PTEN overexpression alters autophagy levels and slows sodium arsenite-induced hepatic stellate cell fibrosis

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Abstract: *Background*: Exposure to inorganic arsenic (iAs) remains a global public health problem. The liver is the main target organ of arsenic, leading to arsenic-induced liver fibrosis. Autophagy is involved. Phosphatase and tensin homology deleted on chromosome ten (PTEN) may participate in arsenic-induced liver fibrosis by regulating autophagy, but the exact mechanisms remain unclear. We established a mouse model of arsenic poisoning through the drinking water, and a fibrosis model using the huma stellate cell (HSC) line LX-2, which was exposed to $NaAsO_2$ for 24h. HE and Masson staining was adopted to observe the degree of liver fibrosis. The cells were transfected using PTEN overexpression plasmid. Western blot and qRT-PCR were used to determine the levels of protein/mRNA expression. The in vivo results were confirmed in HSCs exposed to $NaAsO_2$, with changes suggesting fibrosis as seen in mice. $NaAsO_2$ upregulated the expression of the autophagic markers microtubule-associated protein light chain A/B (LC3), recombinant human autophagy effector protein (Beclin-1), hairy and enhancer of split homolog-1 (HES1), but downregulated PTEN. α-smooth muscle actin (α-SMA) expression was significantly upregulated in all $NaAsO_2$ groups. PTEN overexpression altered $NaAsO_2$-induced autophagy which LC3, Beclin-1 were downregulated. Notch1, HES1, α-SMA, and collagenIexpression were all downregulated in the $NaAsO_2$ groups. In conclusion, PTEN overexpression might decrease autophagy and inhibit fibrosis progression caused by this toxin. The NOTCH1/HES1 pathway is likely to be involved in this process. Most previous studies did not investigate PTEN and arsenic-induced liver fibrosis specifically. The present study highlights the importance of targeting PTEN for the management of arsenic exposure.

Keywords: inorganic arsenic; liver fibrosis; autophagy; Notch1/Hes1/PTEN

1. Introduction

Arsenic contamination is a serious environmental and geochemical issue endangering human health [1]. Exposure to inorganic arsenic compounds cause not only skin lesions, kidney damage, and peripheral nervous system injury but also liver injury, liver fibrosis, cirrhosis, and liver cancer [2]. Arsenite, which is the trivalent form of arsenic [As (III)]. The liver is the most important target organ of arsenic metabolism [3]. Epidemiological studies linked chronic iAs exposure to an increased risk of liver disease, fibrosis, cirrhosis, and liver carcinogenesis [4-6]. The mechanisms underlying arsenic-

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induced liver fibrosis are multifaceted, including oxidative stress, inflammatory response, apoptosis, necrosis, and methylation [7-9]. In recent years, studies demonstrates that autophagy plays a critical role inarsenic-related carcinogenic mechanisms.

Autophagy is essentially a protein degradation system of the cell's own lysosomes which plays a vital role in removing misfolded proteins and damaged organelles to maintain cellular homeostasis [10-11]. Significantly, arsenic exposure affects autophagy in a dose-dependent manner. Multiple autophagy-related genes (ATGs) and signaling pathways co-regulate autophagy processes and biological functions [12]. As of September 2022, a total of 232 autophagy-related genes were obtained from the HADb Human Autophagy Database, with the key signaling molecules being Beclin-1, VMP1, Atg5-Atg12, Atg4, and LC3, and phosphatidylinositol 3 kinase/protein serine-threonine kinase (PI3K-Akt) pathways also play a role in autophagy.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is one of the tumor suppressor genes which exerts vital effects on cell growth, proliferation, migration, signal transmission, invasion and apoptosis [13]. Existing studies also demonstrated that abnormal PTEN expression is associated with the non-neoplastic disease. Activation of pro-fibrotic signaling pathways through PTEN may lead to fibrosis in the liver, lung, and kidney tissues [14-15]. Some studies found that PTEN may be involved in the regulation of fibrosis in various organs through autophagy. Xun Lai, et al. [16] found that reduced autophagy enhances PTEN expression, while increased autophagy reduces PTEN expression, suggesting that the degradation of PTEN by autophagy via the interaction of PTEN-p62 acts as a novel way of tumorigenesis of HULC. Qing Yin et al [17] in diabetic nephropathy found that miR-155-5p promoted autophagy and attenuated interstitial fibrosis by targeting PTEN. In addition, PTEN is involved in the regulation of multiple pathways. For example, PTEN negatively regulates phosphatidylinositol 3-kinase (PI3K) involved in the regulation of the PI3K-Akt-mTOR pathway [18]. Hairy and enhancer of split 1(Hes1) can affect the transcription level of PTEN mRNA and downregulates the expression of PTEN protein, thereby affecting the Notch1-HES1-PTEN pathway [19]. However, the exact mechanism underlying interaction of PTEN, autophagy, and arsenic-induced liver fibrosis is not fully understood.

