

Original Article

The mRNA levels of PPAR α , HIF-1 α , and VEGF in the liver tissues of rats with alcoholic liver disease

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Abstract: Objective: To investigate the mRNA levels of peroxisome proliferator-activated receptor α (PPAR α), hypoxia-inducible factor-1 α (HIF-1 α), and vascular endothelial growth factor (VEGF) in the liver tissues of rats with alcoholic liver disease. Methods: A total of 50 Wistar rats were randomly divided into a 4-week model group ($n = 10$), an 8-week model group ($n = 10$), a 12-week model group ($n = 10$), a 16-week model group ($n = 10$), and a control group ($n = 10$). The control group got the same volume of distilled water, and the rats in the model groups were given ethanol to establish alcoholic liver disease model. The mRNA levels of PPAR α , HIF-1 α , and VEGF in the rats' liver tissues, the fatty liver degree, and the inflammation degree in each group were examined and compared. Results: The liver tissues in 4 model groups showed a more worsened fatty liver degree and inflammation degree than those in control group ($P < 0.05$). With the extension of the modeling time, the fatty liver degree and inflammation levels were significantly increased ($P < 0.05$). All the model groups showed lower mRNA level of PPAR α , and higher levels of HIF-1 α and VEGF than the control group ($P < 0.05$). With the extension of the modeling time, the relative mRNA level of PPAR α was decreased, while the mRNA levels of HIF-1 α and VEGF increased (all $P < 0.05$). The fatty liver degree and inflammation level were negatively correlated with the PPAR α mRNA level ($r = -0.899, -0.893, P < 0.05$) and positively correlated with the HIF-1 α and VEGF mRNA levels ($r = 0.791, 0.679, 0.744, 0.597, P < 0.05$). The PPAR α mRNA level was negatively correlated with HIF-1 α and VEGF mRNA levels ($r = -0.732, -0.681, P < 0.05$). Conclusion: High PPAR α mRNA levels and low HIF-1 α and VEGF mRNA levels in the liver tissues of rats with alcoholic liver disease may be closely related to fatty liver and inflammation reactions.

Keywords: Alcoholic liver disease, rats, peroxisome proliferator activated receptor α , hypoxia inducible factor-1 α , vascular endothelial growth factor

Introduction

Alcoholic liver disease, a global public health issue, is characterized by the manifestation of hepatic adipocytes, which may develop into hepatitis, liver fibrosis, and cirrhosis, seriously endangering the patients' lives [1]. Long-term alcoholism is the dominant cause of alcohol-related liver disease, but the pathogenesis is complex and unclear. It includes a variety of pathophysiological processes, some of which still lacks a sufficient basis. Factors found to influence alcoholic liver disease have been explored in several studies [1, 2]. Peroxisome proliferator-activated receptor α (PPAR α) is related to various lipid metabolism pathways and liver diseases [2]. Hypoxia inducible factor-

1 α (HIF-1 α) and its downstream vascular endothelial growth factor (VEGF) are highly expressed in non-alcoholic liver diseases and increase with the aggravation of alcoholic hepatitis diseases [3, 4]. It can thus be suggested that PPAR α , HIF-1 α and its downstream VEGF may be the keys to the occurrence and progression of alcoholic liver disease. Still, there is no published study on the relationship among PPAR α , HIF-1 α , and VEGF in alcoholic liver disease in China. This study was set to further investigate the roles of PPAR α , HIF-1 α , and VEGF in alcoholic liver disease. The changes in mRNA levels of PPAR α , HIF-1 α and VEGF in liver tissues were observed by establishing a rat model of alcoholic liver disease in this study.

The significance of PPAR α , HIF-1 α , and VEGF mRNA

Table 1. qRT-PCR primer sequences

Gene name	Primer sequence (5'-3')
PPAR α (Upstream primer)	CACGATGCTGTCCTCCTT
PPAR α (Downstream primer)	TCCAGTTCGAGGGCATTG
HIF-1 α (Upstream primer)	TGCATCTCCACCTTCTACCC
HIF-1 α (Downstream primer)	CTGCTCCATTCCATCCTGTT
VEGF (Upstream primer)	ACTGGACCCTGGCTTTACTGC
VEGF (Downstream primer)	TTGGTGAGGTTTATCCGCATG
β -actin (Upstream primer)	CATCACACTATCGGCAATGAG
β -actin (Downstream primer)	GACAGCACTGTGTTGGCAT

Materials and methods

Animals

A total of 50 SPF-class, male, three-month-old Wistar rats, weighing (180~220) g were provided by the Animal Experimental Center of Hebei Medical University, with the license number SCXK (Ji) 2018-1-002. The temperature of feeding environment was 20~25°C, and the relative humidity was 50%~70%. All experiments in this study complied with the recommendations of the Regulation for the Management of Laboratory Animals of the Ministry of Science and Technology of China.

