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Higher activity of alcohol dehydrogenase is correlated with hepatic fibrogenesis

Na Gao, Jing Li, Ming-Rui Li, Bing Qi, Zhao Wang, Gao-Ju Wang, Jie Gao, Hai-Ling Qiao*

Institute of Clinical Pharmacology, School of medicine, Zhengzhou University, Zhengzhou 450052, China (N.G., J.L., M.-R.L., B.Q., Z.W., G.-J.W., J.G., H.-L.Q.)

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Corresponding author: Hai-Ling Qiao

Address: 40 Daxue Road, Zhengzhou, 450052, China

Tel: 86-371-6665-8363; Fax: 86-371-6665-8363

Email: qiaohl@zzu.edu.cn

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Abbreviations

ADH, alcohol dehydrogenase; ALDH: acetaldehyde dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; PCNA, proliferating cell nuclear antigen.

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Abstract

Hepatofibrosis can progress to cirrhosis and hepatocellular carcinoma (HCC). Prevention, stabilization and reversal disease progression is vital for patients with hepatofibrosis, and identifying risk factors for hepatofibrosis is urgently needed. This study examines the activities of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) in the fibrotic livers from HCC patients (n=88) with comparison to the activities found in patients with normal livers (n=74). A fibrosis-carcinoma rat model was used to study activity of ADH in fibrosis and HCC, and relationship between innate ADH activity and extent of hepatofibrosis or HCC. There was substantial inter-individual variation in the activities of ADH and ALDH in normal livers. The activities of total ADH, ADHI and ADHII in fibrotic livers were significantly higher than that in normal liver ($P < 0.001$), while the activity of ALDH was slightly higher. The positive rates of ADHI and ADHII were 84.1% and 77.3%; the area under the receiver operator characteristics (ROC) curve was 0.943 and 0.912, respectively. For the rat model as compared to the control, ADH activity in liver was significantly increased at the fibrotic and HCC stages and there was no significant difference between ADH activity in liver at these two stages. The innate activity of ADH in serum was well correlated with the extent of hepatofibrosis as indicated by Masson area%, $Ki67^+$%, $PCNA^+$%, and GST-p average density at fibrotic stage but not at HCC stage. Higher activity of ADH is a risk factor for hepatofibrogenesis and it might be a prevention target for hepatofibrosis.

1. Introduction

Hepatofibrosis is a common pathophysiology of chronic liver disease with various causes, and leads to abnormal extracellular matrix deposition and ultimately a pathological process of liver structural and functional abnormalities(Wheeler et al., 2001; Bataller et al., 2004). Hepatofibrosis is mostly asymptomatic in its early stages, but as it progresses it disrupts the liver architecture and function, ultimately leading to cirrhosis and even hepatocellular carcinoma (HCC) (Bataller et al., 2004). Early fibrosis is potentially reversible but it has been widely accepted that liver cirrhosis is irreversible, with no effective therapeutic drug treatment. HCC is a major global health problem as the second leading cause of cancer-related death (Beck et al., 2017). Prevention, stabilization, or reversal of disease progression is very important for patients with hepatofibrosis, and identification of the risk factors for hepatofibrosis is urgently needed.

Alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenases (ALDH) are responsible for the metabolism of a variety of endogenous and exogenous substrates, including ethanol, retinol, aliphatic alcohols, hydroxysteroids and lipid aldehyde (Song et al., 2015). Human ADH is comprised of a family of enzymes that have been grouped into several classes (Maly et al., 1999). In humans, the first two classes are mainly located in the hepatic parenchymal cells. ADH Class I (ADHI) is the classical liver ADH, which represents up to 95% of total activity in this organ and ADH Class II (ADHII) is detected only in the liver. Among all these isoenzymes, ADHI is the main isoenzyme involved in ethanol metabolism. Isoenzymes of ADHI are dimers

composed of α, β, and γ subunits, encoded by *ADH1A, ADH1B* and *ADH1C* loci (Maly et al., 1999). There are some gene polymorphisms for *ADH1B* and *ADH1C*, such as ADH1B polymorphism which was reported to have a marginal effect on alcohol pharmacokinetics (Marshall et al., 2014). The metabolism of ethanol by ADH produces acetaldehyde, which is mainly metabolized by ALDH to acetate(Lieber, 1997; Zakhari and Li, 2007; Jelski and Szmitkowski, 2008). ALDH can be divided into groups according to their Michaelis constant values (Km) for acetaldehyde. Liver mitochondrial ALDHII is mainly responsible for the metabolism of acetaldehyde (Jelski and Szmitkowski, 2008).

According to the International Agency for Research on Cancer (IARC), acetaldehyde associated with consumption of alcoholic beverages belongs to group 1 of human carcinogens. Acetaldehyde can interact with DNA to produce DNA adducts (Yu et al., 2010). The acetaldehyde-DNA adducts can lead to cancer in many tissues including liver, esophagus, and gastrointestinal tract (Song et al., 2015). In addition, recent results show that the lack of ADHIII in hepatic stellate cells and natural killer cells was associated with the inhibition of nonalcoholic hepatofibrosis(Yi et al., 2014). These results suggest that ADH is not only involved in alcohol-induced liver injury through the metabolism of ethanol, but also may play a key role in non-alcoholic liver injury.

At present, a number of studies have focused on the relationship between ethanol metabolizing enzymes (ADHs and ALDHs) and disease, such as renal cell carcinoma, pancreatic cancer and brain cancer(Jelski et al., 2007; Jelski et al., 2008c;

Laniewska-Dunaj et al., 2013; Jelski et al., 2014; Orywal et al., 2015; Orywal et al., 2016). Interestingly, total ALDH activity is not significantly changed in tissue or serum with these cancers. However, Jelski (Jelski et al., 2008a) examined the change of ADH and ALDH activity in 44 cases of HCC and found that total ADH, ALDH and ADH I activities were significantly higher in HCC tissues than that in healthy tissues. Jelski (Jelski et al., 2008b) also reported that ADHI activity in serum was significantly increased in patients with metastatic liver cancer, but there was no change in ALDH activity. So far, changes in ADH activity in fibrotic livers have not yet been reported, nor have changes in ADH activity in serum and liver at different stages of HCC progression. Finally, as ADH and ALDH act sequentially on many substrates, knowledge of the ratio of ADH to ALDH (ADH/ALDH) is likely to be important.

To identify risk factors for hepatofibrosis, our group obtained normal livers mainly from hepatic hemangioma patients and fibrotic livers from patients with HCC and measured the activities of total ADH, ADHI, ADHII and ALDH. The fibrosis-carcinoma rat model induced by diethylnitrosamine (DEN) was used to study the change in activity of ADH in different stages of HCC to determine if high ADH activity was a risk factor for hepatofibrosis.

2. Materials and Methods

2.1 Experiments with human liver

2.1.1 Human liver samples

Human normal liver samples were obtained from 74 patients with normal liver

function and fibrotic liver tissues which were 2 cm distant from the tumor tissues were obtained from 88 patients with HCC between 2012 and 2014 (Gao et al., 2016; Zhou et al., 2016; Gao et al., 2017) (Table 1). All the liver samples were obtained at First Affiliate Hospital of Zhengzhou University, Affiliated Provincial People's Hospital of Zheng Zhou University, and Affiliated Cancer Hospital of Zhengzhou University. Following excision, liver samples were frozen in liquid nitrogen within 30 min. All patients signed informed consent forms and this study was approved by the ethics committee of Zhengzhou University. Compared with normal group, there were more males, smokers, and drinkers in the HCC group ($P < 0.01$).

Liver samples were assigned four stages from S1 to S4 (Zhou et al., 2016). Among the 88 fibrotic livers, 5 were S1, 15 were S2, 23 were S3 and 45 were S4.

