at 4˚C in moist chambers. Following washing with 0.01 mol/l PBS (pH 7.2) three times, slides were incubated with a biotinylated secondary anti-mouse (cat. no. PV-6000; 1:1; OriGene Technologies, Inc.) or anti-rabbit antibodies (cat. no. PV-9000; 1:1; OriGene Technologies, Inc.) for 30 min at 37˚C. Samples were then incubated with HRP-avidin D (cat. no. A-2004; Vector Laboratories, Inc.; Maravai LifeSciences, San Diego, CA, USA) at 37˚C for 30 min and developed using a diaminobenzidine kit (Vector Laboratories, Inc.; Maravai LifeSciences). Sections were counterstained with hematoxylin for 30 sec at room temperature. Staining intensities were determined by measuring the integrated optical density (IOD) with light microscopy (magnification, x200) with associated software (SPOT Basic™ image capture software version 3.2.4; cat. no. SPOT53BE; SPOT Imaging; Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Cell culture and treatment. The human liver cancer cell line HepG2 was obtained from the American Type Culture Collection (cat. no. HB-8065; Manassas, VA, USA). HepG2 cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Abgent Biotech Co., Ltd., Suzhou, China), penicillin and streptomycin (100 U/ml, each) in a humidified environment at 37˚C with 5% CO_2. For inhibitor studies, all inhibitors were dissolved in dimethyl sulfoxide (DMSO) and control experiments were performed with equal volumes of DMSO. Cells were treated for 2 h with MEK inhibitor PD98059 (5 μmol/; Calbiochem; Merck KGaA, Darmstadt, Germany) or phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (5 μmol/l; Calbiochem; Merck KGaA) prior to transformation with the HSD17B4 overexpression plasmid.

Cell proliferation assay. HepG2 cell proliferation assays were performed using a bromodeoxyuridine (BrdU) Cell Proliferation Assay kit (Millipore; Merck KGaA) according to the manufacturer's instructions. Cells were labeled with BrdU for 6 h at 24 h following HSD17B4 plasmid transfection. Optical density readings were performed at 450 nm to measure the incorporation of BrdU. All groups were evaluated with ≥3 separate wells/experiment.

Plasmid constructs and transfection. HSD17B4 cDNA was cloned into the pLL3.7 vector (Addgene, Inc., Cambridge, MA, USA). HepG2 cells were plated at $5x10^6$/well in 6-well culture dishes for 18 h to reach ~70% confluence in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS without antibiotics. Cells were washed twice with serum-free RPMI-1640 and 1 ml serum-free Opti-MEM I (Gibco; Thermo Fisher Scientific, Inc.) was added to each well. A DNA-Lipofectamine 2000 complex was prepared according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 6 μg transfected plasmid and 30 μl Lipofectamine reagent were added to each reaction. The empty vector control is indicated using '-' and the HSD17B4 plasmid is highlighted using '+' in the following. Cells were incubated at 37˚C for 6 h. Following transfection, cells were maintained in DMEM containing 1% FBS for 24 h and the transfection efficiency was determined using western blotting. Cells were collected for further analysis.

Small interfering (si) RNA transfection. siRNA targeting HSD17B4 (siHSD17B4; forward, 5'-GUACCUUUGUAU UUGAGGAdTdT-3' and reverse, 5'-UCCUCAAAUACAAAG GUACdTdT-3') and non-specific siRNA (siNC; forwards, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACG UGACACGUUCGGAGAATT-3'), were purchased from Sigma-Aldrich (Merck KGaA). Transfections with 10 μmol/l siRNA (siNC as '-' and siHSD17B4 as '+') were performed