Group modeling and specimen preparing

All 50 rats were randomly separated into 5 groups: the 4-week model group ($n = 10$), the 8-week model group ($n = 10$), the 12-week model group ($n = 10$), the 16-week model group ($n = 10$) and the control group ($n = 10$). After 1 week of adaptive feeding, the control group was fed an equal amount of distilled water, and the model groups were given ethanol by gavage to establish the alcoholic liver disease models. In weeks 1-4, 30% 5.0 g/kg/d ethanol was given by gavage; in weeks 5-8, 40% 6.0 g/kg/d ethanol was given by gavage; in weeks 9-12, 50% 7.0 g/kg/d ethanol was given by gavage; and in weeks 13-16, 60% 8.0 g/kg/d ethanol was given by gavage. The gavage was administered at 9:00 a.m. every day, qd. 10, 10, 9, and 8 rats in the 4-, 8-, 12-, and 16-week groups were survived, and the 10 rats in the control group all survived. After the modeling, the rats were fasted for 12 hours, and the liver tissues were dissected after anesthesia with 3% pentobarbital sodium.

Main experimental instruments

A UV1901 ultraviolet spectrophotometer was provided by the Shanghai AuCy Scientific Instrument Co., Ltd. A Trizol kit, a reverse transcription kit, and a Tran Start Tip Top Green q PCR SuperMix kit were provided by the Beijing CoWin Century Biotechnology Co., Ltd. Primer sequence was provided by Sangon Biotech (Shanghai) Co., Ltd. An ABI7500 real-time fluorescence quantitative PCR instrument was provided by Applied Biosystems Co., Ltd., USA. A DYCZ-24KS electrophoresis apparatus was provided by the Beijing Liuyi Instrument Factory. The gel imaging system was provided by Alpha Innotech Co., Ltd., USA.

Histological changes of the livers

Liver tissues 1 cm away from the edge of the right lobe were fixed in 4% paraformaldehyde solution, dehydrated with ethanol, made transparent with xylene, and embedded in paraffin. After HE staining, the fatty liver levels (5, FO~4) and the inflammation levels (5 grades, GO~4) in the liver tissues were evaluated according to the *Guidelines for the Diagnosis and Treatment of Alcoholic Liver Diseases* [5], all of which ranged from 0~4 levels. FO: < 5% liver cell steatosis; F1: 5%-30% liver cell steatosis; F2: 31%-50% liver cell steatosis; F3: 51%~75% liver cell steatosis; F4: over 75% liver cell steatosis. The higher the score, the higher the fatty liver degree and the higher the inflammation levels.

The PPAR α , HIF-1 α , and VEGF mRNA levels were measured using PCR (Real-time PCR, RT-PCR)

(1) Extraction of total RNA: The total RNA of liver tissues samples was extracted using the Trizol method by following the instructions. The ultraviolet spectrophotometer showed that the absorbance (A) ratio A_{260}/A_{280} was in the range of 1.7~2.0, which meant that the RNA extraction was successful. (2) Reverse transcription to cDNA: We did the reverse transcription of the total RNA to cDNA according to the reverse transcription kit instructions. (3) RT-PCR amplification: We used cDNA as a template and the β -actin gene as an internal reference to carry out the RT-PCR amplification. The primer sequences are shown in **Table 1**. According to

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Table 2. Comparison of the liver histological changes in the rats in each group ($\bar{x} \pm s$)

Group	n	Fatty level	Inflammation level
Control group	10	0.00 \pm 0.00	0.00 \pm 0.00
4-week MD	10	0.84 \pm 0.40 ^①	0.30 \pm 0.45 ^①
8-week MD	10	2.30 \pm 0.64 ^{①,②}	0.80 \pm 0.40 ^{①,②}
12-week MD	9	3.00 \pm 0.47 ^{①,②,③}	1.33 \pm 0.47 ^{①,②,③}
16-week MD	8	3.57 \pm 0.56 ^{①,②,③,④}	2.00 \pm 0.50 ^{①,②,③,④}
F value		83.315	18.919
P value		< 0.001	< 0.001