2.1.2 Determination of ADH (total ADH, ADHI and ADHII) activities and total ALDH activity

Liver tissues were homogenized in potassium phosphate buffer (0.1 mol/L, pH 7.4), then centrifuged at 1200 g for 20 min at 4℃. The supernatant was used to determine ADH and ALDH activities. The protein concentration was determined by Bradford protein quantification kit and performed according to standard procedures provided by the manufacturer (BOSTER Biological Technology Co., ltd).

The activity of total ADH, ADHI, ADHII and total ALDH were measured according to the method of Skursky, Wierzchowski and Jelski (Skursky et al., 1979; Wierzchowski et al., 1989; Jelski et al., 2008a). The activities of total ADH, ADHI and ADHII were determined with p-nitrosodimethylaniline (NDMA),

4-methoxy-1-naphthaldehyde and 6-methoxy-2-naphthaldehyde as substrates, and the activity of total ALDH was determined with 6-methoxy-2-naphthaldehyde as substrate. Briefly, the reaction mixture contained supernatant, substrate, NAD or NADH, and sodium phosphate buffer. A wavelength of 440 nm for total ADH, and emission wavelengths of 370 nm and 360 nm with excitation wavelength of 316 nm for ADHI and ADHII were utilized, respectively. An emission wavelength of 360 nm used to determine the activity of ALDH with the excitation wavelength of 310 nm.

2.2 Experiments with rats

2.2.1 Establishment of fibrosis-carcinoma in a rat model

51 male Sprague-Dawley (SD) rats weighing 180 ± 20 g were maintained at 22°C with a standard 12-hour light/dark cycle with free access to rodent chow and tap water and were randomly divided into 2 groups: control (n=10) and model (n=41). After feeding, the model group was injected intraperitoneally with 50 mg/kg DEN twice a week for 4 weeks, then injected intraperitoneally with 50 mg/kg DEN once a week to 14 weeks. Ding et al reported that the HCC rat model induced by DEN was characterized by an inflammation stage (week 2–6), a fibrosis stage (week 8–12), and an HCC stage (week 14–20)(Ding et al., 2017). In this experiment, twenty rats in the model group were randomly assigned and sacrificed at 12 weeks, designated “fibrosis group”. The remaining rats in the model group were sacrificed at 19 weeks, and termed “HCC group”. The control rats were injected intraperitoneally with normal saline and also sacrificed at 19 weeks. The diagnosis of fibrosis and HCC were made based on the results of Hematoxylin–eosin (HE) staining and Masson staining. The

results were studied by a pathologist who was blinded to the study. Histopathologic examination showed centrilobular atypia and multiple larger dysplastic foci and there was marked collagen deposition as demonstrated by Masson's trichrome staining in the rats treated with DEN for 12 w. In addition, about half of rats developed HCC by week 19 according to pathology results. Our study was carried out strictly accordance with the Guide for the Care and Use of Laboratory Animals and all the experimental procedures reported here were approved by the Zhengzhou University Animal Care and Use Committee.

Serum from all rats was collected at 0, 8, and 12 w, and the serum of control and rats in HCC group were collected at 16 and 19 w. The liver were collected after scarification.

2.2.2 Preparation of rat liver homogenate

200 mg of liver tissue was homogenized in normal saline and then centrifuged at 2500 r/min for 10 min at 4℃. The supernatant was used to determine enzyme activities. The protein concentration was determined by BCA protein quantification kit (BOSTER Biological Technology Co., ltd).

2.2.3 Determination of alanine aminotransferase (ALT) and aspartate aminotransferase （AST） in serum

ALT and AST activity in serum was determined using a commercial assay kit from Jiancheng Bioengineering Institute (Nanjing, China) according to standard procedures. Results of serum AST and ALT activities are expressed as units per liter (U/L).

2.2.4 Histopathology

Liver specimens for histopathological analysis were obtained from the tissues fixed with 10% formalin and embedded in paraffin. Paraffin sections, 5 μm thick, were stained with hematoxylin and eosin (H&E staining) and Masson staining. Liver specimens were graded according to the Ishak scoring system (Ishak et al., 1995). The percentage of fibrotic area after Masson staining was measured.

2.2.5 Immunohistochemistry

The altered hepatic foci (Kitahara et al., 1984; Sato et al., 1984) were measured by immunohistochemical analysis of glutathione-s-transferase placental form (GST-p) levels in the liver. Hepatocyte proliferation was quantified by immunohistochemical analysis of PCNA and Ki-67. In addition, the activation of hepatic stellate cell was evaluated by immunohistochemical analysis of α-SMA and collagen 1.

Anti-Ki67 antibody (ab 15580, Abcam, UK), anti-PCNA antibody (ab 29, Abcam, UK), and anti-GST pi antibody (ab 138491, Abcam, UK) were used in immunohistochemistry. The proportion of positive cells ($Ki67^+$%, $PCNA^+$%) and average density (GST-p) were quantified with image processing software(Image-pro-plus 6.0). The expression of α-SMA and collagen 1 was assessed by H-score system (histochemistry score) (Yeo et al., 2015). A briefly, the formula for the H-score is: H-SCORE=∑（PI×I）=（percentage of cells of weak intensity×1)+(percentage of cells of moderate intensity×2)+percentage of cells of strong intensity×3).

2.2.6 Determination of the ADH activity

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The ADH activity in serum and liver homogenate of rat was determined using a commercial assay kit from Jiancheng Bioengineering Institute (Nanjing, China) according to standard procedures.

2.3 Statistical analysis

Statistical analysis was performed with SPSS 17.0 software. The data set of human liver were not normally distributed, thus the data were expressed as median and range, and nonparametric methods were generally used for statistical analyses. In the data set from the rat model, statistical differences between different stages were determined using an independent *t*-test or one-way ANOVA since they were normally distributed. The Pearson correlation was used to determine the correlations between the activity of ADH and liver injury-related indexes. The level of the confidence was set at 0.05.

3. Results

3.1 ADH and ALDH activities in human liver

3.1.1 Activity of ADH and ALDH in human normal liver

The activity of total ADH, ADHI, ADHII and total ALDH were detected in 74 normal liver samples using probe drugs known to be specific for each enzyme. The data for these measurements were shown in Table 2 and the results demonstrate substantial individual variations. The two biggest individual variations were evidenced in the activity of ADH and ADH/ALDH, with 450.8 and 809.6-fold differences, followed by that of ADHI/ALDH, ADHII, ALDH and ADHI, demonstrating changes from 14 to 41-fold. The values for ADH activity at the 5th and the 95th percentiles were 0.38 and 50.4 $nmol \cdot min^{-1} \cdot mg^{-1}$ protein, respectively,

exhibiting about a 132-fold variation. There were six outliers (60.91, 71.70, 72.12, 0.16, 0.27 and 0.34 nmol·min^{-1}·mg^{-1} protein) in total ADH activity.

There were no statistically significant differences ($P > 0.05$) in the ADH, ADH I, ADH II and ALDH activities as a function of gender, age, smoking status, or drinking habit (data not shown).

3.1.2 Activity of ADHs and ALDH in fibrotic liver

The median protein concentration in fibrotic liver was significantly lower than those in normal liver tissues ($P < 0.05$). Total ADH, ADHI, ADH II and ALDH activities were detected in 88 fibrotic livers and were shown in Table 2. Just as in normal liver, there were substantial individual variations and the two biggest individual variations were observed with the activity of ADH and ADH/ALDH, varying by 36.5 and 69.9-fold. The results show that individual variation is smaller than that of normal liver. The values of ADH activity at the 5th and the 95th percentiles were 1.9 and 18.8 nmol·min^{-1}·mg^{-1} protein, respectively, exhibiting about an 9.9-fold variation. As with normal liver, there were also no statistically significant differences ($P > 0.05$) in the ADH, ADHI, ADHII and ALDH activities as a function of gender, age, smoking status, or drinking habit (data not shown).

As compared with normal liver, total ADH, ADHI and ADHII activities were significantly increased in fibrotic liver ($P < 0.001$), with 4.0, 3.0 and 2.6-fold changes. The ALDH activity in fibrotic liver also was higher than that of normal liver ($P < 0.01$), but the increase rate was relatively small (1.3-fold). The ratio of ADH/ALDH and ADH I/ALDH also were increased significantly in fibrotic liver relative to that of controls. These results suggested that fibrotic livers might have a greater capability for ethanol metabolism to acetaldehyde.

