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Correlation between HSD17B4 expression in rat liver cancer tissues and inflammation or proliferation

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Abstract. – OBJECTIVE: Pathogenesis and progression of liver cancer are correlated with inflammatory response and estrogen level. 17β-estradiol dehydrogenase IV (HSD17B4) is highly expressed in human liver cancer tissues. HSD17B4 participates in liver cancer cell proliferation via suppressing estradiol (E2) activity. This study generated a rat liver cancer model, on which the correlations between HSD17B4 and tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), proliferating cell nucleus antigen (PCNA) expression were analyzed.

MATERIALS AND METHODS: Male Sprague Dawley (SD) rats were randomly assigned into control and model group (N=30). Diethylnitrosamine was used to induce liver cancer in a rat model. HE staining was used to observe liver injury whilst ELISA was used to measure serum TNF-α and IL-6 levels. The level of serum E2 was quantified by radioimmunoassay. Serum liver function indexes were measured by automatic biochemical analyzer. Protein expressions of HSD17B4, p-Akt, p-ERK and PCNA were measured by Western blot.

RESULTS: The inflammatory infiltration and necrosis of hepatocytes were shown in model group by HE staining, along with aggravated liver indexes. Significantly high phosphorylation level of Akt and ERK, along with the increase of HSD17B3 and PCNA expressions, was found in model group ($p<0.05$ compared to control group). Serum E2 level was statistically decreased, whilst TNF-α and IL-6 were up-regulated ($p<0.05$). HSD17B4 was positively correlated with TNF-α, IL-6 and PCNA expressions (r=0.68, 0.62 and 0.56, $p<0.05$).

CONCLUSIONS: HSD17B4 is over-expressed in rat liver cancer tissues. Its expression was positively correlated with TNF-α, IL-6 and PCNA levels, and probably participates in liver cancer cell proliferation via ERK and Akt signal pathway.

Key Words:
Liver cancer, TNF-α, PCNA, HSD17B4.

Introduction

Liver cancer is a kind of common malignant tumor occurring in digestive tract, with relatively higher incidence and mortality worldwide. Aflatoxins intake and HBV infection are major risks for hepatocellular carcinoma. Currently, pathogenesis mechanism of liver cancer has not been fully illustrated, although viral hepatitis, liver cirrhosis and aflatoxins are considered to be probably involved. Epidemic survey showed that liver cancer incidence in males was higher than that in females, indicating possible correlation between estrogen level and liver cancer pathogenies and progression[1,2]. During pathogenesis process of liver cancer, inflammatory cell infiltration leads to the release of fibrosis and inflammatory factors, necrosis and proliferation of hepatocytes[3,4]. Nuclear factor-kappa B (NF-κB) plays an important role in the progression of inflammation into cancer, as it participates in liver cancer formation via mediating target gene transcription. The activation of NF-κB can facilitate cancer cell proliferation via modulating PCNA, IL-6 and cell cycle protein D1. During onset and progression of hepatocellular carcinoma, TNF-α/NF-κB interacts and works as a bridge[5,6]. HSD17B4 contributes to the regulation of steroid metabolism, as it can inactivate the oxidation of estradiol (E2) into E1. Study showed that E2 could inhibit pathological progression of hepatocellular carcinoma, as E2 metabolic disorder results in pathology condition of bone and cardiovascular system. Also,

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evidence showed that E2 level in hepatocellular carcinoma tissues was lower than normal people. Research demonstrated that HSD17B4 was highly expressed in prostate cancer tissues, and was correlated with cancer malignancy. In liver cancer cells, both HepG2 and HSD17B4 expressions were increased, and the overexpression of HSD17B4 probably participated in cancer progression via modulating E2 levels[7,8]. Moreover, both TNF-α and NF-κB are over-expressed in liver cancer tissues and adjacent tissues. Their levels are significantly higher in HBV-infected liver cancer lesions than that in adjacent or non-cancerous tissues.

Diethylnitrosamine (DEN) is regarded as liver toxic substances. Study on rat model showed that DEN, as similar with n-dimethyl nitrosamine, could also induce cancers via inducting methylation of intracellular protein and nucleic acids, hepatocyte apoptosis, DNA damage and persistent hepatocyte regeneration. Accumulative findings illustrated the development of liver cancer from inflammation and liver cirrhosis to liver cancer pathogenesis[9,10]. This study thus determined the relationship between HSD17B4 and inflammation or proliferation by using a rat model.

