Research Progress and Ideas on the Anti-liver Fibrosis Effect of Ethnic Medicine Plumbagin Based on microRNAs/TLR4/NF-κB and NLRP3 Inflammasome Activation

Mingzhe LU[1△], Qianyu LIU[1△], Yue PENG[2*], Jiang LIN[2*], Weiqian GUO[1], Miao YANG[1]

1. School of Graduate, Guangxi University of Chinese Medicine, Nanning 530200, China; 2. School of Basic Medicine, Guangxi University of Chinese Medicine, Nanning 530200, China

Abstract The core of hepatic fibrosis is the activation of hepatic stellate cells. Through the lipopolysaccharide/TLR4/MyD88/NF-κB signal transduction pathway, the inflammatory response in the liver is directly enhanced, and then returns to promote the activation of hepatic stellate cells. And TLR4/MyD88/NF-κB signaling pathway can directly regulate the activation of NLRP3 inflammasome and is an important pathway for activating hepatic stellate cells. TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway is regulated by upstream microRNAs. These miRNAs can significantly regulate the inflammatory response of the liver and the activation behavior of hepatic stellate cells, affecting the formation of liver fibrosis. Previous studies have found that the active ingredient of Guangxi specialty ethnic medicine, plumbagin, has a definite anti liver fibrosis effect, but its mechanism of action is not clear. This paper provides a review of the research progress on the above issues, and further research ideas have been derived from this, stating that "the anti liver fibrosis effect of plumbagin is achieved by regulating miRNA/TLR4/MyD88/NF-κB inflammatory pathway and activating downstream NLRP3 inflammasome".

Key words Plumbagin, Anti-liver fibrosis, Hepatic stellate cells, TLR4, microRNAs, NLRP3 inflammasome

1 Introduction

The liver is the most important metabolic organ in the human body, and liver fibrosis refers to a chronic liver disease in which the liver is affected by various physical and chemical factors, causing steatosis, inflammation, and necrosis of liver cells, as well as excessive proliferation and abnormal deposition of extracellular matrix (ECM) components in the necrotic area. Hepatic fibrosis is a typical pathological feature of chronic liver disease and has become a necessary pathological process for various chronic liver diseases to develop into cirrhosis[1]. Among them, 25% to 40% can also develop into the final stage of fibrosis, cirrhosis. Its causes are numerous and complex, and almost any factor that can cause chronic liver damage can lead to liver fibrosis, such as chronic hepatitis B, chronic hepatitis C, fatty hepatitis (including alcoholic or non alcoholic), autoimmune liver disease, schistosomiasis liver disease, drug-induced liver disease, and some congenital metabolic diseases. These pathogenic factors can lead to the formation of liver fibrosis and affect the progression of liver fibrosis by acting on liver cells such as hepatic stellate cells (HSCs), hepatic sinusoidal endothelial cells (HSECs), and Kupffer cells (KCs)[2].

According to reports, there are over 30 million chronic liver disease patients in China, with 2.1% developing cirrhosis every year. After 5 years of liver cirrhosis, the probability of cancer transformation reaches 17%, and it exceeds 40% after 10 years. The wide range of its causes and high mortality rate have seriously affected the quality of life and medical expenses of the people[3]. Researchers have realized that liver fibrosis is actually a reversible pathological phenomenon in the early and middle stages. As early as the 1970s, Professor Hans Popper, the founder of contemporary hepatology, pointed out: "whoever can prevent or delay liver fibrosis will be able to treat most liver diseases"[4]. Professor Friedman[5] proposed at the recent Conference on Liver Fibrosis held by the European Society of Hepatology that treating hepatitis B and C can resist fibrosis, proving the reversibility of liver fibrosis. Compared with pulmonary fibrosis, cardiac fibrosis and scleroderma, hepatic fibrosis is easier to reverse. Therefore, it plays a very important role in improving the quality of life and prognosis of patients by preventing or early intervening the pathological process of liver fibrosis, delaying its development or even reversing it. But no satisfactory means have been found to solve this major global health problem to date.

