Over-Expression of *ATPase II* Alleviates Ethanol-Induced Hepatocyte Injury in HL-7702 Cells

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Statistical Analysis **C**
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Background: Excessive alcohol consumption can cause hepatocellular injury. *ATPase II* (*ATP8A1*) can display an ATP-dependent phospholipid translocase activity. However, the function of *ATP8A1* in hepatocyte injury is still unclear. In the present study we explored the effect of *ATP8A1* on ethanol-induced hepatocyte injury.

Material/Method: A human hepatocyte strain, HL-7702, was pretreated by ethanol with gradient concentration for 2, 4, 8, and 12 h, and were then divided into 6 groups after the cells were transfected. We detected cell viability by use of the Cell Counting Kit-8 (CCK-8) assay. Reactive oxygen species (ROS), apoptosis rate, and mitochondrial membrane potential (MMP) were measured using flow cytometry. We used quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot to measure the mRNA and protein expression, respectively.

Results: Ethanol inhibited the viability of HL-7702 cells and suppressed the expression of *ATP8A1* in dose- and time-dependent manners. Furthermore, over-expression of *ATP8A1* reduced the level of ROS and the apoptosis rate and recovered the MMP. Additionally, over-expressed *ATP8A1* regulated the protein and mRNA levels of apoptosis-related molecules. Moreover, over-expression of *ATP8A1* enhanced the phosphorylation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt).

Conclusions: Over-expression of *ATP8A1* alleviated ethanol-induced hepatocyte injury. Moreover, the PI3K/Akt signaling pathway appears to participate in inhibition of ethanol-induced hepatocyte apoptosis and may provide a candidate target for the treatment of alcoholic liver diseases (ALD).

MeSH Keywords: **Apoptosis • Arsenite Transporting ATPases • Hepatocytes • Phosphatidylinositol 3-Kinase**

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Background

Alcoholic liver disease (ALD) is considered to be associated with the alcohol abuse and unhealthy lifestyle [1]. Excessive alcohol intake can trigger hepatocyte injury and death by apoptotic pathways [2]. Moreover, excessive alcohol use can contribute to mitochondrial damage, depressed adenosine triphosphate (ATP) synthesis rate, and an impaired oxidative phosphorylation system [3]. Ethanol can promote the accumulation of reactive oxygen species (ROS) in hepatic mitochondria, which are a group of reactive, short-lived, oxygen-containing species. Excessive ROS impairs some organisms, resulting in cell death for both tumors and healthy cells [4]. Generally, hepatocyte apoptosis is caused by exposure to ethanol [5].

Apoptosis can be caused through the mitochondrial pathway and the death receptor pathway [6]. In the former, cytochrome C (a pro-apoptotic protein) can bind Apaf-1 and procaspase-9 to form an apoptosome. The agminated procaspase-9 can activate caspase-9 and caspase-3 to initiate apoptosis [7,8]. Bcl-2 and Bcl-2-associated X protein (Bax) in the Bcl-2 gene family have opposite effects [9]. Bcl-2 can inhibit cell apoptosis by preventing the release of cytochrome C from the mitochondria to the cytoplasm, while Bax is an antagonist of Bcl-2 [10]. Moreover, as a cleavage enzyme, caspase-3 can induce cell apoptosis [11,12]. Fas and Fas ligand (Fasl) are tumor necrosis factors that can induce the death receptor pathway. Fas/Fasl can recruit Fas-associated death domain-containing protein (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC), and then the autoproteolysis and activation of procaspase-8 can activate Caspase-3, leading to apoptosis [13].

The PI3K/Akt pathway, a vital anti-apoptotic pathway in cells, can suppress apoptosis by regulating the death receptor pathway and the mitochondrial pathway [14]. The PI3K/Akt pathway can regulate the expression of many anti-apoptotic genes by its downstream transcription factor [15]. As a major downstream target of PI3K, Akt can phosphorylate multiple proteins to regulate cell survival [16,17]. A previous study suggested that p-Akt can phosphorylate the death promoter Bad to preserve mitochondrial integrity by promoting Bcl-2 [18]. Meanwhile, Bcl-2 can prevent Bax from conformational changes to inhibit apoptosis [19]. Many studies have revealed that inhibition of the PI3K/Akt pathway activates Fas-mediated apoptosis in gastric, colon, and prostate cancer [20–22]. Briefly, PI3K/Akt signaling may provide an effective strategy to relieve ethanol-induced hepatocyte injury.

ATP8A1 (formally termed *ATPase II*) is an amino phospholipid acyl transferase (APLT), whose activity can be stimulated by transport phosphatidylserine (PS) and phosphatidyl ethanolamine (PE). *ATP8A1* is able to move PS across the leaflets of the vesicle membrane in presence of ATP [23,24]. It has also

been documented that the over-expression of *ATP8A1* helps maintain transmembrane lipid homeostasis in the liver [25]. However, the effect of *ATP8A1* on ethanol-induced hepatocytic injury is still unknown. In the present study, we explored the role of the *ATP8A1* in ethanol-induced hepatocytic injury.

Material and Methods

Cell culture

The human hepatic cell line HL-7702 was obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). HL-7702 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 mg/mL streptomycin at 37°C with 5% CO_2 (all from Invitrogen, Carlsbad, CA, USA).

Transfection of HL-7702 cells

Cells at a density of 4×10^5 cells were seeded into 6-well plates. After culturing for 24 h, the medium was replaced by Opti-MEM (Invitrogen) and cultured. The pIRES2-EGFP-*ATP8A1* and control vector were designed and cloned by Takara Biotechnology (Dalian) Co., Ltd. Plasmids were transfected according to the Lipofectamine 2000 protocol (Invitrogen, Grand Island, NY, USA). After incubation for another 48 h, the treated cells were used for further study.