Materials and Methods

Experimental Animals and Grouping

Healthy males 8-week old Sprague Dawley (SD) rats (body weight 160-180 g) were provided by Laboratory Animal Center, Chinese Medicine Academy (Certificate number: SYXK-2013-0025) and were kept in an SPF grade animal facility with standard food and water. Animals were randomly assigned into control and model group (N=30). Model group received 70 mg/kg DEN via intraperitoneal injection (once a week for 12 consecutive weeks) to construct liver cancer model. Control group received equal volume of saline. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Chinese People's Liberation Army (PLA) General Hospital.

Drugs and Reagents

DEN, proteinase inhibitor, urethane were bought from Sigma-Aldrich (St. Louis, MO, USA). Radioimmunoassay kit, ELISA kits for TNF-α and IL-6 were purchased from Zhongshan Jinqiao (Zhongshan, Guangdong, China). Assay kits for liver function indexes (ALT, ALB, TBI and GGT) were collected from Pointe Biotech (Nanjing, Jiangsu, China). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Billerica, MA, USA). TRIzol reagent was provided from Invitrogen (Carlsbad, CA, USA). Antibodies for p-Akt, p-ERK and PCNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-rat HSD17B4 polyclonal antibody, secondary antibody and DAB kit were provided by Boster (Wuhan, Hubei, China).

Animal Model Preparation

Rat liver cancer model was prepared following previous literatures[11] using intraperitoneal injection of 70 mg/kg DEN once a week for 12 consecutive weeks. Equal volume of saline was given to control group. Rats were fasted for 8 h before sacrifice. Rats were deeply anesthetized by 5% urethane. Blood samples were collected from femoral artery for separating serum by centrifugation. Liver tissues were collected and fixed in formalin solution for observing tissue morphology.

Biochemical Index Assay

At 4, 8 and 12 weeks after model generation, blood samples were collected. Serum TNF-α and IL-6 levels were measured by ELISA. Following manual instruction of test kit, absorbance (A) values were measured on a microplate reader. E2 levels in serum and liver tissues were measured by radioimmunoassay approach. Following manual instruction of test kit, radioimmunoassay counter was used to measure radiation intensity for calculating E2 content. Fully automatic biochemical analyzer was used to test serum liver function indexes including ALT, AST, TBI and GGT following manual instruction of test kit.

HE Staining

At 4, 8 and 12 weeks after model generation, rats were sacrificed for observing general morphology of liver tissues. Liver tissues were immersed in paraformaldehyde and were prepared for 5 μm paraffin-based slices. After hematoxylin-eosin (HE) staining, light field microscope was used to observe tissue morphology.

Western Blot for p-Akt, p-ERK, HSD17B4 and PCNA Protein Expression

Total proteins were extracted from cells. After the protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-

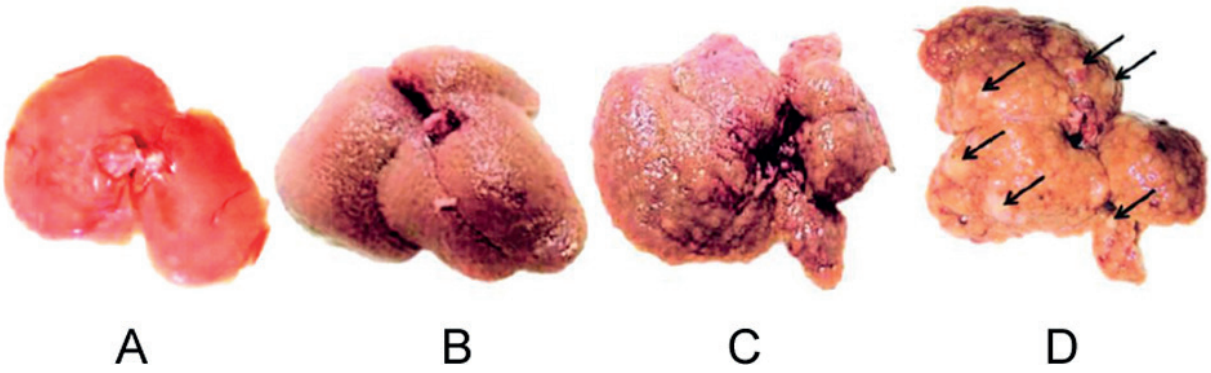

Figure 1. General observation of liver morphology. ***A,*** control group without significant abnormality; ***B,*** 4 weeks after model preparation, showing sparsely distributed small lesions; ***C,*** 8 weeks after model preparation, liver edge became bluntness with abundant cancer lesions. Arrows, whitening tumor lesions.