Note: ^① $P < 0.05$, compared with the control group; ^② $P < 0.05$, compared with the 4-week model group; ^③ $P < 0.05$, compared with the 8-week model group; ^④ $P < 0.05$, compared with the 12-week model group.

the Tran Start Tip Top Green q PCR Super Mix instructions, the culture conditions were as follows: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 15 s, PPAR α annealing at 60°C for 30 s, HIF-1 α annealing at 56°C for 30 s, VEGF annealing at 58°C for 30 s, and extension at 72°C for 45 s, recording the fluorescence values in real-time for 40 cycles. The PCR products were treated with optical density scanning using an electrophoresis and gel image analysis system, and the relative mRNA levels of PPAR α , HIF-1 α , and VEGF were expressed using the 2^{- $\Delta\Delta$ Ct} method [6].

Statistical analysis

SPSS 22.0 statistical software was used to process the data. The measurement data met a normal distribution were expressed by ($\bar{x} \pm s$). The comparisons among multiple groups used a single factor analysis of variance followed with SNK post hoc test. The relationships between the fatty liver degree, the inflammation levels, and the levels of PPAR α , HIF-1 α , and VEGF were analyzed using grade nonparametric correlation analyses. The relationships among the mRNA levels of PPAR α , HIF-1 α , and VEGF were analyzed using Pearson correlation analyses. $P < 0.05$ was considered a statistically significant difference.

Results

Comparison of the histological changes in the rat livers among groups

The degree of fatty liver and inflammation in tissues of each model group were more severe than that in the control group rats ($P < 0.05$). With the prolongation of the modeling time, the fatty and inflammation levels in the liver tissues

worsened significantly ($P < 0.05$). See **Table 2** and **Figure 1**.

Comparison of the relative mRNA levels of PPAR α , HIF-1 α , and VEGF in the rats' liver tissues among groups

The model groups had lower PPAR α mRNA level and higher HIF-1 α and VEGF mRNA levels in the liver tissues than the rats in the control group ($P < 0.05$). With the prolongation of the modeling time, the relative mRNA level of PPAR α decreased, while the relative mRNA levels of HIF-1 α and VEGF increased significantly ($P < 0.05$). See **Table 3**.

Correlation analysis between the rat liver histological changes and the mRNA levels of PPAR α , HIF-1 α , and VEGF

The degree of fatty liver and inflammation in rat livers were negatively correlated with the PPAR α mRNA levels ($P < 0.05$). In contrast, the degree of fatty liver and inflammation in the rat livers were positively correlated with the HIF-1 α and VEGF mRNA levels ($P < 0.05$). See **Table 4**.

Correlation analysis of the PPAR α with the HIF-1 α and VEGF

The PPAR α mRNA levels in the rat livers were negatively correlated with the HIF-1 α and VEGF mRNA levels ($r = -0.732, -0.681, P < 0.05$). See **Figure 2**.

Discussion

Alcoholic liver disease, a type of toxic liver damage, is mainly caused by long-term heavy drinking. A regional epidemiological investigation shows that the incidence of alcoholic liver disease in China has been on the rise in recent years [7]. Alcoholic liver disease has emerged as the main cause of loss of appetite, abdominal pain, and hemorrhages. Evidence suggests that it would further develop into liver cirrhosis and gastrointestinal hemorrhages, seriously endangering the patients' lives. Under normal circumstances, after entering into liver, the main organ of alcohol metabolism, ethanol will be metabolized into acetaldehyde and acetic acid by ethanol oxidase and acetaldehyde dehydrogenase then into water and carbon dioxide. It is common for excess ethanol to accelerate the degradation of ethanol using

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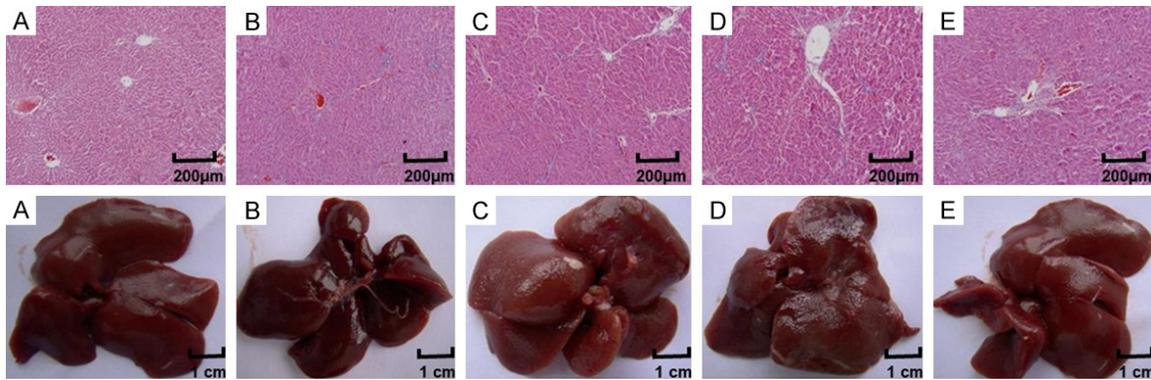