2 HSCs and liver fibrosis

HSCs account for approximately 5% - 15% of the total number of cells in normal liver tissue. The research results indicate that although various cells in the liver can synthesize ECM, HSCs are

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△These authors contributed equally to this work.
*Corresponding author. E-mail: 834908495@qq.com; 1713552545@qq.com

the main source of ECM in chronic liver disease[6]. Similar evidence has been obtained in liver fibrosis models induced by carbon tetrachloride (CCl4), iron overload, and biliary obstruction. It has been found that activated HSCs significantly increase in liver injury lesions and their surrounding areas, directly leading the occurrence and development process of various etiologies of liver fibrosis[7]. The proliferation and activation of HSCs can synthesize and secrete a large amount of ECM, so the activation of HSCs is the central link in the occurrence of liver fibrosis. When the liver is constantly stimulated by chronic injury, HSCs will transform from their usual relatively static phenotype to an activated phenotype and undergo some functional changes[8]: (i) increased cell proliferation rate; (ii) reduced cell apoptosis; (iii) mobility; (iv) contractility; (v) chemical chemotaxis; (vi) changes in the type and quantity of synthesized ECM; (vii) synthesizing a series of fibrogenic cytokines. Among them, leptin is one of the most important mitosis factors, which can significantly promote the division, proliferation and activation of HSCs.

Currently, multiple signaling factors and pathways have been found to activate HSCs[9], such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), leptin, vascular endothelial growth factor (VEGF); mitogen activated protein kinase MAPK pathway, amino terminal kinase/signaling and transcriptional activator (JNK/STAT), TGF-β1/Smads, NF-κB signaling pathway. The latest research[10] shows that the NLRP3 inflammasome signaling pathway is an important emerging signaling pathway, which is a new research hotspot; the related proteins secreted by the NLRP3 inflammasome activation signaling pathway significantly affect the proliferation, contraction, and other functions of HSCs. The activated HSCs can trigger and promote the occurrence and development of liver fibrosis. Therefore, new research on the inhibitory targets of NLRP3 inflammasome activation related signaling pathways in HSCs will have important value for the effective prevention and treatment of liver fibrosis.

3 NLRP3 inflammasome and liver fibrosis

NLRP3 inflammasome is currently the most widely and comprehensively studied inflammasome. NLRP3 inflammasome is an intracellular multi-protein complex mainly composed of the Nod-like receptor family pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC), and the effector molecule caspase-1 precursor (procaspase-1)[11]. NLRP3 inflammasome has cytoplasmic pattern recognition receptor that recognizes a series of signal changes from pathogen-associated molecular patterns (PAMPs) to dangerous signals[12]. The activation of NLRP3 inflammasome will trigger inflammatory responses, regulating the development of diseases and cell fate. If NLRP3 inflammasome is expressed in liver tissue cells, it can trigger a series of reactions such as HSCs activation, liver cell apoptosis, and pyroptosis, and trigger inflammatory response of liver. Therefore, this inflammasome plays an important role in the occurrence and development of liver fibrosis[13]. HSC is one of the main target cells of the NLRP3 inflammasome pathway[14]. Multiple studies[15-16] have shown that downstream proteins of the NLRP3 inflammasome pathway are expressed in HSC, significantly regulating the function of the cell. HSC directly promotes the formation of liver fibrosis through the NLRP3 inflammasome signaling pathway. Research[17] has shown that NLRP3 inflammasome promotes the formation of liver fibrosis, which is related to up regulation of TGF-β and activation of HSC, and the specific mechanism is achieved through the NLRP3 inflammasome/caspase-1 signaling pathway. The specific approach is as follows[18]: the activated NLRP3 inflammasome undergoes component assembly, and NLRP3 binds to the adaptor protein ASC, which then causes procaspase-1 to self catalyze and activate into caspase-1. The activated caspase-1 accelerates the process of liver fibrosis mainly through two signaling pathways: (i) caspase-1 promotes the maturation of precursors for pro-inflammatory factor interleukins IL-1β and IL-18, and forms IL-1β and IL-18, and leads to the occurrence and development of inflammatory reactions. Although inflammation plays a crucial role in the protective immunity of the body, fibrosis can also occur in the liver under sustained inflammatory pathological stimulation[19]. (ii) Complex cell death program——pyroptosis can be initiated. Pyroptosis is a newly discovered cell necrotic death mode characterized by cell swelling, rapid lysis, plasma membrane permeability, and the release of pro-inflammatory cytokines and cell contents. The entire process from initiation to completion is more rapid than apoptosis.