PAGE) and transferred into polyvinylidene-difluoride (PVDF) membrane, it was blocked in defatted milk powder. Protein antibodies against p-Akt, p-EKR, HSD17B4 and PCNA (diluted at 1:1000, 1:1000, 1:2000, and 1:1000) were added in parallel with anti-β-actin antibody (1:1000) for overnight incubation. Tris-buffered saline-tween (TBST) was used in rinse the membrane, for adding secondary antibody (1:5000 dilution) in 1 h incubation. ECL reagent was used for developing the membrane in dark. Quantity One image analysis system was used to measure protein bands.

Statistical Analysis

SPSS 19.0 software (SPSS Inc., Armonk, NY, USA) was used for statistical analysis. Measurement data were tested for normality and were presented as mean±standard deviation (SD). Comparison of means among multiple groups was performed by one-way analysis of variance (ANOVA) and the between-group comparison was performed by LSD test. Spearman was used for correlation analysis to reveal the relationship between HSD17B4 and TNF-α, IL-6, PCNA. A statistical significance was defined when $p<0.05$.

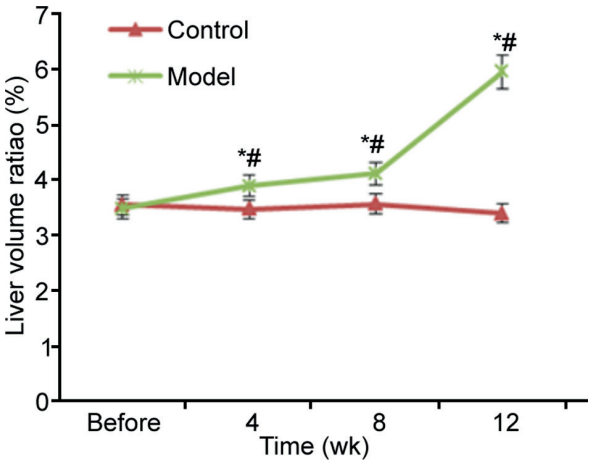

Figure 2. Rat liver volume ratio. ***A,*** control group; ***B,*** model group; *, $p<0.05$ compared to before model preparation; #, $p<0.05$ compared to control group.

Results

General Conditions of All Rats

DEN was used to induce liver cancer in a rat model. General observation showed no abnormality in liver tissues in control group. In model group, roughness was found in rat liver surface after 4 weeks of generation. At 8 weeks post-injection, liver volume was increased, and granules and round lesions on liver surface emerged. At 12 weeks after model generation, unevenly sizes of gray tumor lesions can be observed on liver surface (Figure 1). Liver volume ratio was significantly higher in model group than control group ($p<0.05$), probably due to heavy tumor loading (Figure 2).

HE Staining for Liver Tissue Pathology

HE staining data indicated no significant change of liver tissues in control group, which has complete liver lobular structure. As time went by, inflammatory infiltration, denaturation and necrosis of hepatocytes were shown in model group. At 4 weeks after model preparation, in liver tissues, diffused hepatocyte edema, disrupted liver structure but intact lobular morphology, focal infiltration of inflammatory

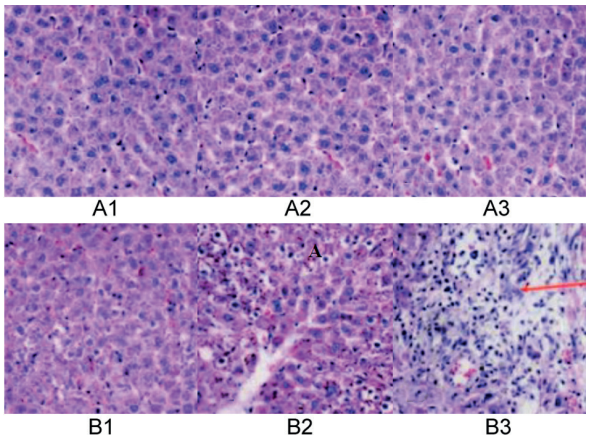

Figure 3. HE staining for liver tissue morphology (X20). ***A,*** control group with normal tissue structure; ***B,*** model group: 1, 4 weeks after model preparation; 2, 8 weeks after model preparation; 3, 12 weeks after model preparation. Arrows, tumor tissues.