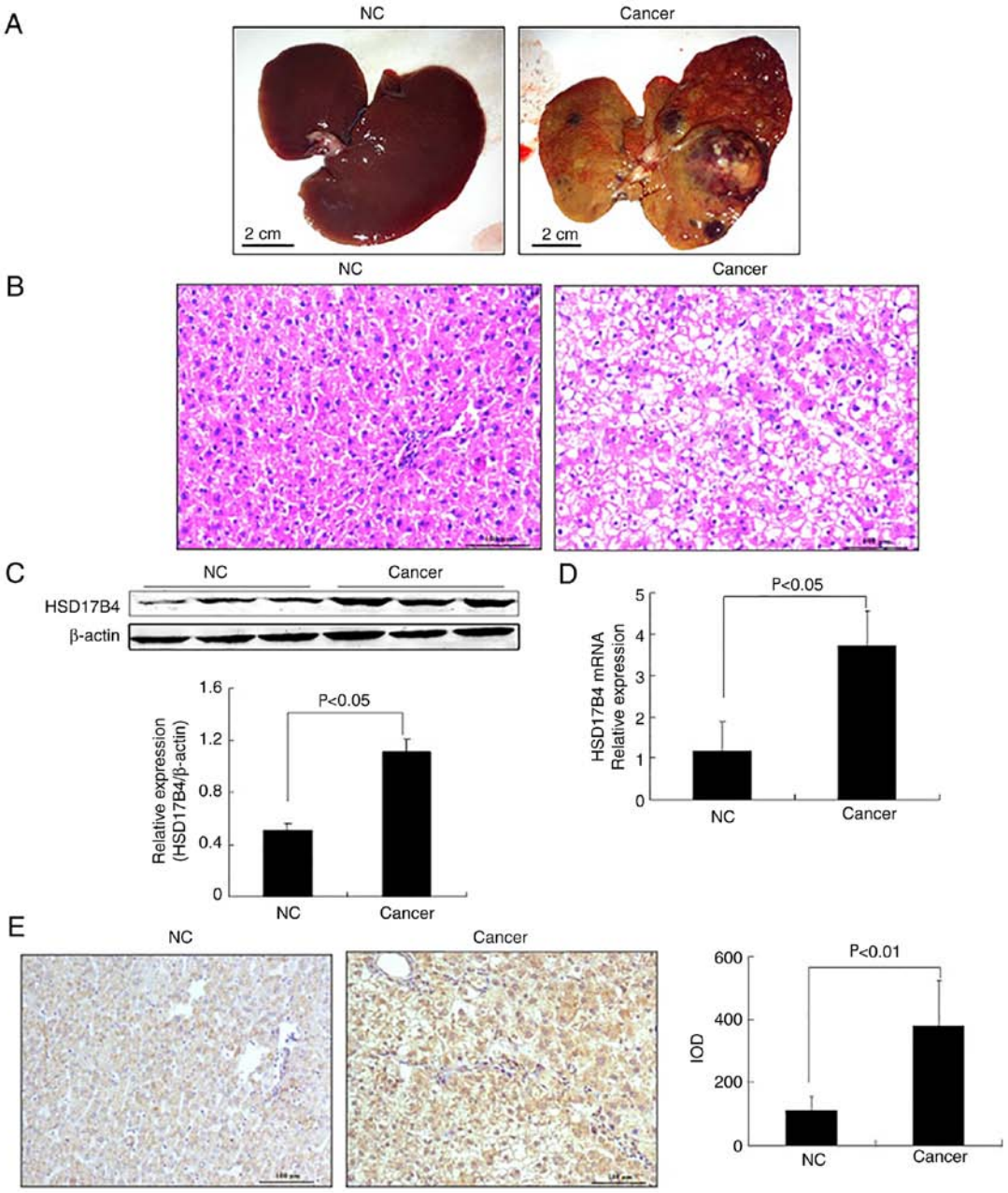

Figure 1. HSD17B4 expression is increased in rats with liver cancer. (A) Images of livers isolated from rats of the NC or the Cancer group. (B) Hematoxylin and eosin analysis of rat livers of the NC or the Cancer group (magnification, x200). (C) HSD17B4 expression in rat liver tissues determined by western blotting (n=8/group). (D) HSD17B4 mRNA expression determined by reverse transcription-quantitative polymerase chain reaction. (E) Immunohistochemistry analysis of HSD17B4 expression in rat livers (magnification, x200). Cancer, rats with diethylnitrosamine-induced liver cancer; NC, negative control; HSD17B4, 17β-hydroxysteroid dehydrogenase 4 ; IOD, integrated optical density.

using the Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions, when cells reached 50-60% confluence. Following 24 h of transfection, the transfection efficiency was determined using western blotting. Cells were harvested and lysed as described for RT-qPCR and western blotting.

Statistical analysis. Data are presented as the mean ± standard deviation of ≥3 independent experiments. All statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Statistical differences between groups were assessed using a one-way analysis of variance, followed by Dunnett's or Bonferroni's multiple comparison tests. Correlation analysis was performed using the Pearson's correlation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HSD17B4 expression is increased in tissues from rats with liver cancer. To evaluate HSD17B4 expression during liver cancer development, rats with DEN-induced liver cancer were investigated. All rats in the Cancer group developed liver cancer while the livers of rats in the NC group exhibited a normal lobular architecture with central vein and radiating hepatic cords (Fig. 1A and B). Hepatocytes were polyhedral in shape and the cytoplasm was granulated with small uniform