CCK-8 assay

Cell viability was performed using CCK-8 (Beyotime, Beijing, China). Cells were cultivated in 96-well plates at a density of 3000 cells, followed by treatment on the fresh media containing 0, 50, 100, 150, 200, 250, and 300 mM of ethanol for 2, 4, 8, 12, and 24 h. When the incubation was over, the CCK-8 was added and cultured for 4 h. The absorbance was detected at 450 nm. After cell transfection, the experiment was divided into 6 groups and the cell viabilities were determined at 12, 24, and 48 h.

Intracellular ROS levels

Using fluorescence-activated cell sorting (FACS) analysis to detect ROS levels in HL-7702 cells treated by ethanol, cells in each group were centrifugated and then incubated in 10 µM diluted 2',7'-dichlorofluorescin diacetate (DCFH-DA) in the dark for 20 min. Cells were washed 3 times, and binding buffer was added. The cells were detected by FACS Calibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) and the results were analyzed by Cyflogic software (Cyflogic Team, Turku, Finland).

Table 1. Summary of the qRT-PCR primers.

Gene	Forward primers (5'-3')	Reverse primers (5'-3')
ATP8A1	ACATGCACTATGAACCCACA	TGAGTTCTGCCCATAAGCAA
Fas	TATCACCACTATTGCTGGAGTCATG	TCAATGTGTCATACGCTTCTTTCTT
Fasl	CTCTGGAATGGGAAGACACCTATG	GCAAGATTGACCCCGGAAGTATA
Bcl-2	GAGCAGACGGATGGAAAAAGGA	CAGGGCAAAGAAATGCAAGTGA
Bax	GGTCTATAATGCGTTTTCCTTACGT	AGAGCTAGGGTCAGAGGGTCATC
Caspase-3	AAGAACTTAGGCATCTGTGGGCA	TTCAGGACAAACATCACAAAACCA
GADPH	TGAACGGGAAGCTCACTGG	GCTTCACCACCTTCTTGATGTC

Analysis of cell apoptosis

The Annexin V-FITC Apoptosis Detection Kit (Biovision, USA) was prepared for cell apoptosis. HL-7702 cells in each group were stained by Annexin V-fluorescein isothiocyanate (FITC) and PI and incubated in the dark at room temperature. Cells were washed with PBS and resuspended. The fluorescence was immediately analyzed by flow cytometry using fluorescence channels FL1H and FL2H. Cells in the lower left quarter represented normal cells. Cells in the right lower quarter and right upper quarter correspond to early apoptotic cells and late dead cells, respectively.

Changes of the mitochondrial membrane potential (MMP)

Changes of MMP were determined by Rh123 (Sigma, St. Louis, MO, USA). HL-7702 cells in each group were resuspended by PBS, followed by incubation with 10 µM Rh123 for 0.5 h in the dark at 37°C. Fluorescence intensity was analyzed by flow cytometry.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) a reverse transcription was performed using the protocol of for SuperScript III reverse transcriptase (Takara, Japan). Primer specificity was verified by NCBI Primer-BLAST. The primers were synthesized (Shanghai Sangon Biological Engineering Technology Company Limited, China) as shown in Table 1. Amplification reactions were performed on the ABI 7500 Real-Time PCR (Applied Biosystems, Foster City, CA, USA). The conditions were: 95°C for 15 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s. GAPDH served as a reference gene and the data were evaluated by the $2^{-\Delta\Delta Ct}$ method. Each sample was detected at least 3 times.

Western blot assay

Total proteins were isolated using lysis buffer (Beyotime, Beijing, China), and the concentrations were measured by BCA protein assay (Thermo Scientific, Rockford, IL, USA). After separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), total proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). for incubation overnight at 4°C with following treatment with specific primary antibodies: anti-ATP8A1 (1: 5000, Abcam), anti-Bcl-2, anti-Bax (1: 100, Santa Cruz Biotechnology), anti-GAPDH (1: 500, Santa Cruz Biotechnology), anti-Fas, anti-Fasl (1: 500, Bioworld technology, Inc., Louis Park, MN), anti-p-PI3K, anti-PI3K, anti-p-Akt and anti-Akt antibody (1: 200, Cell Signaling Technology). After washing in PBS, goat anti-rabbit second antibody (1: 3000, Rockland, Gilbertsville, PA, USA) was incubated with membranes for 1 h. The proteins were detected using the Odyssey Infrared Imaging System (Version 2.1.12, LI-COR, Lincoln, NE).

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical differences were analyzed using the *t* test and one-way analysis of variance (ANOVA). Differences with a *P* value less than 0.05 were regarded as statistically significant.