cells and lesion necrosis were observed. At 8 weeks later, denaturation of fat tissues, with aggravated edema, hyperplasia of bile duct, and formation of pseudo-lobules and mesenchymal tissue hyperplasia were apparent. At 12 weeks after model preparation, we found hemorrhage necrosis, with visible fibrous capsule around cancer tissues (Figure 3).

Liver Function Index in all Groups

Data on liver function indexes showed aggravation in model group, along with significant elevation of serum ALT, TBI and GGT levels (p<0.05 compared to control group at the same time point). ALB level was statistically decreased (p<0.05 compared to control group at the same time points, Figure 4).

E2, TNF-α and IL-6 levels in rat Liver Cancer Model

After model preparation, serum TNF-α and IL-6 levels were significantly elevated (p<0.05 compared to control group at the same time point). Serum and liver tissue levels of E2 were significantly decreased (p<0.05 compared to control group at the same time point, Figure 5 and Figure 6).

Table I. Correlation between HSD17B4 and TNF-α, IL-6 and PCNA expression.

	HSD17B4	
Index	**R-value**	**p-value**
TNF-α	0.68	<0.05
IL-6	0.60	<0.05
PCNA	0.56	<0.05

Protein expression of p-Akt, p-ERK, HSD17B4 and PCNA in Liver Cancer Rats

Compared to control group, growing phosphorylation levels of Akt and ERK, and increasing protein expressions of HSD19B4 and PCNA were shown in model group (p<0.05, Figure 7 and Figure 8).

Correlation Analysis Between HSD17B4 and TNF-α, IL-6 and PCNA Expression in Liver Cancer Rats

Correlation analysis indicated that HSD17B4 protein expression was positively associated with TNF-α, IL-6 and PCNA expression (r=0.68, 0.60 and 0.56, p<0.05, Table I).

Discussion

Major therapeutic approaches include surgical resection, liver transplantation and percutaneous ablation. Compared to other malignant tumors, the treatment efficacy for liver cancer is still unsatisfactory. Inflammation is closely correlated with tumor formation, and liver cancer is frequently occurred in patients with chronic hepatitis or liver cirrhosis patients. Chronic and persistent viral infection cause repeated liver tissue repair-regeneration. At terminal stage of hepatocellular carcinoma, expressions of inflammatory signal transduction and cell-apoptosis related genes are remarkably elevated compared to those in early stage. Liver cancer pathogenesis involves a multi-step progress with the participation of various signal transduction pathways[12,13]. This study successfully generated the rat liver cancer model by the treatment of DEN, and inflammatory infiltration, denaturation, necrosis and aggravated liver function index were shown in model group. Both macrophage and monocyte can produce TNF-α during infection or replication of hepatitis virus, which activates NF-κB signaling pathway and induces cell apoptosis[14,15]. TNF-α exerts to pivotal function in inflammatory cascade response, as hepatocytes can secrete inflammatory factors including TNF-α under pathological conditions. Such inflammatory factors exaggerate inflammatory response