Figure 1. The hematoxylin-eosin (original magnification $\times 100$) of the liver sections and liver morphology in the rats. Note: A. The control group; B. 4-week model group; C. 8-week model group; D. 12-week model group; E. 16-week model group.

Table 3. Comparison of the relative PPAR α , HIF-1 α , and VEGF mRNA levels in the liver tissues of the rats in each group ($\bar{x} \pm s$)

Group	n	PPAR α mRNA	HIF-1 α mRNA	VEGF mRNA
Control group	10	0.98 \pm 0.11	0.26 \pm 0.05	0.43 \pm 0.04
4-week MD	10	0.56 \pm 0.09 ^①	0.33 \pm 0.08 ^①	0.48 \pm 0.05 ^①
8-week MD	10	0.42 \pm 0.08 ^{①,②}	0.44 \pm 0.07 ^{①,②}	0.54 \pm 0.06 ^{①,②}
12-week MD	9	0.35 \pm 0.06 ^{①,②,③}	0.52 \pm 0.08 ^{①,②,③}	0.61 \pm 0.08 ^{①,②,③}
16-week MD	8	0.12 \pm 0.03 ^{①,②,③,④}	0.62 \pm 0.11 ^{①,②,③,④}	0.69 \pm 0.07 ^{①,②,③,④}
F value		19.030	18.451	12.115
P value		< 0.001	< 0.001	< 0.001

Note: ^① $P < 0.05$, compared with the control group; ^② $P < 0.05$, compared with the 4-week model group; ^③ $P < 0.05$, compared with the 8-week model group; ^④ $P < 0.05$, compared with the 12-week model group.

Table 4. Correlation analysis between the rat liver histological changes and the PPAR α , HIF-1 α , and VEGF mRNA levels

Index	Fatty level		Inflammation level	
	r value	P value	r value	P value
PPAR α mRNA	-0.899	< 0.001	-0.893	< 0.001
HIF-1 α mRNA	0.791	< 0.001	0.679	< 0.001
VEGF mRNA	0.744	< 0.001	0.597	< 0.001

catalase and ethanol oxidase [8]. However, in the process of metabolizing it to acetaldehyde and acetic acid, the oxidative coenzymes will be transformed into reductive coenzyme, which will lead to abnormal redox reactions in the liver, exacerbate the oxidative stress reactions, aggravate the inflammatory reactions, and cause damage to liver cells [9]. Consistent with the literature, this study found that the model groups, i.e., the alcoholic rats, had more fat and inflammation in their liver tissues than

those fed with an equivalent amount of pure water. And with the prolongation of the modeling time, the degree of fatty liver and inflammation showed an increasing trend, indicating that long-term heavy drinking will aggravate the degree of fatty liver and inflammation.

PPAR α is widely involved in lipid metabolism and closely related to ethanol metabolism [10, 11]. The literature on PPAR α has highlighted

that ethanol can inhibit liver metabolism and fatty acid transport by inhibiting the PPAR α levels and it can accumulate in the hepatocytes, resulting in abnormal lipid metabolism [12]. Abnormal lipid metabolism will aggravate lipid accumulation in the liver, causing fatty liver, oxidative stress, and inflammatory reactions. It forms a vicious circle to exacerbate the liver damage. Decreased PPAR α level was detected in the liver tissues of the model groups than that in the control rats in this study. With the prolongation of the modeling time, the relative PPAR α mRNA level in the liver tissues decreased, and PPAR α mRNA was negatively correlated with the degree of fatty liver and inflammation in the liver, suggesting that the PPAR α mRNA level was inhibited, thus leading to the progression of alcoholic liver disease. These results provide further support for the hypothesis that the PPAR α levels can be inhibited by ethanol, and the activation of PPAR α is inhibited, leading to the oxidation of fatty acids