This study assessed mouse response to iAs^{3+}, with histological changes suggesting liver fibrosis and the changes in liver protein expression. The results were confirmed in vitro, with HSC responses to different iAs^{3+} doses to characterize the full range of chronic arsenic exposure levels. Then, we investigated overexpression of PTEN during iAs^{3+}-induced fibrosis and exploring the effect of NOTCH1/HES1/PTEN signaling pathway on autophagy. Our data could be used to explore changes in autophagy related-proteins during iAs-induced fibrosis in human HSCs. These results could lay a foundation and provide new insights for further liver fibrosis investigations. These results could be important in areas where arsenic exposure through drinking water and food is significant.

2. Materials and methods

2.1. Reagents and antibodies

The following reagents were used in this study. Sodium arsenite (iAs^{3+}, analytical grade) (No.3 Chemical Reagent Factory, Beijing, China). PTEN overexpression plasmid and control vector plasmid (Jikai Gene Biology Co. Shanghai, China). Fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) (American Hyclone Co). Bicinchoninic acid assay (BCA) protein quantification kit (Thermo Scientific, USA). Electrotransfer and electrophoresis solution (Solarbio Co. Beijing, China). Primer Script™ RT Master Mix kit and QuantiNova SYBR Green PCR Kit (Takara, Japan). Chemiluminescence western blot detection kit (Thermo Scientific). Anti-GAPDH, anti-LC3, anti-Beclin 1, anti-PTEN, anti-HES1, anti-NOTCH1, anti-CollagenI, and anti-α-SMA (Abcam Co, UK). Masson staining kit (Solarbio, Beijing, China)

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2.2. Animals and treatments

Twenty-four healthy sterile C57BL/6 male mice (weighing 20–22 g) were purchased from the Animal Experiment Center of Xinjiang Medical University (Xinjiang, China) and maintained on 24-h adaptive feeding for 1 week at a temperature of 25 °C ± 2°C and a relative humidity of 45% ± 5%. The mice were divided into four groups of six each using a random-number table. The LD50 of sodium arsenite in the mice was 16.2 mg/kg, as determined by the Horn method. We administered 1/15 of the LD50 as a dose in the high-dose group, and the differences among the dose groups were twofold: high-dose group (H), $NaAsO_2$ 1.08 mg/kg; medium-dose group (M), $NaAsO_2$ 0.54 mg/kg; and low-dose group (L), $NaAsO_2$ 0.27 mg/kg; the animals in the normal control group (C) drank deionized water solution for 24 weeks. During the exposure period, the water consumption of the animals was recorded daily, and they were weighed three times a week. The mice were fed in groups for 4, 8, 16, and 24 weeks before they were killed. All animal experiments were granted ethics committee approval and were conducted in accordance with the regulations of the Ethics Committee of Xinjiang Medical College and the Guidelines for the Care and Use of Laboratory Animals of the Chinese National Institute of Health (Ethical approval number: IACUC-20210309-08).