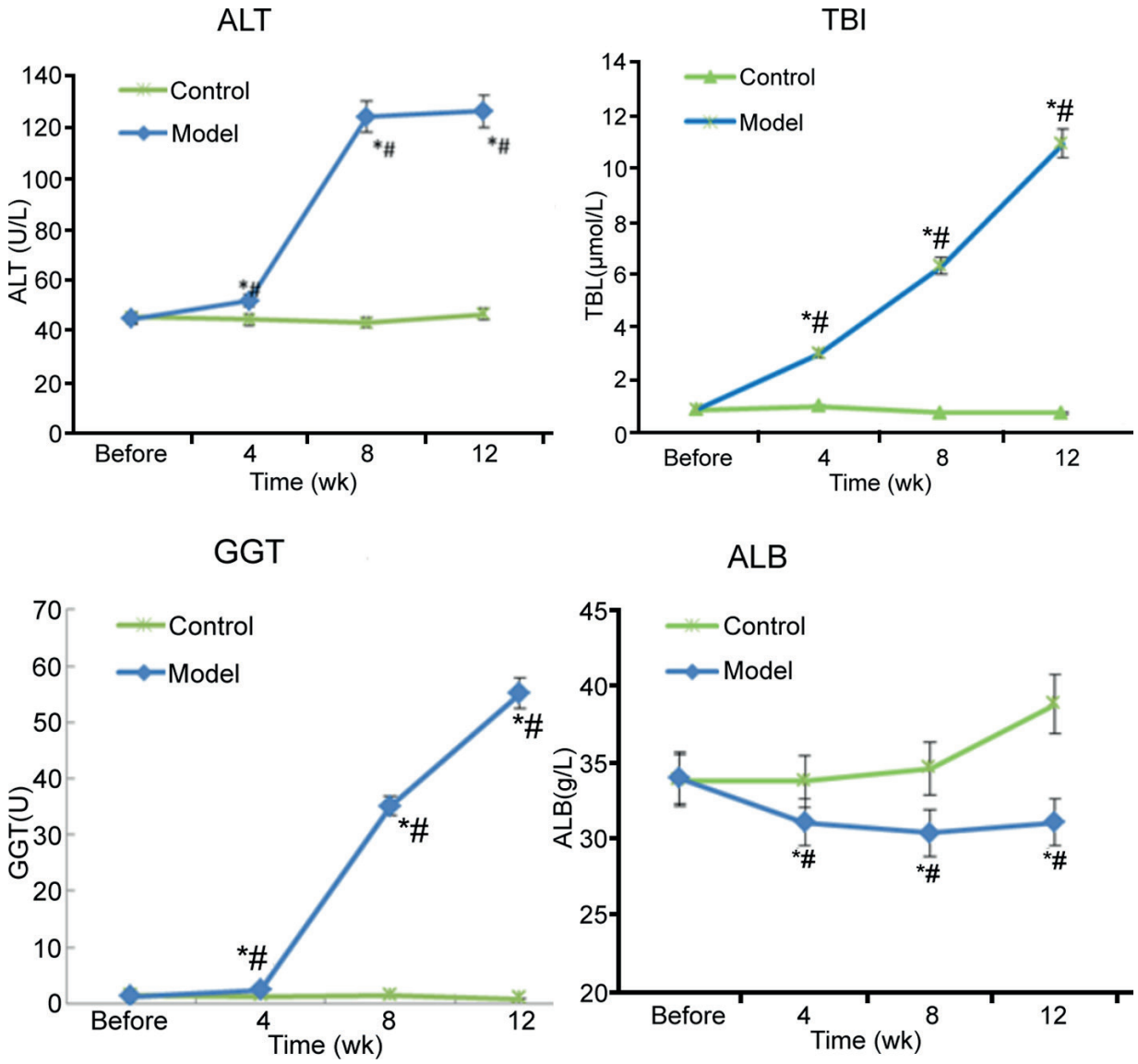

Figure 4. Liver function index ALT, ALB, TBI and GGT. ***A,*** control group; B, model group. *, $p<0.05$ compared to before model preparation; #, $p<0.05$ compared to control group.

in a paracrine or autocrine manner, leading to inflammatory cascade response[16]. TNF-α stimulates focal inflammation of body to facilitate exfiltration of inflammatory factors. ICAM-1 can bind with leukocytes via specific receptors to favor the adhesion and aggregation or adhesion of leukocytes in endothelium of glomerulus, micro-vessel and aggravate liver damage[17]. TNF-α and its activated inflammatory mediators can bind with target cell receptor so as to induce the activation of kinase and transcription factors, or multiple signal pathways and aggravate liver damage[18]. The over expression of IL-6 participates in T cell activation, facilitates neutrophil adhesion, induces trans-vascular migration of neutrophil to inflammatory sites during the process of organ damage[19]. TNF-α and IL-1β lead to NF-κB phosphorylation, which induces a series change of expressions of genes related with inflammatory factors and NO production. As the level of TNF-α was increased in HepG2 cells, HSD17B4 expression was elevated with the reduction of E2 level, which promotes the proliferation of liver cancer cells[20,21]. Evidence also indicated that E2 inhibited liver cancer cell proliferation