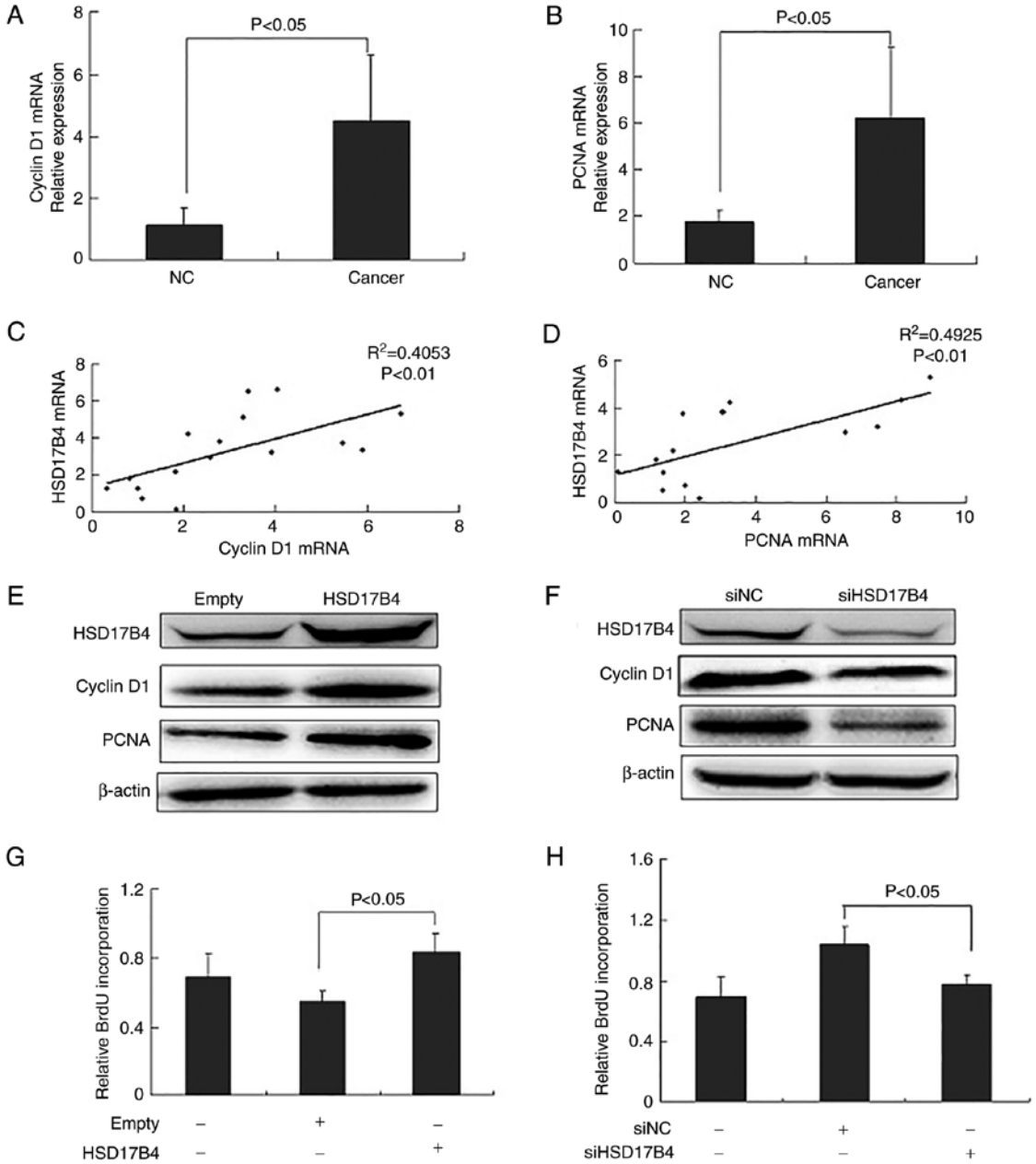

Figure 2. HSD17B4 promotes liver cancer proliferation by enhancing expression of proliferation-associated genes. mRNA expression of (A) cyclin D1 and (B) PCNA in liver tissues isolated from rats of the NC or the Cancer group determined by reverse transcription-quantitative polymerase chain reaction. Correlation analyses of HSD17B4 mRNA expression and (C) cyclin D1 or (D) PCNA mRNA expression in liver tissues isolated from rats. Cyclin D1, PCNA and HSD17B4 protein expression in HepG2 cells treated with (E) HSD17B4 overexpression vector or (F) with siHSD17B4 determined by western blotting. (G) Proliferation of HepG2 cells (G) overexpressing HSD17B4 or (H) treated with siHSD17B4 assessed using the BrdU incorporation assays. Cancer, rats with diethylnitrosamine-induced liver cancer; NC, negative control; HSD17B4, 17β-hydroxysteroid dehydrogenase 4 ; PCNA, proliferating cell nuclear antigen; si, small interfering RNA; Empty, cells transfected with empty pLL3.7 vector.

nuclei. The Cancer group exhibited extensive cell swelling and single cell necrosis; necrotic cells were small with basophilic nuclei and dark cytoplasm (Fig. 1B). These results suggested that the rat liver cancer model using DEN was successfully established.

HSD17B4 expression in the liver tissues of the rats was determined. HSD17B4 protein and mRNA levels were significantly increased in the Cancer compared with the NC group (P<0.05; Fig. 1C and D). DEN administration resulted in HSD17B4 accumulation in the cytoplasm of liver tissues from rats with liver cancer (Fig. 1E). Compared with the NC, the IOD was significantly increased in the Cancer group (P<0.01; Fig. 1E). The results indicated that HSD17B4 expression was increased during liver cancer development.