Results

The effects of ethanol on cell viability and expression of ATP8A1

HL-7702 cells were used to evaluate the stress of ethanol. Compared with the control group, cell viability decreased with increasing concentrations of ethanol in time- and dose-dependent trends (Figure 1A). qPCR results showed that the mRNA expression of *ATP8A1* increased slightly at 50 mM, and then declined (Figure 1B). Western blot results showed that, with
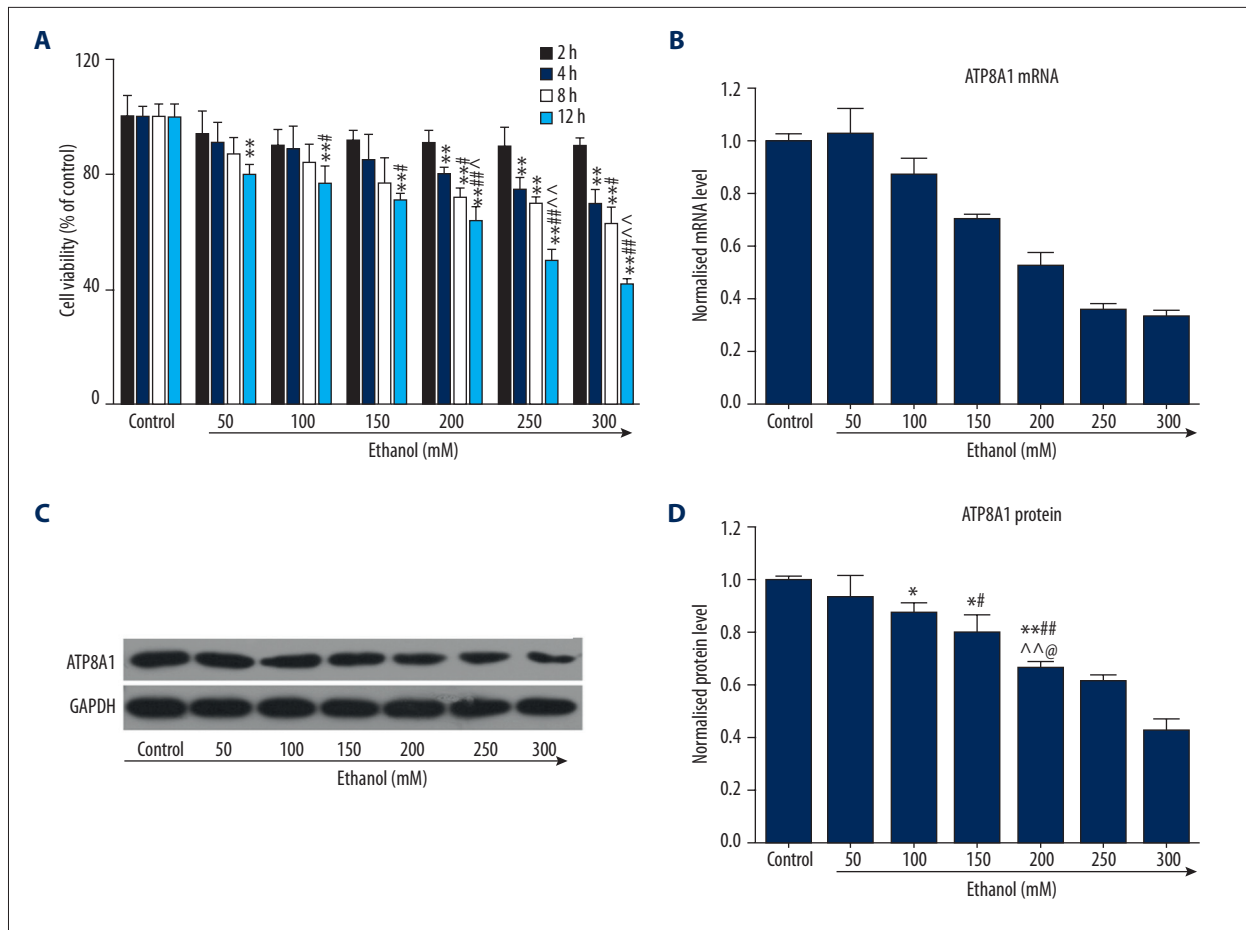
Figure 1. Ethanol improved hepatocyte injury. (**A**) Cells were cultivated with ethanol at gradient concentrations (50, 100, 150, 200, 250, and 300 mM) for 2, 4, 8, and 12 h. CCK-8 assay was used to detect viability of HL-7702 cells. ** $P<0.01$ compared with control group; # $P<0.05$, ## $P<0.01$ compared with ethanol-treated group for 2 h; ^ $P<0.05$, ^^ $P<0.01$ compared with ethanol-treated group for 4 h. (**B**) The mRNA level of *ATP8A1* was concentration-dependent. (**C**) Representative Western blot result showing expression of *ATP8A1* after ethanol treatment for 4 h. (**D**) Protein expression of *ATP8A1* showed differential expression levels under gradient ethanol concentration. * $P<0.05$, ** $P<0.01$ compared with control group; # $P<0.05$, ## $P<0.01$ compared with the group with 50 mM ethanol; ^^ $P<0.01$ compared with the group with 100 mM ethanol; @ $P<0.05$ compared with the group with 150 mM ethanol.

the increased concentration of ethanol, the protein expression of *ATP8A1* was gradually reduced (Figure 1C), and it was decreased significantly when the ethanol dosage reached 200 mM (** $P<0.01$, ## $P<0.01$; Figure 1D). Therefore, the treatment of 200 mM ethanol for 4 h was used in subsequent experiments.

Over-expression of ATP8A1attenuated hepatocyte injury

To analyze the role of *ATP8A1* in hepatocyte injury, we transfected the HL-7702 cells with an *ATP8A1*-plasmid and NC-vector. The efficiency of *ATP8A1* over-expression vector was confirmed by significant enhancement of the *ATP8A1* protein and the mRNA level (Figure 2A, 2B). Cell viability was substantially increased by the over-expression of *ATP8A1* at 12, 24, and 48 h. Interestingly, with the prolongation of the time,

cell viability was gradually increased by *ATP8A1* over-expression (Figure 2C).