3.1.3 Correlation between the activities of total ADH, ADHI, ADHII and ALDH

Correlations between the activities for total ADH, ADHI and ADHII were analyzed. Positive correlation was shown among each of them in normal tissue (Fig.1A) and fibrotic tissue (Fig.1B). Strong correlations ($r \geqq 0.6$) were observed between ADH I and ADH II in normal livers and the remaining correlations were moderate ($0.3 \leqq r < 0.6$). The activity of ALDH correlated only with ADHII, which is participates actively in the degradation of circulating epinephrine and norepinephrine, in normal livers ($P < 0.01$). ALDH activity in fibrotic livers showed a moderate correlation with ADHI ($P < 0.001$).

3.1.4 Correlation between the activities of total ADH, ADH I, ADH II and ALDH and the degree of hepatofibrosis

There was a positive correlation between the activities ADHI, ADHII and the extend of hepatofibrosis ($P < 0.01$, Fig.2B, C). Total ADH activity had no relationship with the extent of hepatofibrosis ($P > 0.05$ Fig.2A). In addition, the ratio of ADHI/ALDH also was positively correlated with the degree of hepatofibrosis ($P < 0.001$, Fig.2F). These results suggest that hepatofibrosis might significantly affect the activity of alcohol-metabolizing enzymes.

3.1.5 Positive rate of total ADH, ADHI, ADHII and ratio of ADHI/ALDH in fibrotic liver tissues

To evaluate whether the increased alcohol-metabolizing enzyme activities or ratios might be potential biomarkers, the positive rates of ADH, ADHI, ADHII, ADH/ALDH, ADHI/ALDH in fibrotic liver of HCC patients were calculated. Values over the P90 (percent 90) of the results for ADH, ADHI, ADHII, ADH/ALDH, ADHI/ALDH in the normal group were considered to be positive. The positive rates

of ADHI, ADHII and ADHI/ALDH activities in fibrotic livers from HCC patients were 84.1, 77.3% and 19.3%, respectively. While the positive rate of total ADH and ADH/ALDH was very low. One reason might be the substantial individual variation. The relationship between diagnostic sensitivity and specificity for ADHI and ADHII is illustrated by the receiver operator characteristics (ROC) curve, with the area under the ROC curve values was a measure of diagnostic accuracy (Fig.3). It showed that the area under the ROC curve for ADHI (0.943) and ADHII (0.912) was very high.

The results indicated that the activities ADHI and ADHII were related to hepatofibrosis and could be good potential biomarkers. However, it was not clear that the increase in ADH activities was a risk factor or a symptom for hepatofibrosis. To address this question the fibrosis-carcinoma rat model was established for further study.

3.2 ADH activity in the rat model

3.2.1 Establishment of the fibrosis-carcinoma rat model

A representative appearance of liver in the different groups were shown in Fig. 4A. The results of H&E staining and Masson staining were shown in Fig. 4B and 4C. The H&E stained sections were quantified by Ishak score and the Masson stained sections were quantified by the percentage of fibrotic area. Compared with the control group, the Ishak scores and the Masson area% of the rats in fibrosis and HCC group were significantly increased ($P < 0.001$). There was no significant difference in Ishak score and Masson area% between fibrosis and HCC rats (Fig.4D and 4E). Moreover, the values of ALT and AST levels in serum were significantly increased in model

group (Fig.4F, $P < 0.001$) compared with the control at different times (8, 12, 16, and 19 w).

3.2.2 Evaluation of hepatocyte proliferation

The percentage of Ki67-positive cells and PCNA-positive cells was significantly increased in rats of fibrosis and HCC groups compared to the control group (Fig. 5B-C, $P < 0.01$). Simultaneously, the hepatic GST-p positive foci was determined. In model groups, the average densities of GST-p positive foci were also significantly increased compared with the control group (Fig. 5D, $P < 0.01$).

3.2.3 Evaluation of activation of hepatic stellate cell

The expression of α-SMA and collagen 1 in liver were significantly higher in rats of fibrosis and HCC groups than that in the control group (Fig. 6, $P < 0.05$). And there was no significant difference in expression of α-SMA between rats in fibrosis and HCC group (Fig.6). However, the expression of collagen 1 was significantly higher in HCC group than that in fibrosis group ($P < 0.01$).

3.2.4 Alteration of ADH activity in a rat model during the progression of HCC induced by DEN

Activities for ADH in serum and liver were measured. Compared with the control group, the activity of ADH in the serum of rats treated with DEN was significantly increased at 16th week (24.21 ± 9.86 U/mL) and 19th week (24.56 ± 9.65 U/mL), with no significant change at 8th and 12th weeks (Fig. 7A, $P>0.05$).

Compared with the control group (3.41 ± 1.12 U/mg protein), ADH activity in liver of rats treated with DEN was significantly increased in rats in the fibrosis groups (4.50 ± 1.05 U/mg protein) and in rats in the HCC group (4.77 ± 2.11 U/mg protein)

(Fig. 7B, $P<0.05$). These results suggest that the activity of ADH in liver at the fibrotic stage is higher than that in control, but from hepatofibrosis to HCC, there was no significant change in ADH activity. Given that the activity of ADH was higher in fibrotic livers from HCC patients than that in controls, we conclude that the activities ADHI and ADHII might be good potential biomarkers for hepatofibrosis.

3.2.5 Relationship between ADH activity and liver injury or hepatocyte proliferation in rats

To confirm that high ADH activity was a risk factor for hepatofibrosis or HCC, we determined the innate activity of ADH in serum.

There was positive correlation between the innate activity of ADH (0 week) in the serum of rats in fibrosis group and the extent of fibrosis as measured by the four indexes (Masson area%, $Ki67^{+}$%, $PCNA^{+}$%, GST-p average density), and the correlation coefficients were 0.453, 0.512, 0.457, 0.450, respectively (Fig. 8A, $P < 0.05$). There was no correlation between the innate ADH activity of rats in HCC group and indices reflecting hepatic lesions ($P > 0.05$, the data was not shown). In addition, there was no significant correlation between the innate activity of ADH and expression of α-SMA and collagen 1 in rats of both fibrosis and HCC groups (the data were not shown). The results indicate that rats with higher ADH activity were more prone to hepatofibrosis, which suggests that the high activity might be a risk factor for hepatofibrosis.

In addition, ADH activity in the liver of rats in the HCC group correlated with the five indexes reflecting hepatic lesions (liver weight/body weight, nodule number,

largest diameter of nodule, cumulative diameter of nodule, Ki67$^+$%), and the correlation coefficients were 0.567, 0.499, 0.438, 0.626, 0.506, respectively (Fig. 8B, $P < 0.05$).

4. Discussion

This study provides the physiological values of ADH and ALDH activities along with the ratio ADH to ALDH in human normal livers. Results showed that ADH activity was significantly higher in fibrotic liver. The positive rates of ADHI and ADHII were higher than 70% and the area under ROC curve was greater than 0.9. The results of the animal experiments further indicated that ADH activity in liver was higher at the fibrotic stage and that there was no significant difference in that measure between fibrosis and the HCC stage. Moreover, there was significant correlation between the innate activity of ADH in serum and the extent of fibrosis as indicated by Masson area%, Ki67$^+$%, PCNA$^+$%, GST-p average density at fibrosis stage. We conclude that elevated activity of ADH may be a risk factor for hepatofibrosis. Moreover, ADH activity also can be a biomarker for hepatofibrosis.