HSD17B4 enhances proliferation and expression of proliferation-associated genes. To evaluate an association between HSD17B4 expression and proliferation-associated genes, mRNA levels of cyclin D1 and PCNA were evaluated. It was observed that expression was significantly increased in rats with liver cancer compared with the NC group (P<0.05; Fig. 2A and B). The correlation analysis between HSD17B4 and cyclin D1 or PCNA mRNA expression revealed a positive correlation (P<0.01; Fig. 2C and D).

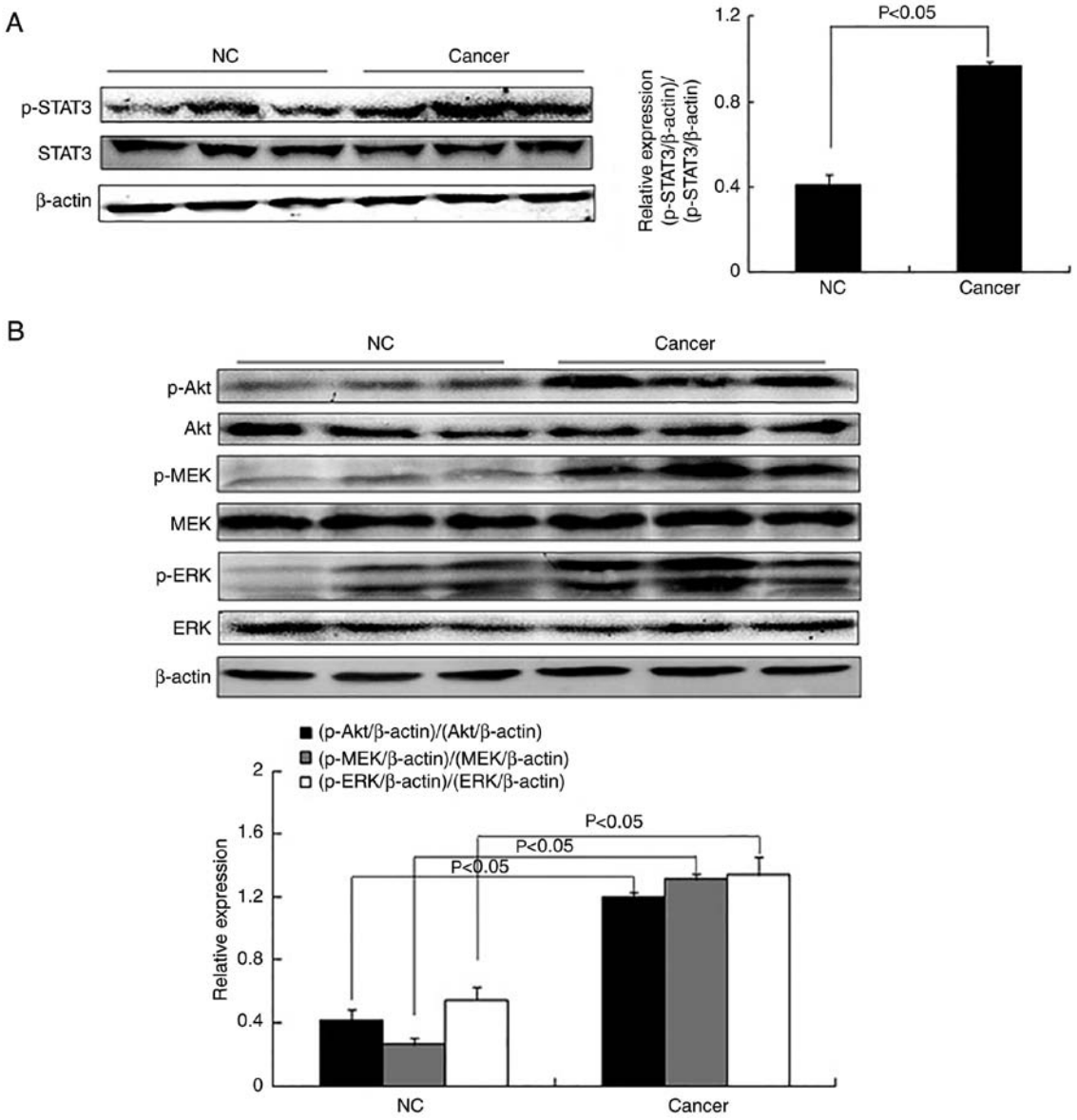

Figure 3. STAT3 activation is increased in liver tissues from rats with liver cancer. (A) STAT3 expression and phosphorylation in liver tissues isolated from rats of the NC or the Cancer group determined (n=8/group). (B) Expression and activation of the Akt, MEK and ERK determined by western blotting. Cancer, rats with diethylnitrosamine-induced liver cancer; NC, negative control; HSD17B4, 17β-hydroxysteroid dehydrogenase 4 ; Akt, protein kinase B; MEK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase.