4 MicroRNAs/TLR4/NF-κB signaling pathway regulating the activation of downstream NLRP3 inflammasome

4.1 Relationship between upstream TLR4/NF-κB pathway and activation of downstream NLRP3 inflammasome Toll-like receptor 4 (TLR4) is a member of the toll-like receptor family. It is a transmembrane protein of mammalian cells, and is mainly related to the recognition of bacterial and viral components (PAMPs and DAMPs). It activates NF-κB by binding to endotoxin lipopolysaccharide (LPS) of gram-negative bacteria[20]. TLR4/NF-κB pathway regulates the activation of NLRP3 inflammasome, triggers liver inflammatory response, and leads to the formation of liver fibrosis. The main mechanisms of TLR4/NF-κB pathway regulating activation of NLRP3 inflammasome are as follows[21]: TLR4 activation, the phosphorylation of interleukin-1 (IL-1) receptor related kinase IRAK-1 is induced by myeloid differentiation factor 88 (MyD88). The phosphorylated IRAK-1 recruits and activates tumor necrosis factor (TNF) receptor 6 (TRAF6), and protein kinase C (PKC), extracellular signal regulated kinase-1/2 (ERK-1/2), and transforming growth factor (TGF-β) activate kinase 1 (TAK-1) and others, and signal factors are gradually activated. Phosphorylationp of p38 MAPK, c-Jun amino terminal kinase (JNK), and I-kappa kinase (IκK) activates IκK and makes p65 NF-κB nuclear translocation and activation, and transcriptional activation of TLR pathway related genes[22]. Activated NF-κB

regulates downstream NLRP3 inflammasome activation through two aspects[23]: (i) regulating the transcription of NLRP3, generating NLRP3 mRNA. NLRP3 mRNA was transcribed into NLRP3 protein, making the activation of NLRP3 inflammasome and the occurrence of inflammatory reactions are provided with material guarantees. (ii) Regulating the transcription of pro-caspase-11, caspase-11 activates NLRP3 inflammasome. NF-κB can also directly regulate the transcription of pro-inflammatory factors pro-IL-1β and pro-IL-18, thereby promoting the occurrence of inflammatory reactions.

4.2 Upstream microRNAs regulating downstream TLR4/NF-κB pathway In recent years, the prevention and treatment research of liver fibrosis has gone deep into the level of microRNAs (miRNAs). miRNA was first discovered by researchers from nematodes, fruit flies, and humans in 2001. This discovery was listed as the first of top ten technological breakthroughs by *Science* (American Journal) in 2001. miRNA is a small non coding RNA molecule that binds to the 3-'untranslated regions (3'-UTRs) of the target mRNA to regulate gene expression. The human genome encodes more than 1 000 miRNAs, and mRNAs in more than 50% of cells are regulated by miRNA. miRNAs play an important regulatory role in many cellular functions, including cell proliferation, differentiation, apoptosis, and stress response[24]. miRNAs play an important role in the occurrence and progression of various liver diseases such as acute liver injury and chronic liver fibrosis by regulating gene expression[25]. Abnormal expression of miRNA-34, miRNA-122, and others in HSC regulates the activation and proliferation of HSC[26]. The expression of miRNA-29b can be significantly decreased in HSC during fibrosis, while miRNA-29b exhibits specific high expression. Research[27] has found that miRNA-29 expression decreases in the liver of liver fibrosis mouse models and chronic viral hepatitis C fibrosis patients, which can enhance the collagen synthesis function of HSC; up regulation of miRNA-29 can significantly reduce collagen secretion in HSC, inhibit proliferation, and hinder the progression of liver fibrosis. The mechanism of miRNA-29 and other interventions in the progression of liver fibrosis is through LPS induced TLRs/NF-κB pathway: as a gene regulator, it targets TLR4/NF-κB pathway through three regulatory mechanisms, regulating inflammatory response, and affecting the formation of liver fibrosis[28]: (i) miRNAs directly target the signaling system components of TLR4; (ii) miRNAs directly activate receptors of TLR4 RNA; (iii) miRNAs feed back and regulate expression of TLR4: in TLR ligand reactions, the expression of several miRNAs is up regulated, and they directly target components of the TLR signaling system, revealing the feedback loop control of miRNAs on TLRs signaling pathway activation. Some special miRNAs such as miRNA-155, miRNA-122, miRNA-146a have also been reported[29] to form positive or negative TLR4/NF-κB signal feedback pathway by regulating receptors, adapter molecules, and regulatory molecules, further affecting the activation of NLRP3 inflammasome. In summary, miRNAs not only exhibit specific expression in HSCs, regulating their morphology and secretion function, but also act on TLR4/NF-κB pathway through upstream regulation, further affecting the activation pathway of the pathway downstream NLRP3 inflammasome. Through three steps, it presents an intervention effect on the progression of liver fibrosis. By targeting TLR4/NF-κB signaling pathway, upstream microRNAs regulates the activation of downstream factor NLRP3 inflammasome, triggers inflammatory responses, continuously stimulates the liver, and leads to the formation of liver fibrosis. All of the above key signaling molecules may become new targets for the treatment of liver fibrosis; the prevention and treatment of liver fibrosis can be achieved through targeted regulation of microRNAs/TLR4/NF-κB signaling pathway, blocking the activation of NLRP3 inflammasome and chronic liver inflammation, and further inhibiting the formation pathway of liver fibrosis.