Up-regulated ATP8A1 protected HL-7702 cells from damage caused by ethanol

Flow cytometry assay showed that *ATP8A1* over-expression reduces the ROS level and apoptosis rate and restored MMP levels (Figure 3A–3C). The over-expression of *ATP8A1* markedly reduced the ethanol-induced ROS generation (Figure 3D). Furthermore, when the cells were exposed to ethanol, the total number of apoptotic cells was increased by approximately 18.33%, while up-regulated *ATP8A1* inhibited cell apoptosis by about 10.4% (Figure 3E). Meanwhile, the over-expression of *ATP8A1* clearly improved the level of MMP impaired by ethanol (Figure 3F).
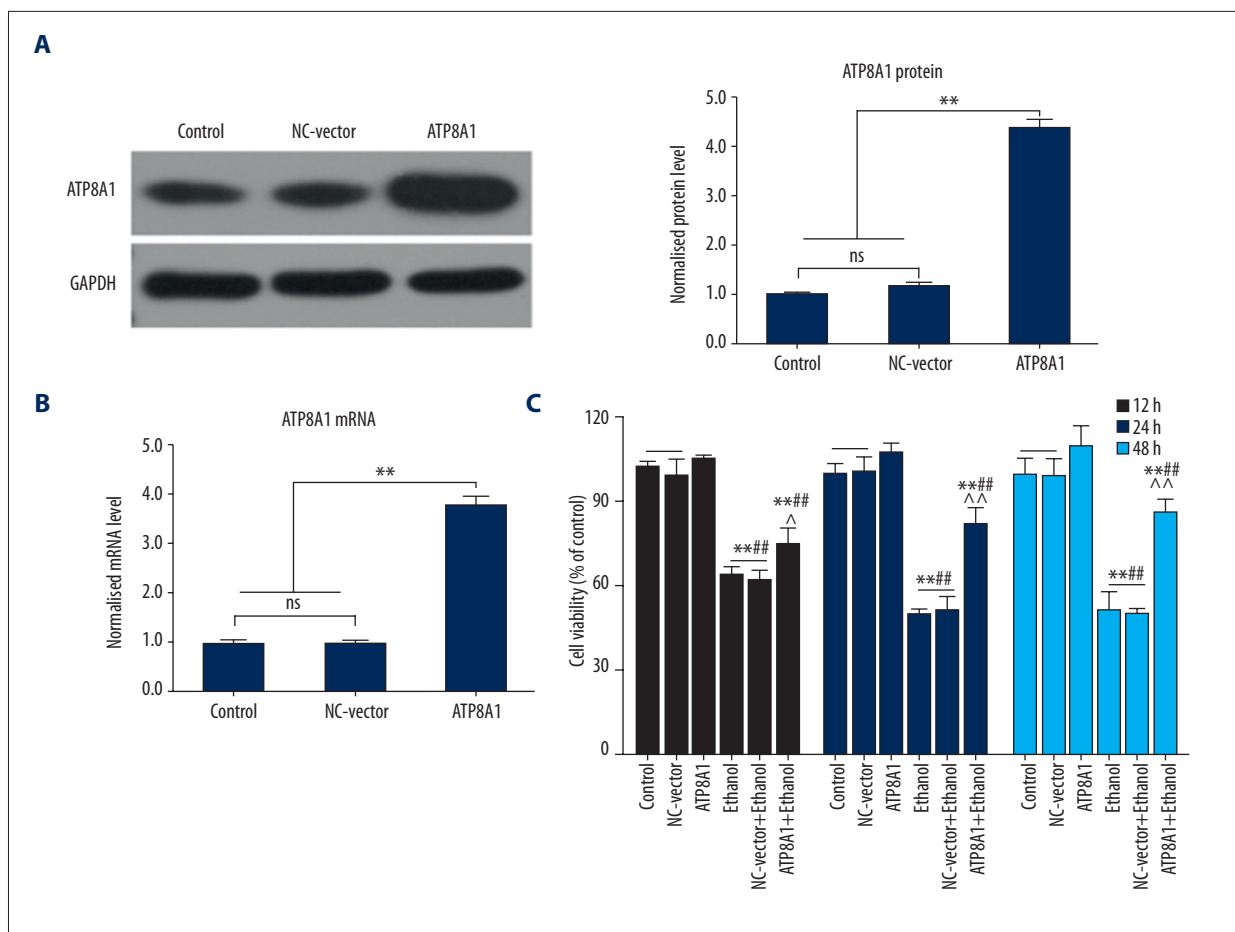
Figure 2. Over-expressed *ATP8A1* recovered the damage induced by ethanol. After cells were transfected with *ATP8A1* and NC-vector, both the protein expression (**A**) and mRNA expression (**B**) of *ATP8A1* in the over-expressed *ATP8A1* group were relatively high compared with control. (** P<0.01 *vs.* control group; ns indicates no statistically significant difference). (**C**) Cell viability was substantially increased by the over-expression of *ATP8A1*. Control group: human hepatocyte line HL-7702; NC-vector group: cells were transfected with NC-vector; *ATP8A1* group: cells were transfected with over-expressed *ATP8A1*; Ethanol group: HL-7702 induced by 200 mM ethanol; NC-vector + Ethanol group: cells transfected with NC-vector were cultured as Ethanol group; and *ATP8A1* + Ethanol group: cells transfected with *ATP8A1* vector were cultured as in the Ethanol group. Cell viability in each group was measured by CCK-8. ** P<0.01 compared with control group; ## P<0.01 compared with ATP8A1 group; ^ P<0.05, ^^ P<0.01 compared with Ethanol group.

Effects of over-expressed ATP8A1 on apoptosis-related molecules in HL-7702 cells

To explore the mechanism of ethanol-induced apoptosis, the levels of mRNAs related to apoptosis were measured (Figure 4A). Fas/Fasl, the death receptors that initiate apoptosis, presented an upward trend when HL-7702 cells were exposed to ethanol. Moreover, over-expressed *ATP8A1* significantly inhibited the ethanol-induced activation of *Fas/Fasl*. The expression of *Bcl-2* (an anti-apoptotic gene) was decreased, while the expression level of *Bax* (a pro-apoptotic gene) and *Caspase-3* was improved after ethanol (200 mM) treatment. However, the expressions of *Bcl-2, Bax,* and *Caspase-3* were largely reversed by the over-expression of *ATP8A1*. In addition, Western blot analysis highlighted a protein trend that was similar to mRNA expression (Figure 4B).