Protein expression of HSD17B4, cyclin D1 and PCNA was determined in HepG2 HSD17B4 overexpression and knockdown cells. Compared with the empty vector, cyclin D1 and PCNA levels were increased in the HSD17B4 overexpressing cells (Fig. 2E). In the siHSD17B4-treated cells cyclin D1 and PCNA expression was downregulated compared with the siNC (Fig. 2F). The results indicated that HSD17B4 expression affected proliferation-associated genes in HepG2 cells.

To further investigate the role of HSD17B4 in proliferation, BrdU incorporation assays were performed with HSD17B4 overexpression and knockdown HepG2 cells. Proliferation of the HSD17B4 overexpressing cells was significantly increased compared with the empty vector control (P<0.05; Fig. 2G) and BrdU incorporation was significantly decreased in the siHSD17B4-treated cells compared with the siNC (P<0.05; Fig. 2H). The results indicated that HSD17B4 promoted HepG2 proliferation by affecting proliferation-associated genes.

STAT3 activation is increased in liver tissues from rats with liver cancer. STAT3 phosphorylation contributes to liver cancer progression (5-7). STAT3 phosphorylation was significantly increased in the Cancer compared with the NC group (P<0.05; Fig. 3A). As STAT3 is involved in various signaling pathways (20,21), proteins associated with the Akt and the MEK/ERK signaling pathways were investigated. Western blot analysis revealed that levels of p-Akt, p-ERK and p-MEK were significantly increased in tissues from the Cancer compared with the NC group (P<0.05; Fig. 3B).

HSD17B4 overexpression increases STAT3 activation. A recent study revealed that HSD17B4 is upregulated in patients with liver cancer and HSD17B4 over-expression promotes HepG2 proliferation (16). To determine whether HSD17B4 serves a role in liver cancer progression, HSD17B4 expression in adjacent and tumor tissues from patients with liver cancer

