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Original Article

The activation of hepatic stellate cells in non-alcoholic steatosis hepatitis rats by osteopontin antibody

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Abstract: Non-alcoholic steatosis hepatitis (NASH) is one important step in the progression of non-alcoholic fatty liver disease (NAFLD), with the activation of hepatic stellate cells (HSCs) as its key mediator for liver fibrosis. Osteopontin (OPN) is closely related with fibrosis and is important for cell adhesion, migration, proliferation and rearrangement of cytoskeleton. This study thus observed OPN expression in NASH model rats and its correlation with smooth muscle actin (α-SMA), in order to investigate the relationship between OPN and HSC activation. NASH model rats were fed with high-fat diet. RT-PCR and Western blotting methods were used to detect gene expression of OPN in liver tissues. Anti-OPN antibody was injected via tail veins. Liver function indexes including ALT, AST, ALP and GCT were tested, along with α-SMA gene expression. Immunohistochemistry was employed to detect the positive expression of α-SMA. HE and Masson staining were used to observe pathological conditions of liver tissues. OPN gene expression was gradually increased with elongated time ($P<0.001$). Model rats had significantly elevated serum ALT, AST, ALP and GCT levels ($P<0.05$). After OPN antibody injection, liver function was improved. NASH rats had elevated α-SMA expression, which was down-regulated by OPN antibody. HE and Masson staining showed disrupted liver morphology and fibrosis, which can be partially improved by OPN antibody. High-fat diet induced liver OPN over-expression, which was closely related with NASH. OPN antibody can decreased α-SMA expression and inhibit liver fibrosis by inhibiting HSF activation.

Keywords: Osteopontin, antibody, non-alcoholic steatosis hepatitis, hepatic stellate cells

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one important public health issue. It mainly refers to a family of clinical symptoms related with insulin resistance (IR) and genetic factors. The pathological alternation is similar to alcoholic liver disease (ALD) but without drinking history [1]. Based on different grades of disease, NAFLD can be divided into non-alcoholic fatty liver (NAFL), non-alcoholic steatosis hepatitis (NASH), liver cirrhosis and liver cancer [2]. NASH therefore is one important step in the progression of NAFLD. Previous study believed that NASH was a benign disease. Recent studies, however, realized that the fatty denature of hepatic cells was a dynamic process, in which related factors could activate hepatic stellate cells (HSCs). The activation of HSC is one critical factor governing extracellular mechanism (ECM) to facilitate the progression of NASH into liver fibrosis and cirrhosis [3]. Study has shown that 30%~40% obesity patients with NASH had liver fibrosis [4]. Although patho-physiological mechanism of NASH is still unclear, certain study has shown that NASH and early liver cirrhosis were reversible [5], making the early intervention necessary.

Osteopontin (OPN) is one phosphorylated glycoprotein that can be synthesized and secreted by various cells. It includes Arg-Gly-Asp integrin binding domain. It is widely distributed in multiple tissues including cytoplasm, serum and urine, and can participate in various pathological and physiological conditions [6]. Previous study has shown that OPN was closely related with fibrosis, and is important for cell adhesion, migration, proliferation and rearrangement of cytoskeleton proteins. For example, OPN could

Table 1. Primer sequence

Gene	Forward primer	Reverse primer
OPN	ACTACAACCATGAGACTGGCAGTGGTTTGC	GAACTCTCTAATTCATGAGAAATGCGGAATTTCAGATAC
α-SMA	CTGTGCTATGTCGCTCTGGA	ATAGGTGGTTTCGTGGATGC
GAPDH	GACATCAAGAAGGTGGTGAAGC	TGTCATTGAGAGCAATGCCAGC

facilitate fibrosis and heart failure via accelerating differentiation of myocardial fibroblast and depressing collagen degradation [7]. However, the study of OPN in NASH pathogenesis and molecular mechanism has not been reported. *In vitro* study has shown OPN was the major component of non-collagen glycoprotein in extra cellular matrix (ECM), and could facilitate the expression of TGF-β receptor II (TGF-βRII) for inducing HSC secreting collagen [8]. This study constructed a rat NASH model, on which OPN gene expression was monitored during disease progression. OPN antibody was injected via tail veins for intervention. The change of serum liver function indexes was observed, along with smooth muscle actin (α-SMA) expression which is related with HSC activation, and HE and Masson staining for pathological changes, in order to investigate the effect of OPN on the inhibition of HSC activation during NASH occurrence and progression, and to find novel targets for treating NASH.

Materials and methods

Materials

A total of 48 male SD rats (6 weeks old, body weight at 200 g±20 g) were fed with high-fat diet (Jinan University, Jinan China). Rabbit anti-rate α-SMA antibody and OPN primary antibody were purchased from Cell Signaling (US). GAPHD primary antibody was purchased from Santa Cruz (US). Immunohistochemistry (IHC) staining kit was purchased from Boster Bio (China). OPN antibody, RNA extraction kit, reverse transcription kit and SYBR kit were all purchased from Toyobo (China). Primers for OPN, α-SMA and GAPDH in RT-PCR were purchased from Yingjun Bio (China).