The protective effect of ATP8A1 over-expression in HL-7702 cells was associated with the PI3K/AKT signaling pathway

To further explore the molecular mechanism underlying the protective effect of over-expressed *ATP8A1* in HL-7702 cells, we examined the expression of proteins, including PI3K, p-PI3K, Akt, and p-Akt, by Western blot analysis (Figure 5A). After ethanol treatment, the p-PI3K/PI3K and p-Akt/Akt levels were significantly inhibited. Over-expressed *ATP8A1* restored the PI3K phosphorylation suppressed by ethanol in HL-7702 cells. Moreover, ethanol inhibited the phosphorylation of Akt and was recovered by over-expressed *ATP8A1*, which is similar to the pattern of PI3K phosphorylation (Figure 5B).
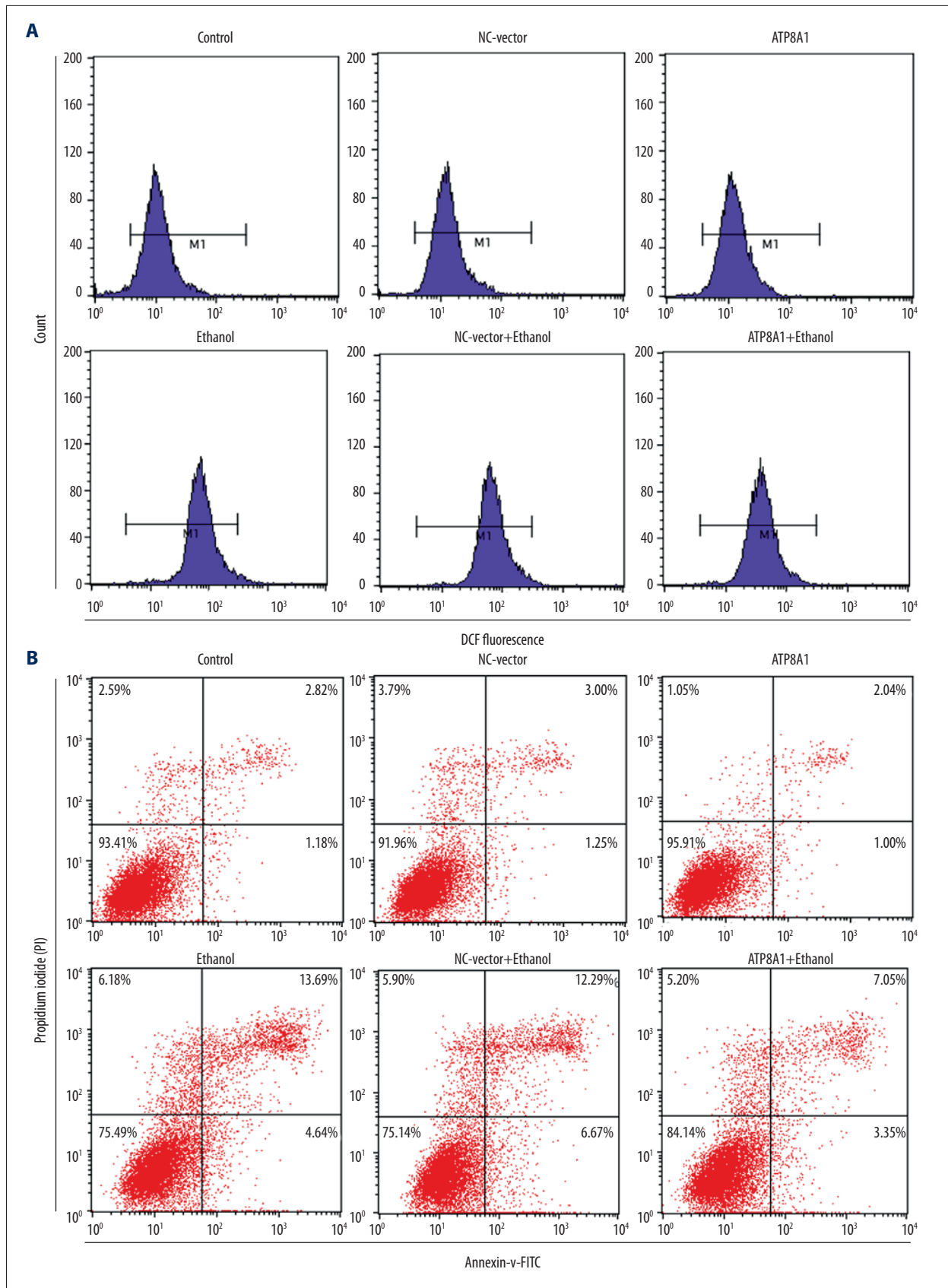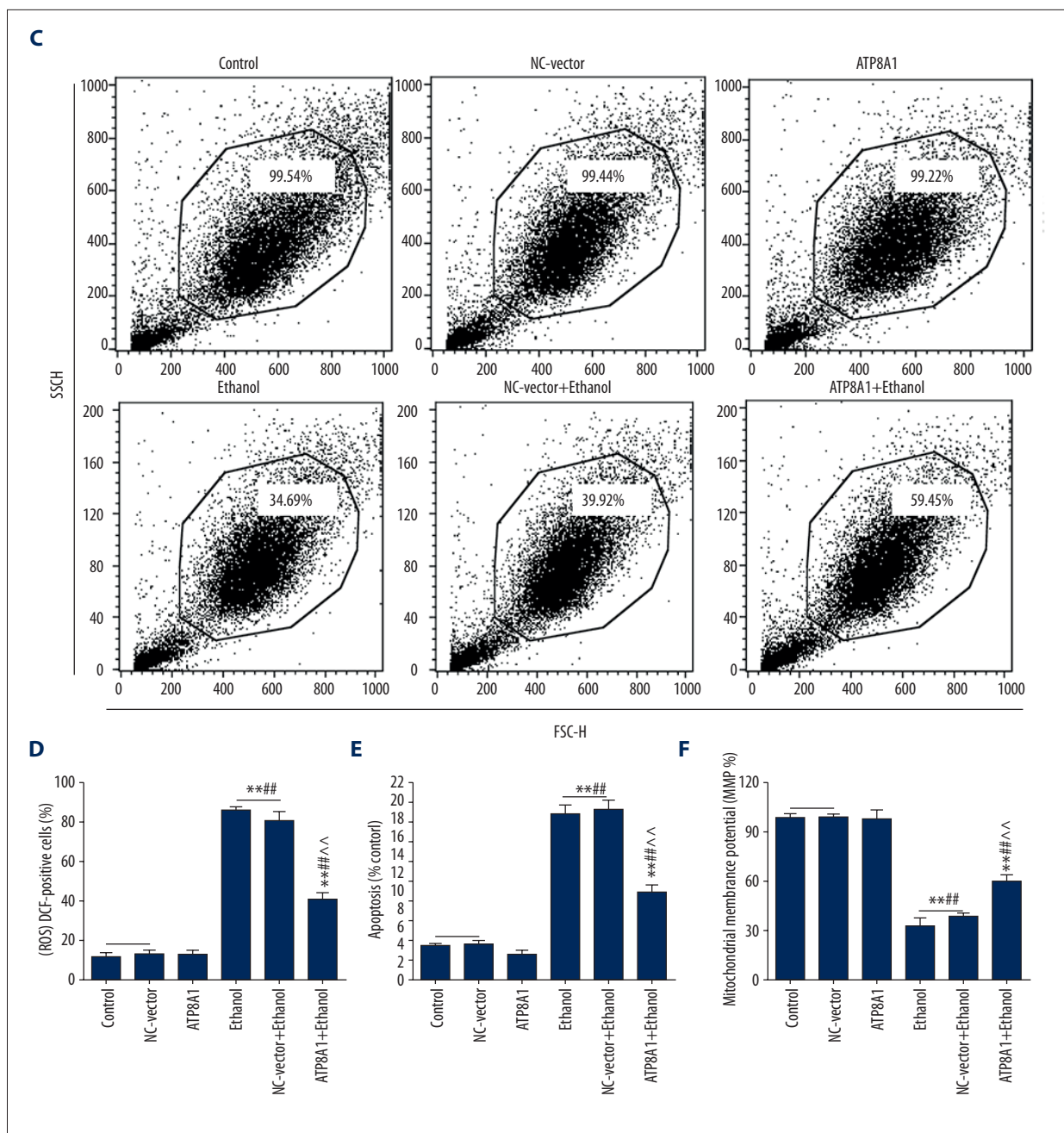
Figure 3. *ATP8A1* over-expression maintained survival of the ethanol-induced hepatocytes. (**A–C**) The levels of ROS, apoptotic cells, and MMP in each group were measured by flow cytometric analysis; (**D–F**) The statistical results of flow cytometric analysis are represented by the histogram. ** P<0.01 compared with control group; ## P<0.01 compared with *ATP8A1* group; ^^ P<0.01 compared with Ethanol group.