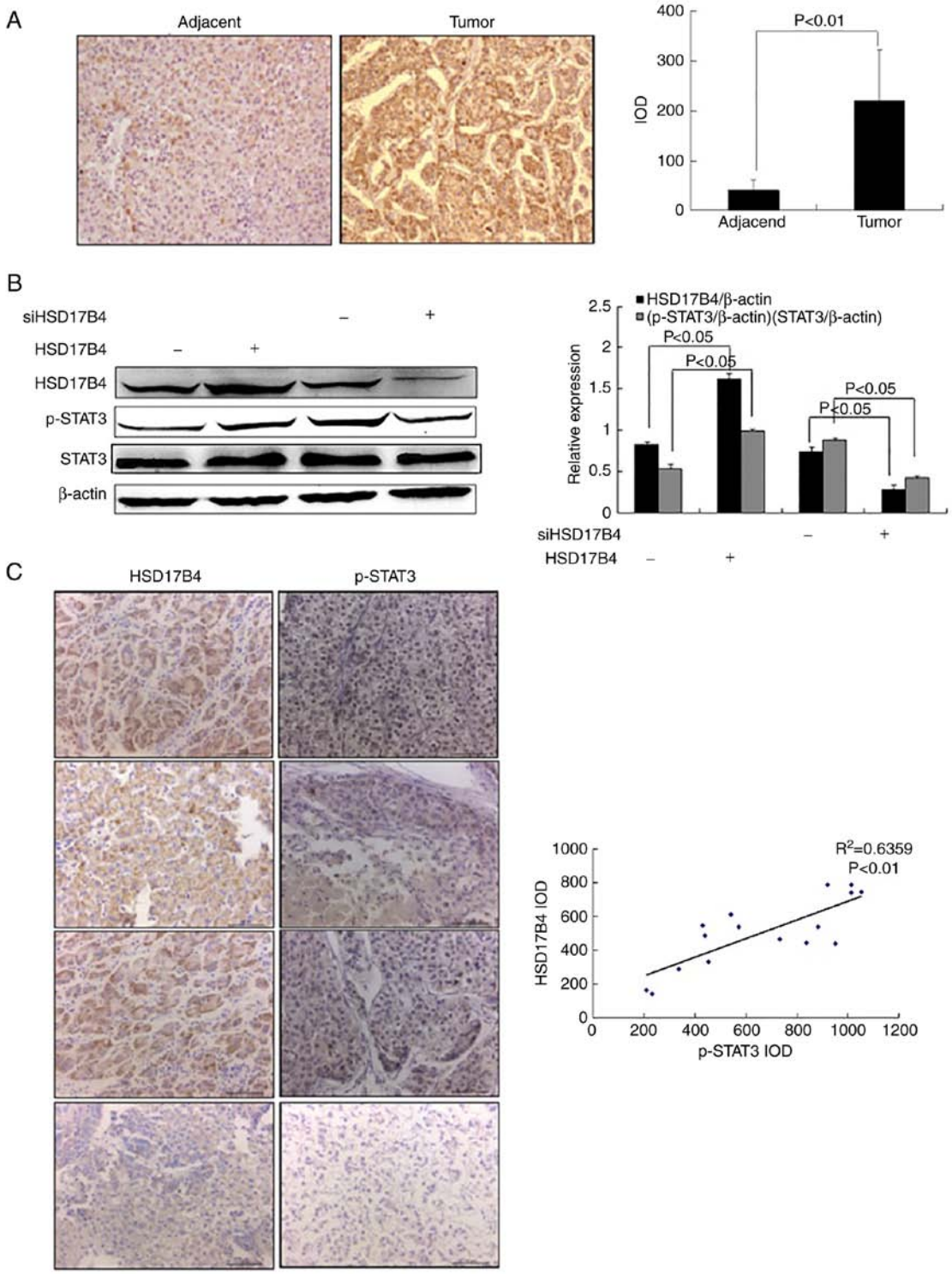

Figure 4. HSD17B4 expression induces STAT3 activation. (A) IHC staining targeting HSD17B4 in liver tumor and adjacent normal tissues from patients with liver cancer (n=16; magnification, x200). (B) STAT3 activation in HepG2 cells treated with HSD17B4 overexpression vector or treated with siHSD17B4 determined by western blotting (n=3/group). (C) IHC analysis of HSD17B4 and p-STAT3 levels in liver tumor and adjacent normal tissues from patients with liver cancer (n=16; magnification, x200) and Pearson's correlation analysis of IODs. HSD17B4, 17β-hydroxysteroid dehydrogenase 4 ; IOD, integrated optical density; si, small interfering RNA; p, phosphorylated; STAT3, signal transducer and activator of transcription 3; NC, negative control; -, transfected empty vector or siNC; +, transfected HSD17B4 vector or siHSD17B4.

were evaluated. IHC images revealed that HSD17B4 expression was increased in tumor tissues compared with adjacent normal tissues obtained from patients with liver cancer and the determined IOD was significantly increased in the cancerous tissues (P<0.01; Fig. 4A).

An association between HSD17B4 expression and activation of STAT3 was evaluated in HSD17B4 overexpression and knockdown HepG2 cells. As presented in Fig. 4B, HSD17B4 overexpression significantly increased STAT3 phosphorylation in HepG2 cells compared with the empty vector