5 Related studies on the anti liver fibrosis effect of plumbagin

Plumbago zeylanica L. is mainly distributed in tropical and subtropical regions. In China, it is mainly distributed in Guangxi, Guangdong, and Yunnan. It is widely distributed in Guangxi, with abundant resources. *P. zeylanica* L. is a medicinal plant that is a specialty of Guangxi. Feature and taste: pungent, bitter, astringent, warm, and toxic. Function: dispelling wind, dispersing blood stasis, and detoxifying. It is called "dien bang" or "godon" by Guangxi Zhuang medicine. Its leaves are used to treat injuries caused by falls, carbuncle, swelling, and hepatosplenomegaly. It is called as "baec pioux ban" or "baec pioux ban" by Guangxi Yao medicine. According to literature records[30], *P. zeylanica* L. has the effect of treating hepatitis, liver cirrhosis, and sprains caused by falls. It is called as bidbipeg by Yunnan Dai medicine, and its dry root is effective in treating rheumatic pain, hyperostosis, liver pain, hepatosplenomegaly and other diseases. Modern medical research[31] has shown that *P. zeylanica* L. has the effects of treating hepatitis, liver cirrhosis, and sprains; significant inhibitory effect on the growth of liver cancer cells. The experiment[32] studied Fuzheng Huayu Jiedu Recipe with *P. zeylanica* L. as the main component, and confirmed its anti hepatic fibrosis, blood activating and blood stasis removing effects by improving the liver blood flow velocity, inhibiting HSC activation, and interfering with the formation of hepatic sinus basement membrane. Whether from the records of folk applications in various ethnic groups, the therapeutic effects of single or compound clinical applications, or the results of modern scientific experimental research, the pharmacological effects shown by *P. zeylanica* L. are effective in treating and reversing liver fibrosis. The main active component of anti liver fibrosis drug of *P. zeylanica* L. is plumbagin, which has the effects of promoting blood circulation and removing blood stasis. According to research report[33], the natural naphthoquinone, plumbagin (molecular formula $C_{11}H_8O_3$; molecular weight 188.18), separated from the root of the medicinal plant *P. zeylanica* L., has a variety of biological activities (Fig. 1),

such as anti-tumor, anti pulmonary fibrosis, anticoagulation, anti atherosclerosis, anti-inflammatory, *etc.* The content of plumbagin is consistent with its strength in promoting blood circulation and resolving blood stasis. The experiment[34] proved that plumbagin has the highest distribution in the liver of experimental mice and has shown definite anti liver fibrosis effects. This medicine is undoubtedly an active ingredient of natural medicinal herb for the treatment of blood stasis syndrome and anti fibrosis, with great potential and application prospects.