Discussion

The major result of this study is that over-expression of *ATP8A1* markedly improved the viability of HL-7702 cells exposed to ethanol (200 mM) for 4 h. Intracellular reactive oxygen species levels and ethanol-induced apoptosis of HL-7702 cells were significantly decreased and MMP was recovered by

ATP8A1 over-expression. The effects of the *ATP8A1* over-expression on ethanol-induced apoptosis appear to not only involve down-regulation of Fas/Fasl and Caspase-3 and the increase of Bcl-2/Bax ratio, but also enhanced phosphorylation of PI3K and Akt.
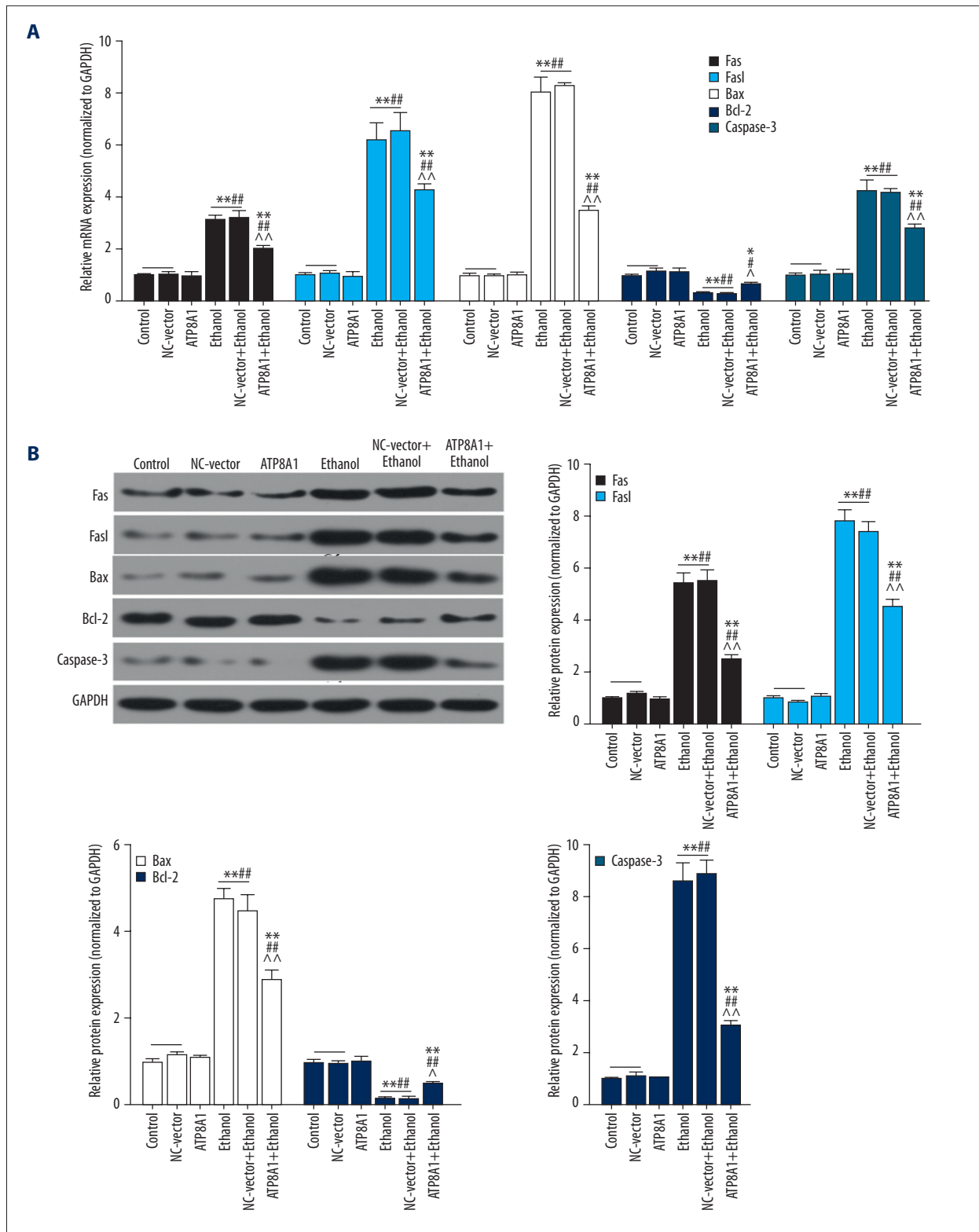
Figure 4. The protective effect of up-regulating *ATP8A1* was related to apoptosis-related proteins. (**A**) The mRNA