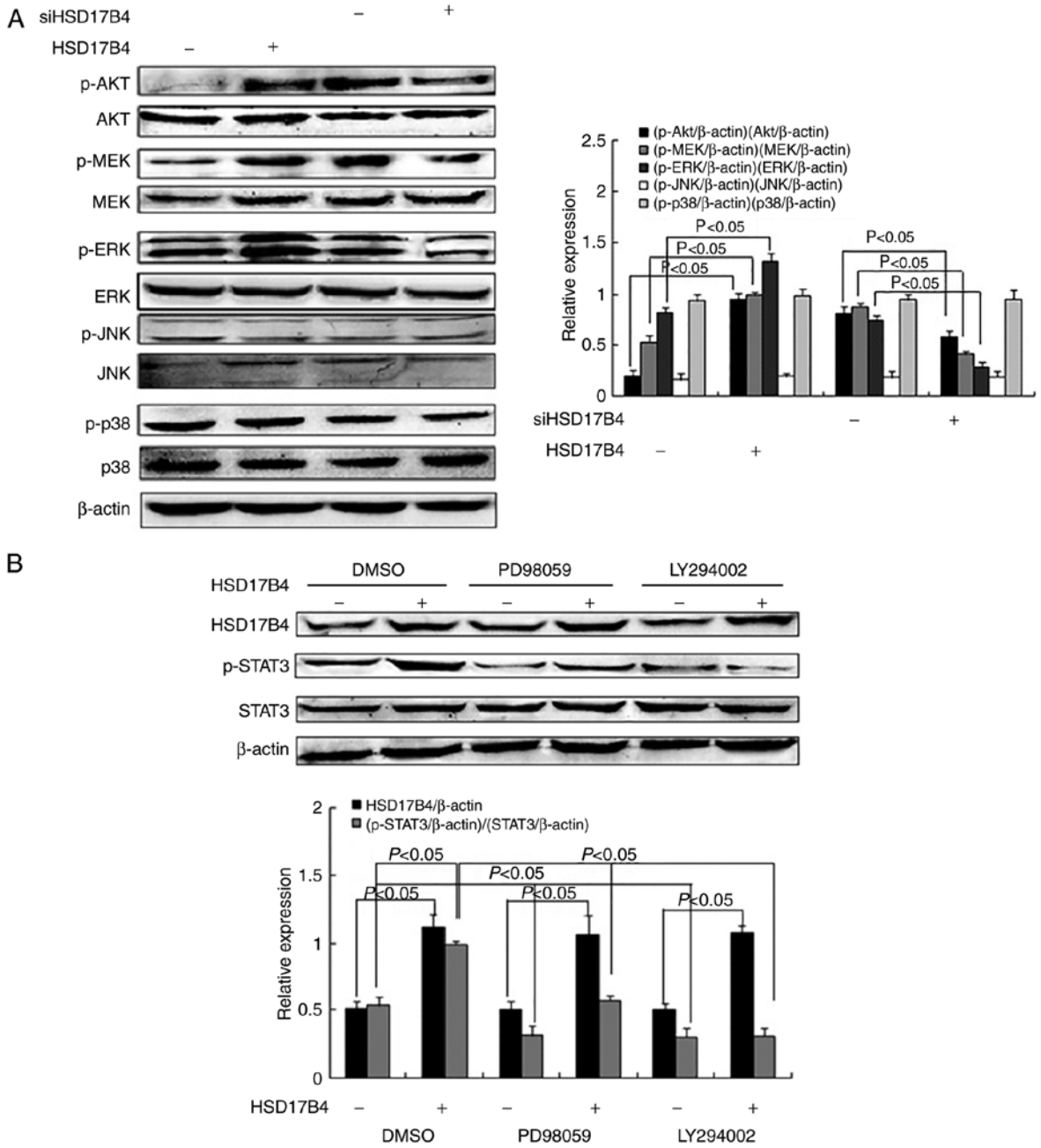

Figure 5. HSD17B4 expression is associated the Akt and the MEK/ERK signaling pathways. (A) Expression and activation of the Akt, MEK, ERK, JNK and p38 in HepG2 cells treated with HSD17B4 overexpression vector or treated with siHSD17B4 determined by western blotting (n=3/group). (B) HSD17B4 over-expressing HepG2 cells were pretreated with LY294002 and PD98059 inhibitors and HSD17B4 expression and STAT3 activation were assessed by western blotting (n=3/group). HSD17B4, 17β-hydroxysteroid dehydrogenase 4 ; si, small interfering RNA; p, phosphorylated; STAT3, signal transducer and activator of transcription 3; Akt, protein kinase B; MEK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; LY294002, phosphoinositide 3-kinase inhibitor; PD98059, MEK inhibitor; NC, negative control; -, transfected empty vector or siNC; +, transfected HSD17B4 vector or siHSD17B4.

group (P<0.05). In contrast, p-STAT3 levels were significantly decreased in the siHSD17B4-treated cells compared to the siNC group (P<0.05; Fig. 4B).

To verify these finding, HSD17B4 and p-STAT3 expression in tumor and adjacent normal tissues of patients liver cancer were evaluated using IHC. IODs determined for HSD17B4 and p-STAT3 suggested a positive correlation between the HSD17B4 expression and STAT3 activation (P<0.01; Fig. 4C). The results suggested that HSD17B4 expression induced STAT3 activation.

HSD17B4 expression induces Akt and MEK/ERK activation. To determine the mechanism by which HSD17B4 promotes STAT3 activation in liver cancer, activation of various signaling pathways involved in liver cancer progression were assessed. HSD17B4 overexpression increased the phosphorylation of Akt, MEK and ERK in HepG2 cells compared with the empty vector group (P<0.05; Fig. 5A). In siHSD17B4-treated cells, Akt, MEK and ERK phosphorylation was significantly decreased compared with the siNC group (P<0.05; Fig. 5A). Activation of JNK and p38 were not affected by the induction

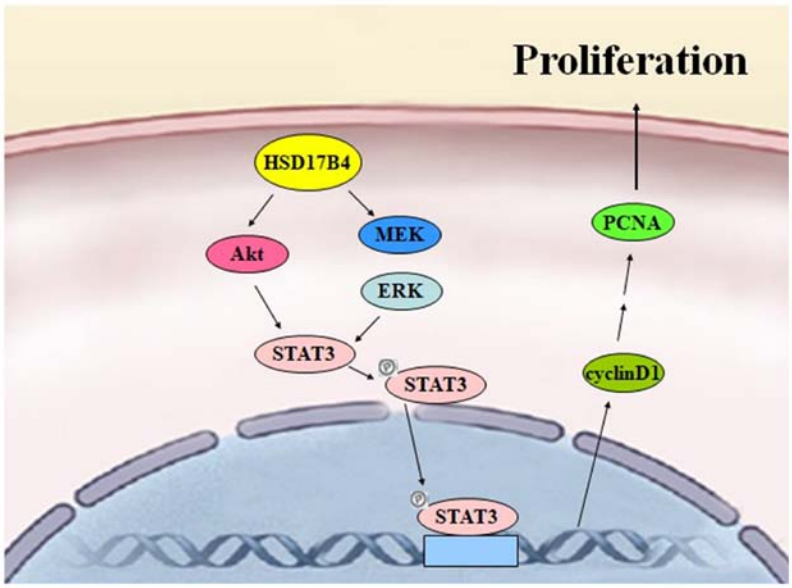

Figure 6. Proposed mechanism for the involvement of HSD17B4, STAT3 and proliferation-associated genes in liver cancer. HSD17B4, 17β-hydroxysteroid dehydrogenase 4 ; p, phosphorylated; STAT3, signal transducer and activator of transcription 3; Akt, protein kinase B; MEK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated kinase; PCNA, proliferating cell nuclear antigen.

or knockout of HSD17B4 expression (P>0.05; Fig. 5A). The results suggested that activation of the Akt and the MEK/ERK signaling pathways was associated with HSD17B expression.