2.3. HE staining for detecting the pathological changes of mouse liver, Masson staining for detecting the liver fibrosis

Fresh liver tissues isolated from C57BL/6 mice were fixed in 10% formalin, dehydrated, embedded in paraffin and sliced. Then, liver sections (4-5μm) were dewaxing and rehydrated, stained with hematoxylin for 10 min and eosin for 3 min, dehydrated with a graded alcohol series, and re-immersed in xylene. According to the standard procedure of Masson staining kit, tissue staining was performed. After sealed with gum, the sections were observed and photographed using an optical microscope.

2.4. Cell culture

LX-2 cells (Punosai Life Technology Co., Wuhan, China) were cultured at 37 °C in an atmosphere containing 5% CO_2 and 95% air at 100% humidity in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% heat-inactivated FBS, 100 mg mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin. When cells reached 80%–90% confluence, they were trypsinized in 0.25% trypsin and passaged into 6-well plates for iAs trioxide exposure.

2.5. Cell transfection

LX-2 cells were cultured in cell medium prepared with 90% DMEM medium /10% FBS/1% dual antibody. Transfection was carried out using a six-well plate until the growth of the cells reached 70%–80%. A volume of 500 μL of DMEM culture medium without antibiotics and serum was transferred into a 1.5-mL sterile centrifuge tube. Then, we added 5 μg plasmid DNA and 8 μL of PEI, followed by mixing and shaking. Further, the mixture was left to stand for 10 min at room temperature, followed by the addition of 1.5 mL of DMEM medium without antibody and serum to the well plate. When the reaction time was completed, the transfection reagent and a DNA mixture were added to the whole well in a uniform drop at 500 uL per well, and were then gently mixed. To achieve the highest transfection efficiency, cells had to be replaced with fresh complete culture medium after 4–6 hours of post-transfection incubation.

2.6. Quantitative real-time PCR (qRT-PCR analysis)

Total RNA was isolated from LX-2 cells and liver tissues using the RNAiso kit (TianGen, China) following the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using the Primer Script™ RT Master Mix kit (Takara, Japan). Then, qRT-PCR was performed using the SYBR ® Premix Ex Taq™II (2×) mix (Takara, Japan) using a CFX 96-type RT fluorescence quantitative PCR instrument (Bio-Rad, USA). GAPDH was used as a reference gene. Cycle thresholds were determined

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for each sample and each gene amplification. Based on the 2-ΔΔCt method, relative target gene expression was calculated. Primer sequences are shown in Table 1. The PCR procedure is shown in Table 2

2.7. Western blot

Total protein was extracted from cells and liver tissues using ice-cold RIPA lysis buffer (plus phenylmethylsulfonyl fluoride). Next, the total protein concentrations were determined by a bicinchoninic acid assay (BCA) protein quantification kit (Thermo Scientific). Protein samples (30 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were further transferred to 0.22-μm polyvinylidene fluoride membranes (Sangon Biotech, Shanghai, China). After blocking in 5% skimmed milk for 1 h at 37 °C, the blots were incubated overnight at 4 °C with the following primary antibodies: LC3 antibody (1:1000) (Proteintech, 14600-1-AP), Beclin-1 (dilution 1:2000) (Bioss, bs-1353R), α-SMA antibody (1:1000), CollagenI (1:1000) (Abcam, ab124964), HES1 antibody (1:1000), PTEN (1:1000) (CST, 11988S), NOTCH1 (1:500) (Abcam, ab52627), GAPDH (1:10,000), and β-actin (1:10,000). The blots were rinsed three times with Tween (Sangon Biotech) plus PBS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sangon Biotech) (1:8000) for 1h. Immunoreactivity was visualized using an alkaline phosphatase color development kit (Sangon Biotech). Image analysis software ImageJ v1.51 (National Institutes of Health, USA) was employed to calculate relative protein expression.

2.8. Statistical analysis

SPSS 25.0 software was utilized for statistical analysis. The quantitative data that were normally distributed were expressed as mean ± standard deviation (x ± s). One-way ANOVA was applied for comparison between groups, and LSD was used for further pairwise comparisons. The median and interquartile spacing [M (QR)] were implemented to describe the non-normal distribution, and the Kruskal Wallis H test was used for comparison between groups.