levels of Fas, Fasl, Bax, Bcl-2, and caspase-3 in each group were measured by qRT-PCR. (**B**) These apoptosis-related proteins expression levels were measured by Western blot. * P<0.05, ** P<0.01 compared with control group; # P<0.05, ## P<0.01 compared with *ATP8A1* group; ^ P<0.05, ^^ P<0.01 compared with Ethanol group.
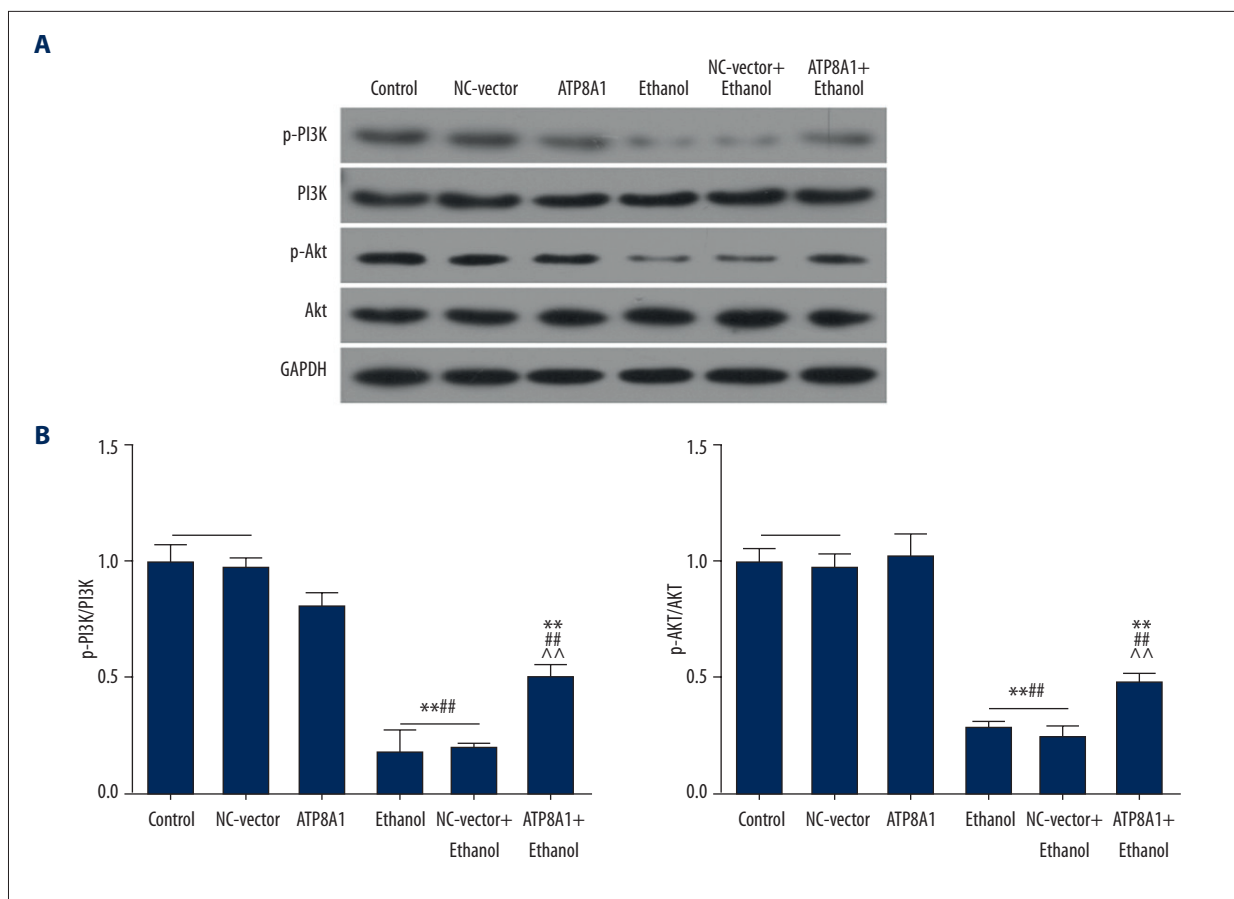
Figure 5. Over-expression of *ATP8A1* might initiate the PI3K/Akt signaling pathway suppresses apoptosis. (**A**) The Western blot result of p-PI3K, PI3K, p-Akt, and Akt. (**B**) Quantitative analysis of p-PI3K/PI3K and p-Akt/Akt ratio. ** P<0.01 compared with control group; ## P<0.01 compared with *ATP8A1* group; ^^ P<0.01 compared with Ethanol group.