Alcohol drinking has been recognized as one of the major risk factors for hepatofibrosis and HCC, together with hepatitis B virus and hepatitis C virus infection (Jelski et al., 2008b). It is accepted that ethanol is metabolized to acetaldehyde by ADH and acetaldehyde is oxidized by ALDH to acetic acid in the liver. Acetaldehyde, the intermediate product of ethanol, is toxic. Therefore not only ADH and ALDH but also the ratio of ADH and ALDH is very important, especially ADHI/ALDH, due to ADHI being mainly responsible for ethanol metabolism. Up to now there have been no reports on the physiological values, or reference ranges for the activities of the

various isozymes of ADH. Hence, determination of the activity and individual variations in ADH activity over a large number of samples is needed in order to provide reliable physiological parameters for in vivo and in vitro research.

Our study was the first to report the physiologic values of ADH activity, ALDH activity and the ratio of ADHI and ALDH in 74 normal human liver samples and found that they vary by 14- to 809-fold in different individuals. Dannenberg *et al* reported that the expression of ADHI in HepG2 cells may regulated by an epigenetic mechanism, such as methylation and histone deacetylation (Dannenberg et al., 2006). The factors that are involved in the activities of ADH will be explored in a future study.

Our results showed that all ADH isozyme activities in fibrotic livers were significantly higher than that in normal livers. The positive rates of both ADHI and ADHII were higher than 70%. Most HCC develop from hepatofibrosis and the potential curative treatment options are very limited (Kimhofer et al., 2015), so it is very important to look for biomarkers for hepatofibrosis. It has been reported that some substances, such as angiopoietin-like protein 2 or serum biglycan can be biomarkers for liver fibrosis, but the area under the ROC curve for the reports were lower than 0.9(Ciftciler et al., 2017; Deng et al., 2017). In this study, the area under the ROC curve for ADH I and ADHII was higher than 0.9. Moreover, the value of ADHI showed good correlation with the degree of hepatic fibrosis. As a result, ADHI and ADHII could be potential biomarkers for hepatofibrosis. It is well known that though some substances can be used as a biomarker for some diseases, most of them

cannot be a risk factor. In this study, we found that the ADH activity was not only a biomarker but also a risk factor for hepatofibrosis.

The correlations among ADH isozymes and ALDH were analyzed. We found that there were significant correlation among total ADH, ADHI and ADHII in normal and fibrotic livers. Amino acid sequence identity between ADH isozymes are at the 60% level (Jelski and Szmitkowski, 2008). The marked correlations among ADH isozyme activities provided clear evidence for the co-regulation of ADH forms. In addition, the correlations between ADH isozymes and ALDH in fibrotic liver were different from that in normal liver. In normal liver, the activity of ALDH had obvious correlations with ADHII. In fibrotic liver from HCC, it was changed to ADHI, which is mainly responsible for metabolism of ethanol. The results suggested that the liver disease significantly affected the activity of the alcohol-metabolizing enzyme.

To further confirm that higher activity was the symptom or a susceptibility factor for hepatofibrosis or HCC, we established a hepatic fibrosis-carcinoma rat model induced by DEN. Our results also found that the rats treated with DEN at 12w and 19w showed the characteristic histological changes of fibrosis and HCC, respectively. Some indices of liver injury, including Masson area%, $Ki67^{+}$%, $PCNA^{+}$%, GST-p average density, also were measured; we found that the innate activity of ADH in serum showed moderate correlations with four indices in the fibrosis stage but not in the HCC stage. Thus the increase in innate activity of ADH may be a susceptibility factor for hepatic fibrosis but not for HCC. Recent research showed that the lack of ADH III in hepatic stellate cells and natural killer cells was associated with inhibition

of hepatofibrosis(Yi et al., 2014), which is similar to our results. In addition, our results indicated that the expression of α-SMA and collagen 1 was significantly higher in rats of fibrosis and HCC groups than found in the control group, which was similar with the results of Ding et al (Ding et al., 2017). However, there was no correlation between ADH activity and content of α-SMA and collagen 1. The detailed mechanism has not been established and should be further explored.

In summary, total ADH, ADH I, ADH II activities were significantly higher in human fibrotic livers than that in normal liver. In a rat liver model, there was a causal relationship between the innate activity of ADH in serum and hepatofibrosis. The results suggest that the higher activity of ADH may be risk factor for hepatic fibrosis and it is possible for ADH to be viewed as a target for hepatic fibrogenesis.

Authorship Contributions

Participated in research design: Hai-Ling Qiao

Conducted experiments: Na Gao, Jing Li, Bing Qi, Zhao Wang, Gao-Ju Wang, Jie Gao

Performed data analysis: Na Gao, Jing Li, Ming-Rui Li

Wrote or contributed to the writing of the manuscript: Na Gao, Jing Li

References

Bataller R, Paik YH, Lindquist JN, Lemasters JJ and Brenner DA (2004) Hepatitis C virus core and nonstructural proteins induce fibrogenic effects in hepatic stellate cells. *Gastroenterology* **126**:529-540.

Beck M, Schirmacher P and Singer S (2017) Alterations of the nuclear transport system in hepatocellular carcinoma - New basis for therapeutic strategies. *J Hepatol* **67**:1051-1061.

Ciftciler R, Ozenirler S, Yucel AA, Cengiz M, Erkan G, Buyukdemirci E, Sonmez C and Esendagli GY (2017) The importance of serum biglycan levels as a fibrosis marker in patients with chronic hepatitis B. *Journal of clinical laboratory analysis* **31**.

Dannenberg LO, Chen HJ, Tian H and Edenberg HJ (2006) Differential regulation of the alcohol dehydrogenase 1B (ADH1B) and ADH1C genes by DNA methylation and histone deacetylation. *Alcoholism, clinical and experimental research* **30**:928-937.

Deng YQ, Zhao H, Zhou JY, Yan LL, Wang GQ and Asses CH-RF (2017) Angiopoietin-like protein as a novel marker for liver fibrosis in chronic hepatitis B patients with normal to minimally raised ALT. *Bmc Infect Dis* **17**.

Ding YF, Wu ZH, Wei YJ, Shu L and Peng YR (2017) Hepatic inflammation-fibrosis-cancer axis in the rat hepatocellular carcinoma induced by diethylnitrosamine. *Journal of cancer research and clinical oncology* **143**:821-834.

Gao J, Wang GJ, Wang Z, Gao N, Li J, Zhang YF, Zhou J, Zhang HX, Wen Q, Jin H and Qiao HL (2017) High CYP2E1 activity correlates with hepatofibrogenesis induced by nitrosamines. *Oncotarget* **8**:112199-112210.

Gao N, Tian X, Fang Y, Zhou J, Zhang H, Wen Q, Jia L, Gao J, Sun B, Wei J, Zhang Y, Cui M and Qiao H (2016) Gene polymorphisms and contents of cytochrome P450s have only limited effects on metabolic activities in human liver microsomes. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences* **92**:86-97.

Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, Desmet V, Korb G, MacSween RN and et al. (1995) Histological grading and staging of chronic hepatitis. *J Hepatol* **22**:696-699.

Jelski W, Chrostek L and Szmitkowski M (2007) The activity of class I, II, III, and IV of alcohol dehydrogenase isoenzymes and aldehyde dehydrogenase in pancreatic cancer. *Pancreas* **35**:142-146.

Jelski W, Laniewska-Dunaj M, Orywal K, Kochanowicz J, Rutkowski R and Szmitkowski M (2014) The activity of alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) in the sera of patients with brain cancer. *Neurochemical research* **39**:2313-2318.

Jelski W and Szmitkowski M (2008) Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in the cancer diseases. *Clinica chimica acta; international journal of clinical chemistry* **395**:1-5.

Jelski W, Zalewski B and Szmitkowski M (2008a) The activity of class I, II, III, and IV alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) in liver cancer. *Digestive diseases and sciences* **53**:2550-2555.

Jelski W, Zalewski B and Szmitkowski M (2008b) Alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) activity in the sera of patients with liver cancer. *Journal of clinical laboratory analysis* **22**:204-209.

Jelski W, Zalewski B and Szmitkowski M (2008c) Alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) activity in the sera of patients with pancreatic cancer.

Digestive diseases and sciences **53**:2276-2280.