3. Results

3.1. Autophagy and fibrosis were established in the liver tissues of mice exposed to different concentrations of sodium arsenite

HE staining showed that the hepatocytes in the control group were uniform in size and morphology, with hepatocytes centered on the central vein and extending radially to the periphery. The hepatic cords were neatly arranged, with no degeneration, necrosis, inflammatory cell infiltration, and fibrous tissue proliferation in the intact liver lobules, and no hepatocyte degeneration. At 4 weeks of sodium arsenite exposure, aqueous degeneration and steatosis were observed in the hepatocytes in the low-dose group, with occasional inflammatory cell infiltration, but the structure of the liver lobules was still intact. In the high-dose group, hepatocyte steatosis was observed in the liver tissue, with obvious interspersed distribution of lipid droplets, scattered punctate and sheet necrosis, and local inflammatory cell infiltration in the visual field. In the high-dose group, the pathological changes were significantly aggravated at the 24th of the experiment. Pseudo-lobules were formed in the hepatic portal area segmented by the fibrous septum, with obvious punctal necrosis and abnormal proliferation of the hepatocytes, as well as edema and inflammatory cell infiltration. (Figure 1).

Masson staining verifies the establishment of a model of liver tissue fibrosis in mice induced by sodium arsenite: As shown in the figure, the normal group only had a small amount of blue-stained collagen fibers distributed in the portal area, without obvious collagen hyperplasia. For short-term (24 hours) arsenic exposure, a small amount of vacuoles formed by necrotic liver cells could be seen in the low-dose sodium arsenite group. The blue-stained fibrous tissue extended outward from the portal area or the central vein; in the high-dose sodium arsenite group, the wall of the central vein

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was thickened, and a large number of blue-stained collagen fibers appeared and surrounded the portal area (Figure 2).

Transmission electron microscope analysis showed that the hepatocytes in the control group were normal and irregular. The nucleus was round or oval with clear nuclear envelope and nucleolus. Organelles, such as the mitochondria, the endoplasmic reticulum, and the ribosomes were with normal morphology and scattered in the cytoplasm. Compared with the control group, the "false dirichlet gap" can be seen between the LX-2 cells in the high-dose sodium arsenite exposure group, and the gap is widened. Compared with the control group, the number of autophagosomes in the high-dose sodium arsenite group was significantly increased after 48 hours of treatment(Figure 3).

3.2. PTEN is involved in the autophagy and fibrosis in the mouse liver tissue caused by sodium arsenite treatment

In order to determine whether PTEN involved in iAs^{3+} induced autophagy and fibrosis in the mouse liver tissue，we examined LC3, Beclin-1, PTEN, α-SMA，HES1 mRNA expression levels in LX-2 cells after exposure to $NaAsO_2$. As shown above, $NaAsO_2$ exposure in mice increased liver LC3, α-SMA, and Beclin-1 mRNA expressed, decreased PTEN expression.

When we compared the normal and all iAs^{3+} treatment groups, LC3 mRNA expression was significantly higher than that of the control at 24 weeks ($P < 0.05$); Beclin1 expression was significantly higher in the L group than in the control group ($P < 0.05$). Similar to Beclin-1, α-SMA mRNA expression levels increased in L group. In all treatment groups, PTEN mRNA expression levels tended to initially increase, then decrease with increasing As^{3+} doses (low iAs^{3+}: 1.79±0.17; medium iAs^{3+}: 0.84±0.10; $P < 0.001$) (Table 3).

At 24 weeks, the LC3, Beclin-1 protein expression level varied among the treatment groups ($P < 0.05$) (Table 4, Fig. 1). LC3 protein expression in the high-concentration group was lower than that in the control group ($P < 0.05$). Compared with the control group, the expression level of Beclin-1 protein in the L group and H group was significantly increased, and the difference was statistically significant ($P < 0.05$). PTEN protein expression in the H group was lower than that in the control group at 24 weeks ($P < 0.05$). HES1 protein expression in the L group was higher than that in the control group ($P < 0.05$), Comparison among each dose group showed that the α-SMA protein expression level increased with prolonged exposure time, and the difference compared with the control group was statistically significant at 24 weeks ($P< 0.05$). Under the different dose at same time points, PTEN mRNA and protein expression levels were negatively correlated with dose (Table 4, Figure 4).