To investigate which signaling pathways are involved in STAT3 activation, HepG2 cells were incubated with PD98059 and LY294002, inhibitors of the MEK/ERK and the PI3K/Akt signaling pathway, respectively, prior to transfection with the HSD17B4 overexpression plasmid (22). STAT3 phosphorylation was determined and it was observed that levels significantly decreased in HSD17B4 overexpressing cells in the presence of MEK/ERK and PI3K/Akt signaling pathway inhibitors compared with the DMSO treated cells and a significant decrease was further observed for empty vector-transfected cells in the presence of inhibitors compared with the DMSO-treated cells (P<0.05; Fig. 5B). The results suggested HSD17B4 expression promoted STAT3 activation via the PI3K/Akt and the MEK/ERK signaling pathways.

Discussion

As an important oxidoreductase, HSD17B4 is widely distributed in peroxisomes of mammalian cells (11-13). HSD17B4 is ubiquitous and increased levels were detected in mammalian liver, heart, brain and prostate tissues under normal physiological conditions (12,13). Initial research focused on HSD17B4 function associated with the inactivation of the estrogen metabolism (23,24). It was further discovered that HSD17B4 is an important enzyme in the fatty acid β-oxidation pathway in peroxisomes; it is involved in the oxidative decomposition of very long-chain fatty acids and branched-chain fatty acids and in the biosynthesis of docosahexaenoic acid (25). An increase of HSD17B4 was observed in a variety of tumor cells and tissue suggesting that HSD17B4 may serve a role in tumor development (26-28).

In a previous study, it was demonstrated that HSD17B4 expressed is increased in patients with liver cancer (16). In the current study, it was observed that HSD17B4 expression was upregulated rats with liver cancer compared with healthy control animals. It was revealed that HSD17B4 overexpression and knockdown in HepG2 increased and decreased expression of proliferation-associated genes, respectively. The current study further revealed a positive correlation between HSD17B4 and cyclin D1 and PCNA mRNA expression in rats with liver cancer. The results suggested that HSD17B4 may promote liver cancer proliferation and may serve a crucial role in liver cancer development.

STAT3 is a nuclear transcription factor that binds to specific sequences of target gene promoters (29,30) and induces cancer cell proliferation by upregulating the expression of various genes (4,31). STAT3 promotes liver tumor formation by mediating multiple cellular processes and enhancing the development of liver cancer (32,33). With accumulating research, STAT3 has become an attractive target for the treatment and prevention of human liver cancer (34,35). The current study suggested that HSD17B4 upregulation mediated STAT3 activation and a correlation was determined between these factors in tumor and adjacent normal tissues from patients with liver cancer.

STAT3 is active in liver tumor cells and is involved in various signaling pathways (20,21). STAT3 phosphorylation is involved the MEK and mitogen-activated protein kinase kinase 4 signaling cascade and is induced independently of ERK-1 or JNK-1 activity by interleukin-6 (36). Sorafenib inhibits liver cancer growth by blocking the MEK/ERK/STAT3 and the PI3K/Akt/STAT3 signaling pathways, independent of Janus kinase 2 and tyrosine-protein phosphatase non-receptor type 11 activation (37). Various studies have confirmed that the MEK/ERK/STAT3 and the PI3K/Akt/STAT3 signaling pathways serve important roles in promoting liver cancer cell proliferation (37,38). Experiments using HepG2 revealed that HSD17B4 induced STAT3 phosphorylation through the MEK/ERK and the PI3K/Akt signaling pathways without

affecting the JNK and the p38 signaling pathways. The specific mechanism by which HSD17B4 affects the signal pathways requires further investigation.

In conclusion, the data collected in the current study indicated that HSD17B4 may be a novel proliferation-promoting protein and the following mechanism is proposed: HSD17B4 overexpression promotes activation of STAT3 via the PI3K/Akt and the MEK/ERK signaling pathways, which stimulate STAT3 binding to the response element. In turn, cell proliferation is promoted via cyclin D1 and PCNA upregulation (Fig. 6). The presented results may describe an experimental basis for novel approaches in the prevention and treatment of liver cancer using HSD17B4.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XL and PM performed the majority of the experiments, collected the clinical samples and acquired patients' information. LK, XW and WL made substantial contributions to acquisition, analysis and interpretation of data. LJ conceived, designed the study and revised the manuscript. All authors read and approved the final version of the manuscript.

Ethical approval and consent to participate

Experiments involving human samples and animal experiments were approved by the Ethics Committee of Hebei Medical University (Shijiazhuang, China). All experiments were conducted according to relevant national and international guidelines. Written informed consent was obtained from all participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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