Kimhofer T, Fye H, Taylor-Robinson S, Thursz M and Holmes E (2015) Proteomic and metabonomic biomarkers for hepatocellular carcinoma: a comprehensive review. *British journal of cancer* **112**:1141-1156.

Kitahara A, Satoh K, Nishimura K, Ishikawa T, Ruike K, Sato K, Tsuda H and Ito N (1984) Changes in molecular forms of rat hepatic glutathione S-transferase during chemical hepatocarcinogenesis. *Cancer research* **44**:2698-2703.

Laniewska-Dunaj M, Jelski W, Orywal K, Kochanowicz J, Rutkowski R and Szmitkowski M (2013) The activity of class I, II, III and IV of alcohol dehydrogenase (ADH) isoenzymes and aldehyde dehydrogenase (ALDH) in brain cancer. *Neurochemical research* **38**:1517-1521.

Lieber CS (1997) Role of oxidative stress and antioxidant therapy in alcoholic and nonalcoholic liver diseases. *Advances in pharmacology* **38**:601-628.

Maly IP, Toranelli M and Sasse D (1999) Distribution of alcohol dehydrogenase isoenzymes in the human liver acinus. *Histochemistry and cell biology* **111**:391-397.

Marshall VJ, Ramchandani VA, Kalu N, Kwagyan J, Scott DM, Ferguson CL and Taylor RE (2014) Evaluation of the influence of alcohol dehydrogenase polymorphisms on alcohol elimination rates in African Americans. *Alcoholism, clinical and experimental research* **38**:51-59.

Orywal K, Jelski W, Werel T and Szmitkowski M (2015) The activity of class I, II, III and IV alcohol dehydrogenase isoenzymes and aldehyde dehydrogenase in renal cell carcinoma. *Experimental and molecular pathology* **98**:403-406.

Orywal K, Jelski W, Werel T and Szmitkowski M (2016) The diagnostic significance of serum alcohol dehydrogenase isoenzymes and aldehyde dehydrogenase activity in renal cell cancer patients. *Experimental and molecular pathology* **100**:416-420.

Sato K, Kitahara A, Satoh K, Ishikawa T, Tatematsu M and Ito N (1984) The placental form of glutathione S-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. *Gan* **75**:199-202.

Skursky L, Kovar J and Stachova M (1979) A sensitive photometric assay for alcohol dehydrogenase activity in blood serum. *Analytical biochemistry* **99**:65-71.

Song B-J, Akbar M, Jo I, Hardwick JP and Abdelmegeed MA (2015) Chapter Ten - Translational Implications of the Alcohol-Metabolizing Enzymes, Including Cytochrome P450-2E1, in Alcoholic and Nonalcoholic Liver Disease, in *Advances in pharmacology* (Hardwick JP ed) pp 303-372, Academic Press.

Wheeler MD, Kono H, Yin M, Nakagami M, Uesugi T, Arteel GE, Gabele E, Rusyn I, Yamashina S, Froh M, Adachi Y, Iimuro Y, Bradford BU, Smutney OM, Connor HD, Mason RP, Goyert SM, Peters JM, Gonzalez FJ, Samulski RJ and Thurman RG (2001) The role of Kupffer cell oxidant production in early ethanol-induced liver disease. *Free radical biology & medicine* **31**:1544-1549.

Wierzchowski J, Dafeldecker WP, Holmquist B and Vallee BL (1989) Fluorometric assays for isozymes of human alcohol dehydrogenase. *Analytical biochemistry* **178**:57-62.

Yeo W, Chan SL, Mo FK, Chu CM, Hui JW, Tong JH, Chan AW, Koh J, Hui EP, Loong H, Lee K, Li L, Ma B, To KF and Yu SC (2015) Phase I/II study of temsirolimus for patients with unresectable Hepatocellular Carcinoma (HCC)- a correlative study to explore potential biomarkers for response. *BMC cancer* **15**:395.

Yi HS, Lee YS, Byun JS, Seo W, Jeong JM, Park O, Duester G, Haseba T, Kim SC, Park KG, Gao B and Jeong WI (2014) Alcohol Dehydrogenase III Exacerbates Liver Fibrosis by Enhancing Stellate

Cell Activation and Suppressing Natural Killer Cells in Mice. *Hepatology* **60**:1044-1053.

Yu HS, Oyama T, Isse T, Kitagawa K, Pham TT, Tanaka M and Kawamoto T (2010) Formation of acetaldehyde-derived DNA adducts due to alcohol exposure. *Chemico-biological interactions* **188**:367-375.

Zakhari S and Li TK (2007) Determinants of alcohol use and abuse: Impact of quantity and frequency patterns on liver disease. *Hepatology* **46**:2032-2039.

Zhou J, Wen Q, Li SF, Zhang YF, Gao N, Tian X, Fang Y, Gao J, Cui MZ, He XP, Jia LJ, Jin H and Qiao HL (2016) Significant change of cytochrome P450s activities in patients with hepatocellular carcinoma. *Oncotarget* **7**:50612-50623.

Footnotes

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Figure legends

Fig.1 Correlations among the activities of ADH, ADHI, ADHII and ALDH in normal (A, n=74) and fibrotic (B, n=88) human livers.

Fig.2 Correlations between the degree of hepatofibrosis and activities of ADH, ADH I, ADH II and ALDH and in fibrotic human livers (n=88).

S1: portal area fibrosis expand, and confined in the hepatic sinus and lobule (5); S2: fibrosis around portal area, fibrous septums form, lobule structure keep (15); S3: fibrous septums and lobule structure disorder, without cirrhosis (23); S4: early stage cirrhosis (45).

Fig.3 Receiver-operating-characteristic (ROC) curves for ADH I and ADH II activity in human fibrotic livers.

The area under the ROC curve for ADH I and ADH II were 0.943 and 0.912, respectively.

Fig.4 Macroscopic and histological hepatic changes in a fibrosis-carcinoma rat model induced by diethylnitrosamine (DEN). (A) Representative rat livers. (B) and (C) Representative haematoxylin-eosin (H&E, magnification 400×) and Masson staining (magnification 400×) of liver tissue. (D) and (E) Ishak score according to H&E staining and quantification of Masson staining area from each rat. (F) Serum ALT (F-1) and AST (F-2) levels. *** *vs* control group $P < 0.001$. Date represent means ± SD.

Control (n=10): the rats were treated with normal saline and sacrificed at 19 w. Fibrosis group (n=20): the rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 w, then injected intraperitoneally with 50 mg/kg DEN once a week to 12 w and sacrificed at 12w. HCC group (n=21): the rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 w, then injected intraperitoneally with 50 mg/kg DEN once a week to 14 w and sacrificed at 19 w.

Fig.5 Immunohistochemistry of Ki67, PCNA and GST-p in a fibrosis-carcinoma rat model induced by diethylnitrosamine (DEN). (A) Representative photographs of immunohistochemistry in liver sections stained for Ki67, PCNA, and GST-p in different groups (magnification 200×). (B), (C) and (D) Quantification of Ki67, PCNA and GST-p staining in different groups. ** *vs* control $P < 0.01$, *** *vs* control $P < 0.001$. Date represent means ± SD.

Control (n=10): the rats were treated with normal saline and sacrificed at 19 w. Fibrosis group (n=20): the rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 w, then injected intraperitoneally with 50 mg/kg DEN once a week to 12 w and sacrificed at 12w. HCC group (n=19): The data of 19 rats in HCC group were determined. The rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 w, then injected intraperitoneally with 50 mg/kg DEN once a week to 14 w and sacrificed at 19 w.

Fig.6 Immunohistochemistry of α-SMA and collagen 1 in a fibrosis-carcinoma rat model induced by diethylnitrosamine (DEN). (A) Representative photographs of immunohistochemistry of liver sections stained for α-SMA (magnification 100×) and collagen 1α (magnification 100×). (B)&(C) H-score of α-SMA and collagen 1α in different groups. * *vs* control $P < 0.05$, ** *vs* control $P < 0.01$, *** *vs* control $P < 0.001$, ## *vs* fibrosis group $P < 0.01$. Date represent means ± SD.