3.3. PTEN overexpression can reduce autophagy induced by sodium arsenite

To confirm that PTEN and identify a relationship between autophagy, we investigated the overexpression of PTEN in LX-2 cells after treatments. Compared with the blank and High-arsenic + blank plasmid group, PTEN mRNA levels were decreased. In the PTEN overexpression group, compared with High-arsenic + blank plasmid group, PTEN mRNA levels were increased. ($P < 0.05$) (Table 5).

The effects of iAs^{3+} on LC3, Beclin-1 protein expression in LX-2 cells after plasmid infection were analyzed. When we compared the blank and High-arsenic + blank plasmid group, LC3 and Beclin-1 protein expression levels were significantly increased. In the PTEN overexpression group, compared with high-arsenic + blank plasmid group, LC3 and Beclin-1 protein expression levels were significantly decreased. ($P < 0.05$) (Table 5, Figure 5).

The protein expression level of Notch1 in the high-concentration sodium arsenite group + blank plasmid group was significantly increased ($P<0.05$) in comparison with that in the control group. Furthermore, the expression level of Notch1 protein in the PTEN overexpression group was significantly downregulated ($P<0.05$) as compared with that in the high-concentration sodium arsenite group + blank plasmid group. The expression level of HES1 protein in the high-concentration sodium arsenite group + blank plasmid group was significantly higher ($P < 0.05$) than that in the control group. Compared with high-concentration sodium arsenite group + blank plasmid group, the

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expression level of HES1 protein in the PTEN overexpression group was significantly downregulated (P<0.05). (Figure 5 and Table 5).

3.4. PTEN overexpression can reduce fibrosis induced by sodium arsenite

After the sodium arsenite exposure, the expression level of α-SMA protein in the high-concentration sodium arsenite group + blank plasmid group was significantly higher (P<0.05) than that in the control group. Compared with the high-concentration sodium arsenite group + blank plasmid group, the expression level of α-SMA protein in the PTEN overexpression group was significantly downregulated (P<0.05). The protein expression level of collagen I in the high-concentration sodium arsenite group + blank plasmid group was significantly higher (P<0.05) than that in the control group. As visible in Figure 6 and Table 6, the protein expression level of collagen I in the PTEN overexpression group was significantly downregulated (P < 0.05) compared with that in the high-concentration sodium arsenite group + blank plasmid group. (Figure 6 and Table 6).

4. Discussion

This study aimed to explore the effects of PTEN with $NaAsO_2$ on liver fibrosis and autophagy. We preliminarily found that the abnormal expression of PTEN was correlated with autophagy in the process of liver fibrosis induced by sodium arsenite in mice. The overexpression of PTEN reduceed the occurrence of autophagy, and slowed down the liver fibrosis caused by sodium arsenite. NOTCH1/HES1/PTEN might involved in the regulation and affected the occurrence of autophagy and fibrosis. This study aims to identify new effective targets for clinical prevention of arsenic poisoning and alleviation of side effects of arsenic in combination therapy of tumor chemotherapy.

Epidemiological investigations reveal links between arsenicosis and various malignant tumors such as lung cancer, skin cancer, liver cancer, gallbladder and gastrointestinal tumors, lymphoma [20-22]. Liver inflammation due to inorganic arsenic can result in liver fibrosis, leading to cirrhosis and carcinogenesis [23]. Hepatic stellate cells (HSCs) are the key effector cells mediating the occurrence and development of liver fibrosis. HSCs activation mainly characterized by fibroblast proliferation, excessive collagen synthesis, and extracellular matrix deposition (including Collagen I and Collagen III) and overexpression of α-SMA [24]. We established an arsenic poisoning model in mice through the drinking water and a fibrosis model in the HSC line LX-2 after exposure to $NaAsO_2$. HE and Masson staining also indicated liver fibrosis in modle. Both of them, arseninic exposure led to significant liver fibrosis, with an upregulated expression of the Collagen I and α-SMA.