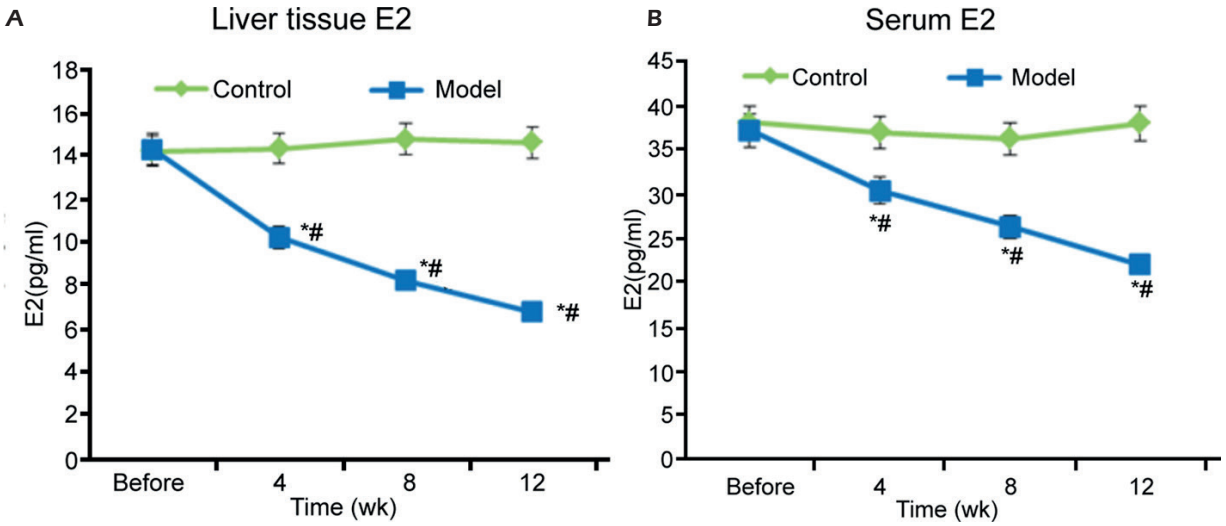

Figure 5. Levels of E2 in serum and liver tissue. ***A,*** control group; ***B,*** model group. *, $p<0.05$ compared to before model preparation; #, $p<0.05$ compared to control group.

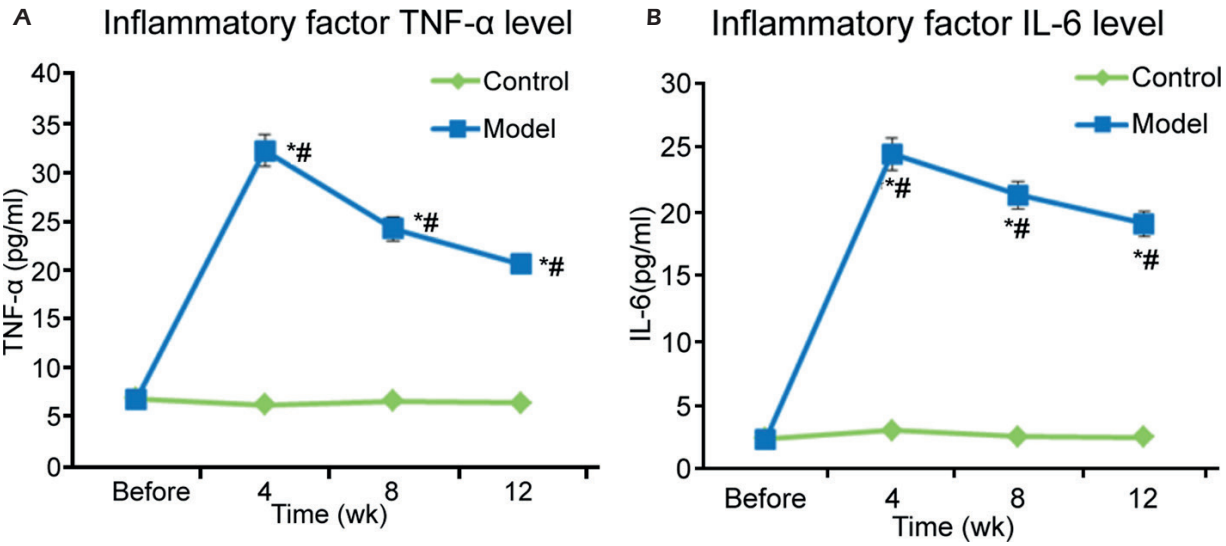

Figure 6. Serum inflammatory factors TNF-α and IL-6. ***A,*** control group; ***B,*** model group. *, $p<0.05$ compared to before model preparation; #, $p<0.05$ compared to control group.