Control (n=8): the rats were treated with normal saline and sacrificed at 19 w. Fibrosis group (n=10): 10 rats were chosen at random from fibrosis group. The rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 weeks, then injected intraperitoneally with 50 mg/kg DEN once a week to 12 w and sacrificed at 12w. HCC group (n=12): 12 rats were chosen at random from HCC group. The rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 weeks, then injected intraperitoneally with 50 mg/kg DEN once a week to 14 weeks and sacrificed at 19 w.

Fig.7 Activities of ADH in serum (A) and liver tissues (B) in a fibrosis-carcinoma rat model induced by diethylnitrosamine (DEN). * *vs* Control $P < 0.05$. Date

represent means ± SD.

Control (n=10): the rats were treated with normal saline and sacrificed at 19 w. Fibrosis group (n=20): the rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 w, then injected intraperitoneally with 50 mg/kg DEN once a week to 12 w and sacrificed at 12w. HCC group (n=21): the rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 w, then injected intraperitoneally with 50 mg/kg DEN once a week to 14 w and sacrificed at 19 w

Fig.8 Correlations between index of hepatic lesions and ADH activity in a fibrosis-carcinoma rat model induced by diethylnitrosamine (DEN). (A) **Correlations** between liver injury-related indicators and ADH activity in serum at 0 w of the fibrosis group. (B) **Correlations** between liver injury-related indicators and ADH activity in rat liver from the HCC group.

Fibrosis group (n=20): the rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 weeks, then injected intraperitoneally with 50 mg/kg DEN once a week to 12 w and sacrificed at 12w. HCC group (n=21): the rats were injected intraperitoneally with 50 mg/kg DEN twice a week for 4 w, then injected intraperitoneally with 50 mg/kg DEN once a week to 14 w and sacrificed at 19 w. The Ki67 of 19 rats in HCC group was determined.

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Tables

Table1 Demographic and laboratory data of patients

	Normal	HCC	*P*-value*
Age			
average ± S.D	49 ± 11	53 ± 11	
≤44 (n, %)	23	20	
45~64 (n, %)	45	51	
≧65 (n, %)	6	17	
Gender			< 0.001
male (n, %)	24	75	
female (n, %)	50	13	
Smoking			< 0.001
yes (n, %)	13	45	
no (n, %)	61	43	
Alcohol intake			< 0.01
yes (n, %)	12	32	
no (n, %)	62	56	
body mass index (BMI)[#]	23.7±3.4	24.2±3.0	
Diagnosis	liver hemangioma, metastatic carcinoma, cholelithiasis, gallbladder cancer	HCC	
Pathology classification	normal	S1, S2, S3, S4.	
Total	74	88	

*Gender, age, smoking status, or drinking habit had no significant effect on ADH, ADH I, ADH II and ALDH activities ($P > 0.05$). [#] Both data of height and weight of 44 patients in control and 59 patients in HCC group were got and the BMI were calculated.

S1: portal area fibrosis expand, and confined in the hepatic sinus and lobule (5); S2: fibrosis around portal area, fibrous septums form, lobule structure keep (15); S3: fibrous septums and lobule structure disorder, without cirrhosis (23); S4: early stage cirrhosis (45).

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Table 2 Activity of ADH and ALDH in normal (n=74) and fibrotic livers (n=88)

	Normal tissue (median, range) ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein)		Fibrotic tissue (median, range) ($nmol \cdot min^{-1} \cdot mg^{-1}$ protein)	
	median, range	ratio	median, range	ratio
ADH	1.81, 0.16~72.12	450.8	7.27, 1.18~43.07***	36.5
ADH I	0.44, 0.09~1.26	14.0	1.34, 0.37~7.10***	19.2
ADH II	0.18, 0.03~0.53	17.7	0.46, 0.14~1.66***	11.9
ALDH	0.12, 0.02~0.32	16.0	0.15, 0.03~0.35**	11.7
ADH/ALDH	17.2, 1.6~1295.3	809.6	44.61, 10.4~726.5***	69.9
ADH I/ALDH	3.8, 1.02 ~ 41.88	41.1	8.96, 1.90 ~ 49.21***	25.9

** *vs* normal tissues, $P < 0.01$, *** *vs* normal tissues, $P < 0.001$

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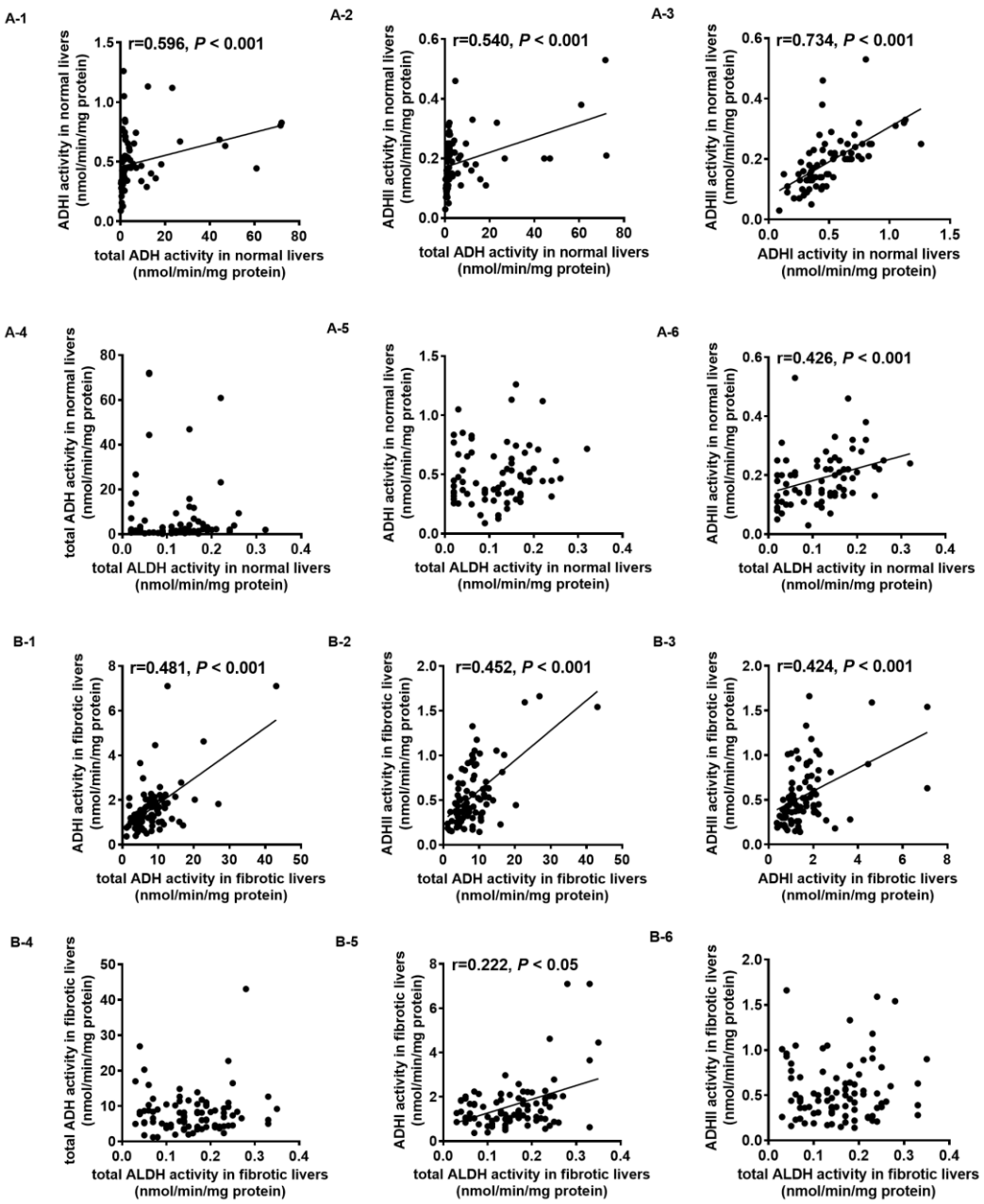