In the past 10 years, the research group in which the author works has systematically conducted research on the effects of plumbagin on promoting blood circulation, resolving blood stasis, and resisting liver fibrosis. It received four scientific research grants related to plumbagin, including the National Natural Science Foundation of China and the Guangxi Natural Science Foundation. Here is a brief summary of the preliminary research results of the research group: (i) the research project of Guangxi Natural Science Foundation showed that *P. zeylanica* L. decoction can significantly inhibit the acute liver damage caused by CCl4 in rats, reduce the levels of ALT and AST, increase plasma albumin, protect the liver and reduce enzymes, inhibit lipid peroxidation of liver tissue, and improve the liver protein synthesis[35]; it can also reduce the levels of HA, LN, and PCIII in plasma. Masson staining observation confirmed the effect of reducing fibroproliferation in liver tissue[36]. (ii) In the research project of the National Natural Science Foundation of China, the research was conducted based on the core cell of liver fibrosis lesion-human hepatic stellate cell line (HSC-LX2), and it was observed from several aspects such as cell proliferation rate, cell apoptosis, collagen synthesis and secretion, and expression of profibrotic cytokines[37-38]. The results showed that the drug could significantly inhibit the cell proliferation rate of HSC-LX2; block the entry of cells into the proliferation cycle and induce cell apoptosis; reduce the synthesis and secretion of fibrotic components α-SMA, type I, and type III collagen in HSC-LX2 strain; reduce the expression level of fibroblasts such as TGF-β1, VEGF. At the same time, it can also increase the expression of MMP1 and MMP13 enzymes that degrade fibrotic components. The above clarifies the anti liver fibrosis effect of plumbagin based on inhibiting the cell viability and secretion function of HSC-LX2. (iii) The research project of the National Natural Science Foundation of China focused on human hepatic sinusoidal endothelial cells (HSECs), and it can be observed that plumbagin can significantly reverse the "hepatic sinusoidal capillarization phenomenon" in the liver[39]. It can reopen the closed window pores in the HSECs, and significantly increase the total area of the window pores responsible for ventilating substances in the HSECs. At the same time, it can reduce the area and thickness of the continuous basement membrane formed outside the HSECs. It can also decrease expression levels of various cytokines in the liver, such as ET-1, TGF-β1, CTGF and ColIV, that cause fibrosis and disrupt liver microcirculation[40]. The results of various levels of scientific research projects above have confirmed that plumbagin can indeed improve liver microcirculation by inhibiting "hepatic sinusoidal capillary lesions", thereby achieving the "promoting blood circulation and resolving blood stasis" effect of traditional Chinese medicine. These experimental results can partially explain the mechanism of the pharmacological effect of plumbagin on liver fibrosis.

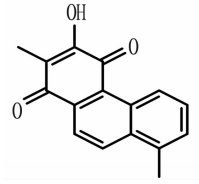

Fig. 1 Structural formula of plumbagin (2-methyl-5-hydroxyl-1, 4-naphthoquinone; $C_{11}H_8O_3$)

5 Conclusions and prospects

By studying the recent research progresses on the mechanism of liver fibrosis, the occurrence mechanism of liver microcirculation disorders, the structure and function of HSEC, and the pharmacological effects of plumbagin, combined with the preliminary research results of the research group, it has been confirmed that the active ingredient of Guangxi specialty ethnic medicine, plumbagin, has definite pharmacological effects on liver fibrosis. Further observation found that the drug could inhibit hepatic sinusoid capillarization and improve liver microcirculation. Based on these results, a deeper exploration can be conducted and a hypothesis can be proposed that the promoting blood circulation, resolving stasis, and anti liver fibrosis effects of plumbagin are achieved by regulating miRNAs/TLR4/NF-κB inflammatory pathway and downstream NLRP3 inflammasome activation. If further research is conducted, a mice liver fibrosis model with NLRP3 gene knockout can be established, as well as cell models of NLRP3 inflammasome and TLR4/NF-κB pathway activation. Through *in vivo* animal and *in vitro* cell experiments, the relationship between NLRP3 inflammasome, TLR4/NF-κB pathway activation related proteins and hepatic fibrosis HSC could be studied, to provide scientific reference for the prevention and control of liver fibrosis. It could study, search, and select upstream targeting miRNAs of the TLR4/NF-κB pathway. And the effects and mechanisms of curcumenol on upstream miRNAs of TLR4/NF-κB pathway, the activation of NLRP3 inflammasome, and its mediated HSC structure or secretion function could be studied. By the research on the expression changes of proteins related to miRNAs/TLR4/NF-κB pathway and NLRP3 inflammasome activation, combined with the previous research of the research group, it can comprehensively elucidate and develop a new mechanism of plumbagin against liver fibrosis from multiple perspectives of the gene and protein levels, such as microRNAs/TLR4/NF-κB and NLRP3 inflammasome activation. These studies can provide richer scientific basis for the further development and utilization of the active ingredient of a Guangxi specialty ethnic medicine, plumbagin, in the treatment of liver fibrosis diseases. Moreover, it can provide new targets and ideas for clinical treatment of chronic liver disease, cirrhosis and portal hypertension.

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