Recently, it has been found that the inhibition of ethanol-induced hepatocytic injury is significant for the treatment of ALD, as well as other methods [26]. It has been documented that *ATP8A1* contributes to maintaining transmembrane lipid homeostasis in liver cells [25]. Therefore, we selected *ATP8A1 as the research object*. We explored the possible role of ATP8A1 in liver injury. In this study, the protective effect of over-expressed *ATP8A1* in response to superfluous ethanol was evaluated. We found that the cell viability and the expression of *ATP8A1* in hepatic HL-7702 cells showed a time- and dose-dependent decrease. We also showed that transfection of *ATP8A1* significantly improved the cell proliferation, indicating that up-regulated *ATP8A1* exerts protective effects on ethanol-induced stress.

Acute and constant exposure to alcohol can result in excessive ROS in the liver mitochondrion [27], which can release pro-apoptotic factors and generate free radicals. Overproduction of intracellular free radicals causes the collapse of MMP [26]. In the present study, we found that excessive ethanol exposure caused over-accumulation of ROS, increased the apoptosis

rate, and depressed MMP. Interestingly, over-expressed *ATP8A1* ameliorated accumulation of ROS, which can lead to mitochondrial damage in the liver [27]. Additionally, *ATP8A1* over-expression suppressed the apoptosis rate and restored the collapsed MMP, suggesting that over-expression of *ATP8A1* might preserve mitochondrial integrity.

We also detected some molecules involved in an apoptosis pathway. The pro- and anti-apoptosis members of the Bcl-2 family were reported to be pivotal regulators of cell apoptosis [28]. In the present study, we found that ethanol remarkably up-regulated *Bax* expression and down-regulated the level of *Bcl-2*, which might make collapsed MMP release Cytochrome C to activate Caspase-3. However, *ATP8A1* over-expression inhibited the expression of Bax and promoted the expression of Bcl-2, indicating that cell apoptosis might be inhibited by *ATP8A1* over-expression. Meanwhile, *ATP8A1* over-expression decreased Caspase-3 activity, which further demonstrated that over-expressed *ATP8A1* can attenuate ethanol-induced hepatocyte apoptosis by the mitochondrial pathway. Fas/FasL shows a promotive effect on cell apoptosis [29]. In the present research,

the levels of Fas and Fasl were distinctly inhibited by over-expressed *ATP8A1*, proving the over-expression of *ATP8A1* can inhibit apoptosis by suppressing Fas/Fasl. *ATP8A1* translocates PS to the cytosolic leaflet of membranes and PS is essential for endosomal membrane traffic [30]. A high PS concentration in the cytoplasm side is essential to the activation of Akt [30,31]. Thus, we speculated that PI3K/Akt might be involved in ethanol-induced apoptosis. Once the phosphorylation of PI3K was activated by receptor tyrosine kinase, the p-PI3K finally activated Akt [32]. Activated Akt can activate downstream transcription factors to regulate apoptosis-related gene expression [33,34]. To explore the anti-apoptotic role of the PI3K/Akt signaling pathway, the levels of PI3K, p-PI3K, Akt, and p-Akt were detected. We found that ethanol could sharply attenuate the phosphorylation of PI3K and Akt. However, the p-PI3K/PI3K and p-Akt/Akt ratio were positively correlated with *ATP8A1* over-expression, revealing that the PI3K/Akt signal pathway might participate in the suppression of ethanol-induced apoptosis in HL-7702 cells.

According to a previous study, the decreased expression of *ATP8A1* can lead to the exposure of phosphatidyl ethanolamine to the cell surface [35]. Membrane lipid bilayer asymmetry caused by the exposure of phosphatidyl ethanolamine is one of the signs of apoptosis [36,37]. Therefore, the possible mechanism for the protective effect of ATP8A1 may be by inhibition of PS externalization. The reduced PS externalization then led to the decrease of ROS accumulation, the reduction of apoptosis, and activation of the PI3K/Akt pathway, and the ethanol-induced injury was finally mitigated. This speculation needs further confirmation and we also do not rule out the possibility of other mechanisms.

Our study was limited in that our data were all from *in vitro* experiments. Deeper *in vivo* investigations or in other cell lines were worth undertaking to establish the role of ATP8A1 in hepatocytic injury.

Conclusions

Over-expression of *ATP8A1* may provide a critical protective effect on ethanol-induced hepatocytic injury mediated by both the mitochondrial pathway and the death receptor pathway. Furthermore, the PI3K/Akt signaling pathway might be associated with the inhibition of ethanol-induced apoptosis. These results show that *ATP8A1* may be a candidate gene in treatment of ethanol-induced liver toxicity.

Conflict of interest

None.

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