Fig. 1

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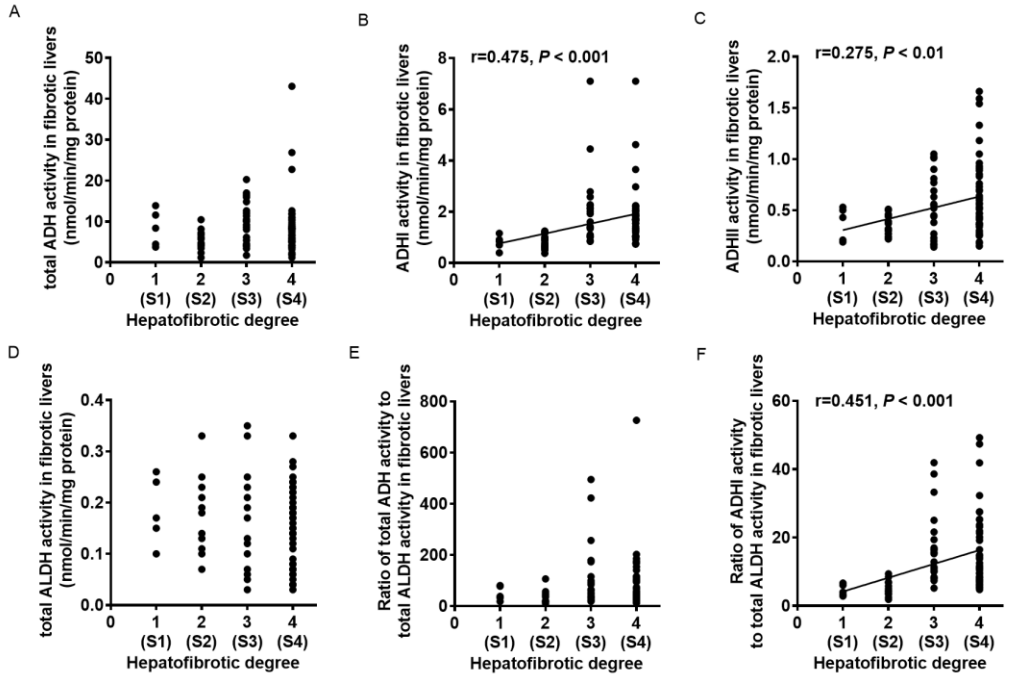

Fig. 2

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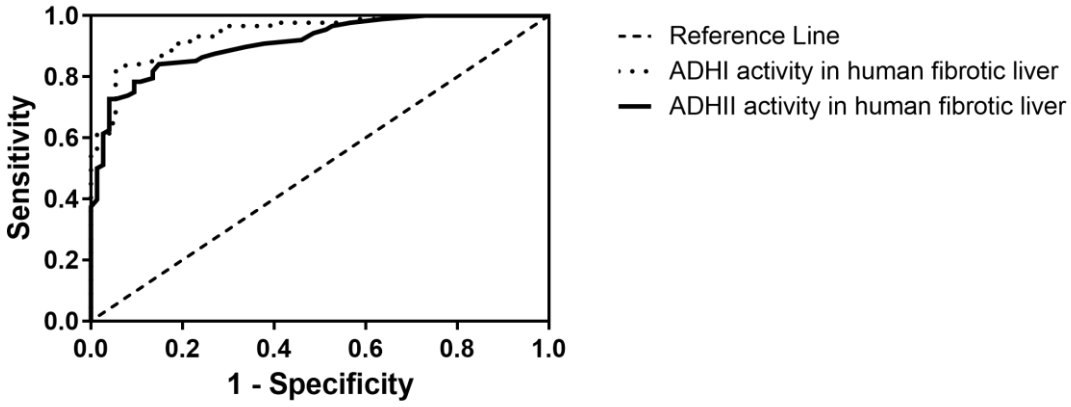

Fig.3

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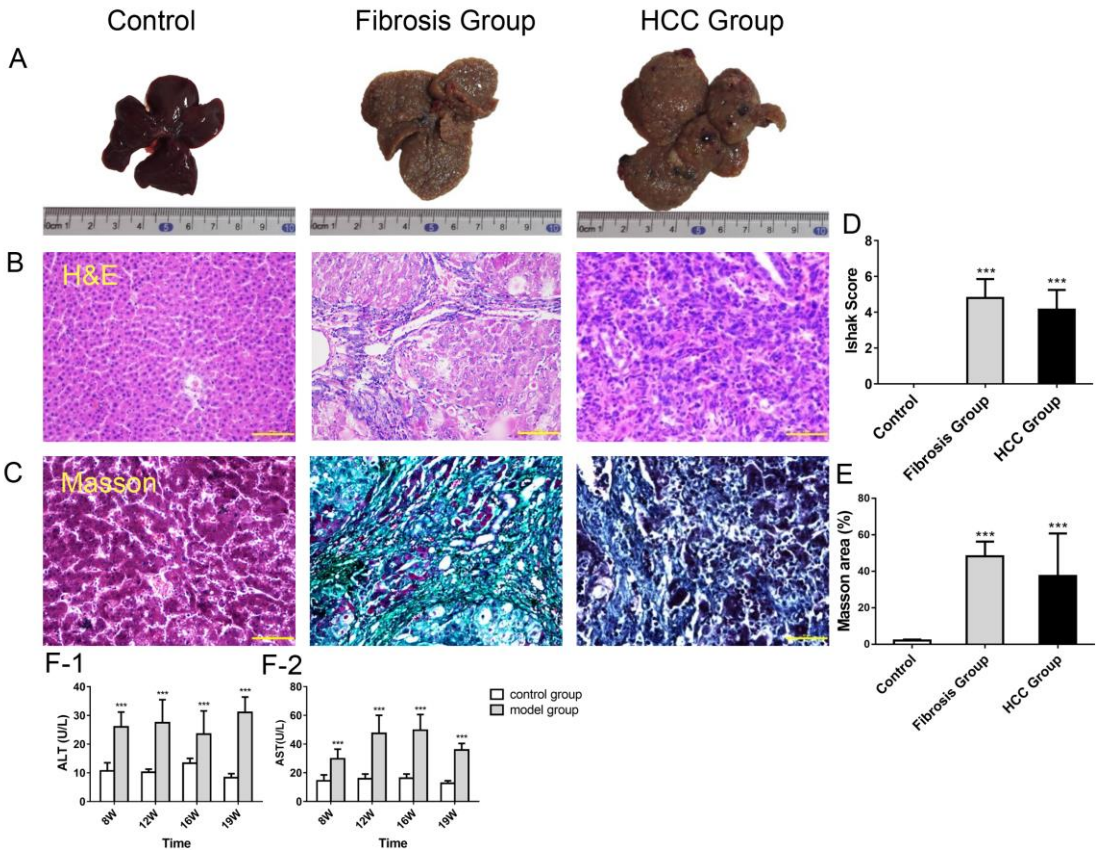