via suppressing IL-6 expression[22,23]. Recent study showed that the abnormal expressions of HIF-1alpha and KISS-1 are closely related to the development and progression of liver cancer, indicating that HIF-1alpha and KISS-1 have important research values in liver cancer, and the expressions of HIF-1alpha and KISS-1 can be used as the index of deterioration degree of liver cancer[24]. This work demonstrated significantly elevating levels of serum TNF-α and IL-6 in liver cancer rats, whilst E2 expression was remarkably decreased in serum and liver tissues. Additionally, we also found higher phosphorylation level of Akt and ERK, as well as the rising expression of HSD17B4 and PCNA in liver cancer tissues, indicating potentiated activity in Akt and ERK signal pathways in liver cancer rat model. Inflammation and proliferation are accompanied with onset and progression of liver cancer. HSD17B4 is aberrantly differentially expressed between lesion and normal tissues, such as low expression in

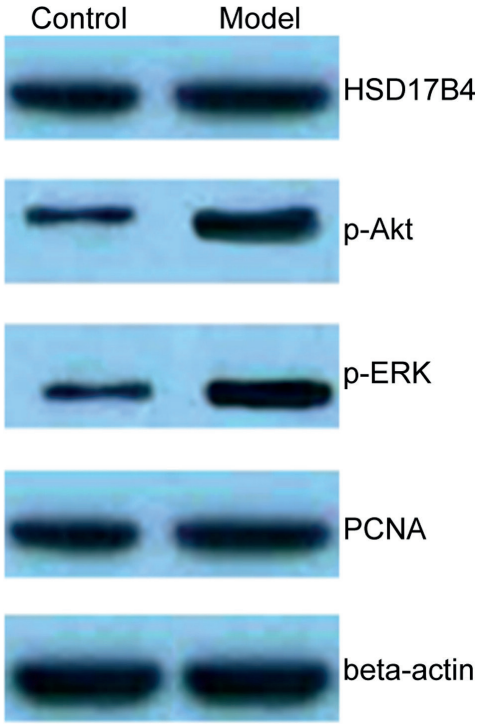

Figure 7. Protein expressions of p-Akt, p-ERK, HSD17B4 and PCNA.

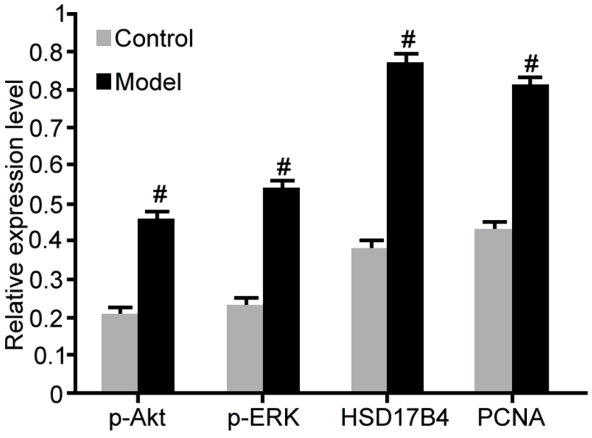

Figure 8. Relative protein expressions of p-AKT, p-ERK, HSD17B4 and PCNA in liver tissues. ***A***, control group; ***B***, model group. #, $p<0.05$ compared to control group.

breast cancer tissues and high in prostate cancer tissues compared to normal tissues. In this study, HSD17B4 protein expression was up regulated in tissues of liver cancer rats, which was positively correlated with TNF-α, IL-6 and PCNA expression, indicating probably significant role of HSD17B4 in tumor transformation of hepatocellular carcinoma.

Conclusions

We demostrated that HSD17B4 was over-expressed in rat liver cancer tissues and the expression is positively correlated with the levels of TNF-α, IL-6 and PCNA, suggesting the involvement of HSD17B4 in inflammation and proliferation of liver cancer cells via ERK and Akt signal pathways, which provides a new clinical basis for diagnosis and treatment of liver cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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