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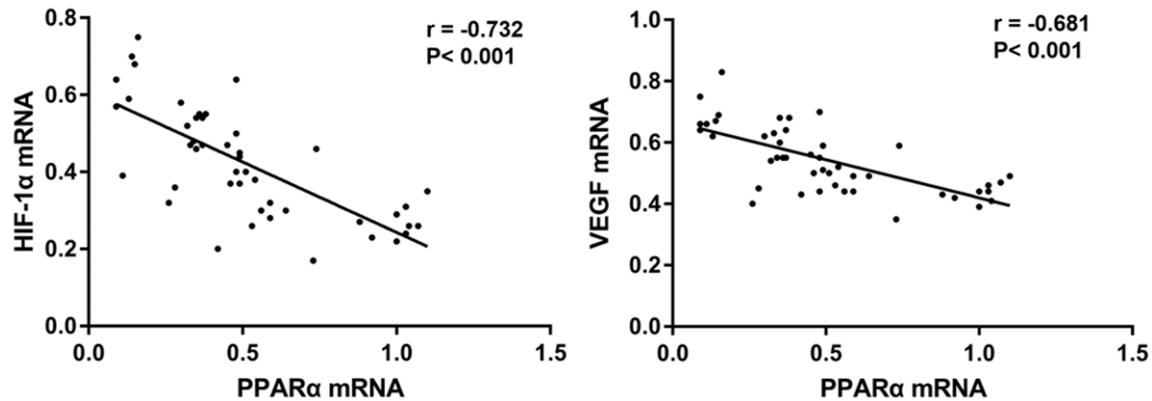


Figure 2. Correlation analysis of PPAR α mRNA with the HIF-1 α and VEGF mRNA levels.

and the inhibition of lipid synthesis. It leads to lipid metabolism disorder, an increase in the accumulation of fat in the hepatocytes, and an aggravation of fatty and inflammatory damage [13, 14].

Under normal circumstances, the oxygen supply and consumption in the liver can maintain a balance. However, the long-term intake of a large amount of alcohol will increase the burden on the liver, inducing a high metabolism state, and increasing its oxygen consumption. Thus, it increases the oxygen gradient of the portal vein and the central vein, and the liver becomes hypoxic [15]. Previous research findings [16] have revealed that alcohol metabolism can increase the liver's oxygen consumption, making the liver hypoxic. Hypoxia is not conducive to alcohol metabolism; it leads to the further development of alcoholic liver disease and to cirrhosis. HIF-1 is a transcription factor that plays a specific role under hypoxia [17]. HIF-1 α , a subunit of HIF-1, participates in regulating the oxygen balance in the micro-environment. Under hypoxia, the HIF-1 α level increases and binds specifically with HIF-1 β and promotes the downstream gene VEGF, performing an adaptive protection and hypoxic regulation pathway. This also agrees with our research that ethanol gavage resulted in higher levels of HIF-1 α and VEGF in the liver tissues of each of the model groups. With the prolongation of the modeling time, the relative mRNA levels of HIF-1 α and VEGF increased. It is speculated that ethanol and its metabolites in the liver may activate phagocytes on the inner surfaces of the hepatic sinuses, activate many transcription factors, and increase the HIF-1 α levels. A large amount of ethanol entering the

body will trigger oxidative stress, resulting in the up-regulation of HIF-1 α and VEGF. In addition, liver hypoxia caused by alcohol intake can activate HIF-1 α and singularize VEGF [18, 19]. This research also shows that the fatty liver degree and inflammation levels in rat liver are positively correlated with the mRNA levels of HIF-1 α and VEGF. It is speculated that the high HIF-1 α and VEGF levels may promote the release of the inflammatory factors, resulting in liver damage, a disorder of the liver fat metabolism, the degeneration and necrosis of liver cells, and inflammatory infiltration [20]. In this research, the relationship between PPAR and HIF-1 α and VEGF was further analyzed. It is found that the PPAR α mRNA level in rat livers was negatively correlated with the mRNA levels of HIF-1 α and VEGF. It is speculated that low PPAR α mRNA level will aggravate lipid metabolism disorder, which will cause liver damage, inhibit ethanol metabolism, lead to the accumulation of a large amount of ethanol in the liver, raise oxygen consumption, increase the oxygen gradient, aggravate the hypoxia level in the liver, and stimulate high HIF-1 α and VEGF levels. A critique of our study is that it is an animal study with a small sample size. We believe that clinical studies with larger sample size should be conducted in the future.

To sum up, PPAR α mRNA is decreased, while HIF-1 α and VEGF are increased in the liver tissues of rats with alcoholic liver disease, which may be closely related to the fatty liver and inflammation levels.

Disclosure of conflict of interest

None.

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