Fig. 4

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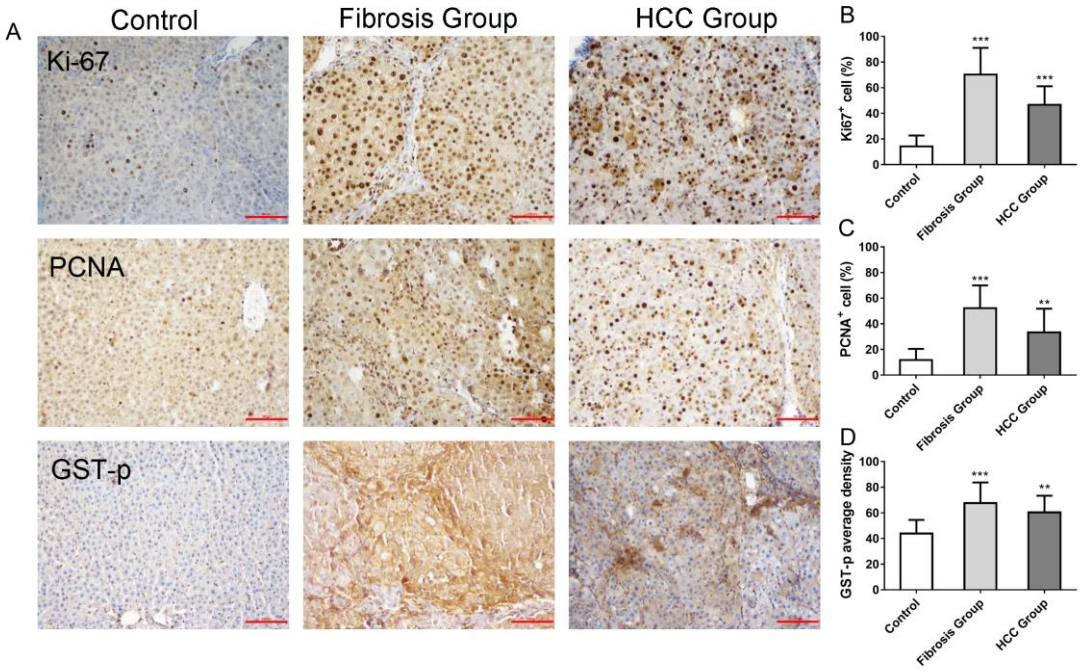

Fig. 5

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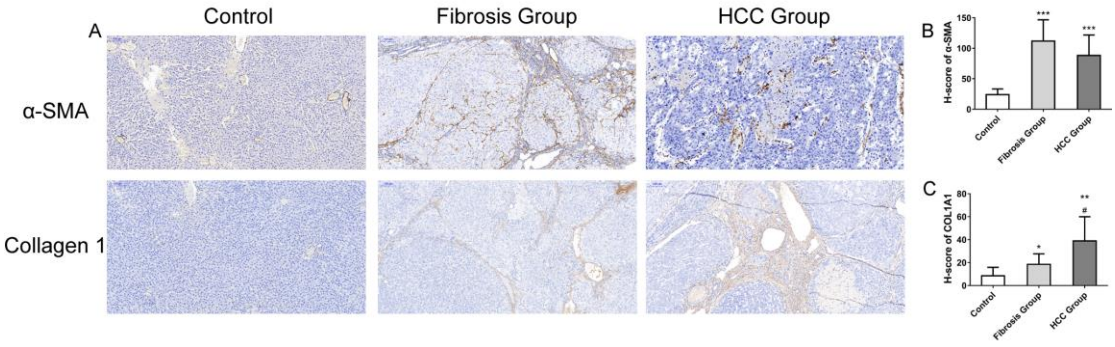

Fig. 6

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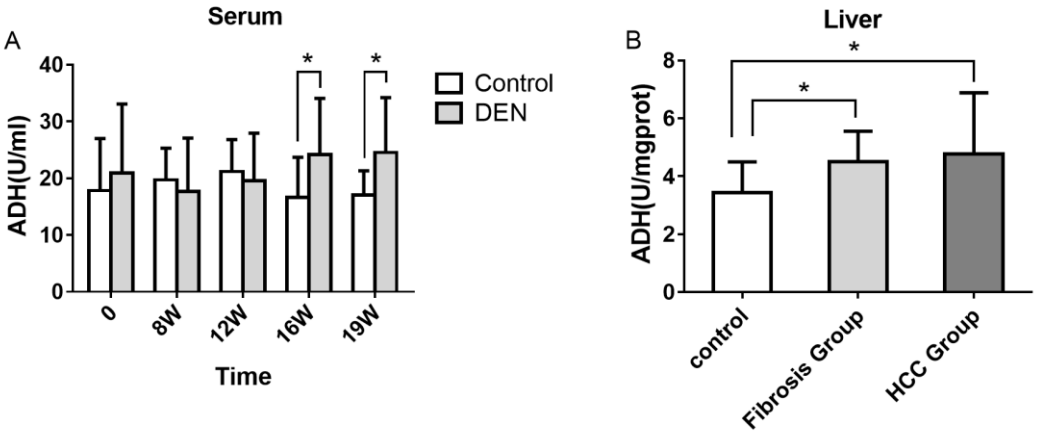

Fig. 7

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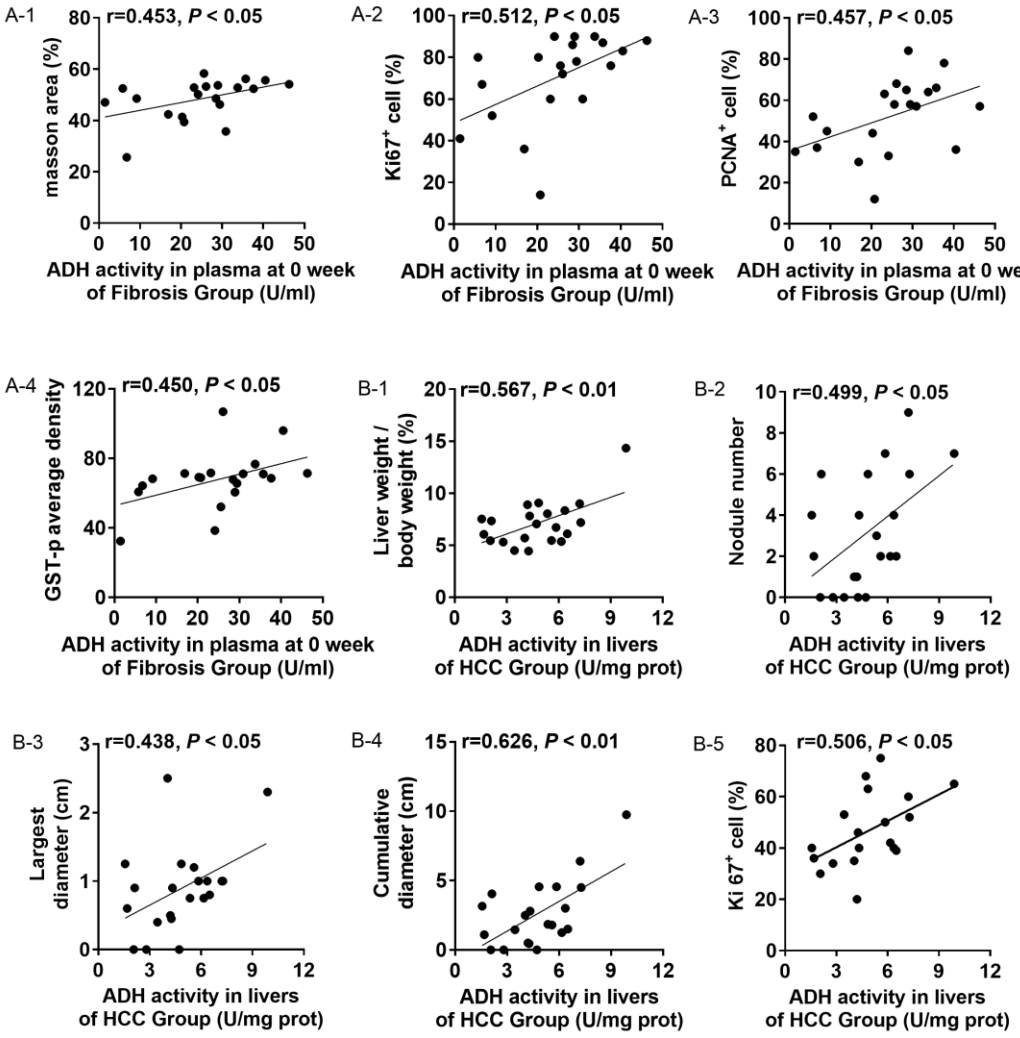

Fig. 8