In recent years, autophagy has been associated with key roles in several human diseases. Autophagy maintains cellular homeostasis by regulating various physiological processes, including cytokine formation, pathogen clearance, antigen presentation, inflammatory responses, and innate and adaptive immune responses [25]. Autophagy activity and biological functions are regulated by several ATGs and associated proteins, including LC3, SQSTM-1/P62, Beclin-1, ATG4, ATG5 and ATG8. LC3 is involved in the formation of autophagosomes and dissociates from lysosomal structures to digest damaged materials. Beclin1 is a key regulator of autophagy and an inactive or dysfunctional Beclin1 leads suppressed autophagic process [26]. In our study, we observed autophagosomes in a mouse fibrosis model by transmission electron microscopy and found that with the increase of infection time and dose, the interstitial space of the mouse hepatocytes widened significantly, the number of lipid droplets and autophagic vesicles with double-layered membranes were increased. Based on our data, in both animal and cellular experiments, both at the genetic level and at the translational level, it was shown that LC3, Beclin1 positively correlated with autophagic activity at the transcriptional and translational levels with increasing dose and duration of sodium iAs^{3+} exposure.

As an important regulator of liver fibrosis, PTEN participates extensively in the process of liver fibrosis by regulating the activities of hepatocytes, hepatic stellate cells, and macrophages. Research indicates that low expression or loss of PTEN was previously observed in the fibrotic liver tissues of rats treated with activated hematopoietic stem cells and CCl4 [27]. Targeting PTEN alleviated liver

fibrosis, while saponin A promoted the expression of PTEN by binding with DNMT1, thereby reducing liver fibrosis [28]. Bueno et al. [29] also found that after knocking out the PTEN gene in alveolar epithelial cells, the degree of pulmonary fibrosis was aggravated Additionally, PTEN is associated with autophagy: Research indicates that SLC9A3R1 increases the expression of PTEN via interaction with PTEN, PTEN increases autophagy, whereas the loss of PTEN results in the inhibition of autophagy [30];Inhibition of autophagy increases PTEN, whereas induction of autophagy decreases PTEN [31] .In this study, PTEN gene and protein levels were downregulated in each iAs^{3+} dose group. However, after the PTEN overexpression plasmid was transferred into human hepatic stellate cells, the level of liver fibrosis and autophagy was downregulated. The results of our study are consistent with those of the above studies. Therefore, the PTEN gene may be an important target to alleviate the progression of fibrosis, and interfering with the expression of PTEN can help reduce fibrosis. The main difference from these earlier studies is that we found that PTEN expression was associated with autophagy, and when PTEN overexpression enhanced the occurrence of autophagy, the increased level of autophagy further contributed to the remission of fibrotic lesions. Therefore, overexpression of PTEN can inhibit autophagy during autophagosome formation and maturation, but autophagy does not inhibit the response to ATG binding. Therefore, the dual role of PTEN gene in enhancing the degree of autophagy and alleviating liver fibrosis deserves more scientific attention.

We also found that Notch1/HES1 may be involved in sodium arsenite exposure-induced liver fibrosis and autophagy, and PTEN can regulate Notch1/HES1 to affect autophagy and fibrosis. Liu et al. [32] found that Notch1 regulated the expression of PTEN, inhibited autophagy through interaction with Hes1, and aggravated renal tubulointerstitial fibrosis in diabetic nephropathy. Our data showed that compared with the high-concentration sodium arsenite group + blank plasmid group, the protein expression levels of Notch1 and HES1 in the PTEN overexpression group were significantly down-regulated. In addition, we used Illumina Human Methylation 850K genome-wide methylation microarray for detection of fibrosis in the stained hepatic stellate cells, in which we utilized high concentrations of sodium arsenite, as well as in the autophagy models group. Using these analyses, we aimed to detect differentially methylated genes at the epigenetic level and further investigate the mechanism of PTEN involvement in the regulation of arsenic-induced liver fibrosis and autophagy. The present study highlights the importance of targeting PTEN for sodium arsenite-induced liver fibrosis and autophagy. Further research on the mechanistic issues of HSC management of arsenic exposure is warranted.

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