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of The First Affiliated Hospital of Jinan University.

Animal grouping and modeling

SD rats were randomly divided into normal control (N=10), model (N=18), placebo (N=10) and OPN antibody (N=10) groups. Control group was fed with normal diet while the other three groups received high-fat diet (88% normal diet + 10% lard oil + 2% cholesterol) as previously documented [9]. Rats were singly housed in a facility at (22±2)°C with 12 h/12 h light cycle and food and water *ad libitum*. Placebo group received equal volume of saline via tail veins at every other day for 2 weeks (0.15 ml each). OPN antibody group had intravenous injection of OPN antibody (titer: 1:32) at every other day for 2 weeks (0.15 ml each). After treatment, chloral hydrate (3 ml/kg) was injected intraperitoneally. Animals were sacrificed following eye ball venous blood collection. Liver tissues were collected for further experiments.

Real-time PCR

Rat liver tissues were rinsed in PBS, and were mixed with 1 ml Trizol for homogenization. Total RNA was extracted by phenol-chloroform. RNA purity and concentration were tested by UV spectrometer. Reverse transcription kit was used to synthesize cDNA. RT-PCR test kit was then used to detect the expression of target genes OPN, α-SMA and GAPDH using specific primers (**Table 1**). RT-PCR was performed on a quantitative fluorescent PCR cycler (ABIstepone, US) under the following conditions: 95°C pre-denature for 20 sec, flowed by 45 cycles each containing 95°C nature for 5 sec, and 60°C annealing for 30 sec. Ct value and copy number were calculated to analyze the relative expression level of target gene OPN and α-SMA using GAPDH as the internal reference. Results were expressed as $2^{-\Delta\Delta Ct}$ values.

Western blotting

Liver tissues were homogenized in lysis buffer. After centrifugation at 4°C for 15 min, total proteins were extracted from the supernatants and were kept at -80°C for further use. Protein concentration was determined by BCA method. 20 μg proteins were employed for SDS-PAGE separation, and were transferred to NC membrane under 300 mA electrical field. The mem-

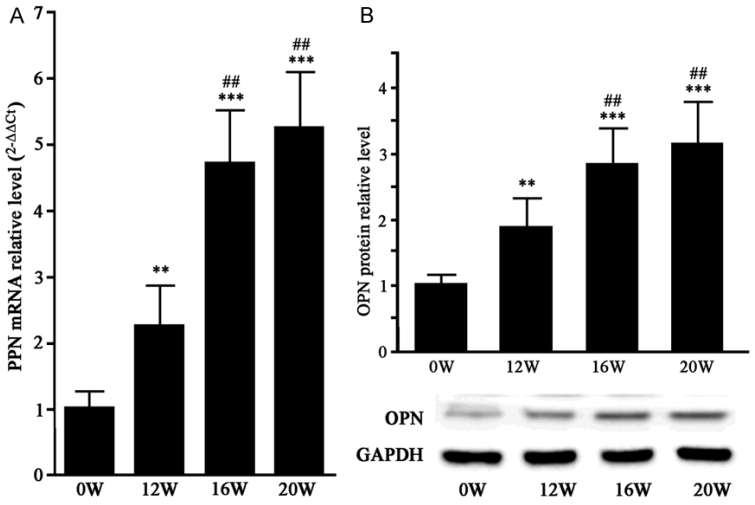

Figure 1. OPN expression in NASH rat liver tissues. **, P<0.01, ***, P<0.001 compared to 0 w group. ##, P<0.01, ###, P<0.001 compared to 12 w group.

brane was blocked by 5% defatted milk powder for 2 h, and was treated with primary antibody against OPN (1:1000), α-SMA (1:1000) or GAPDH (1:2000) at 4°C overnight. After TBST rinsing, HRP-conjugated secondary antibody (1:5000) was added to incubate the membrane for 2 h, followed by rinsing and ECL development. Gel imaging analysis system was used to analyze optical density of each band. Using GAPDH as the internal reference, the relative expression of OPN and α-SMA was calculated.

Serum index

Blood samples were collected from eye ball veins, and were incubated at room temperature for 2 h. The blood was centrifuged by 3000 r/min under 4°C for 5 min to collect serum, which was aliquot and stored at -70°C for further use. Liver function indexes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyltranspeptidase (GGT) following manual instruction of test kits. Automatic biochemical analyzer was used to collect and analyze results.

Immunohistochemistry (IHC)

Liver tissues were prepared for paraffin slides, which were processed by IHC staining using test kits and previous documents under the same condition [10]. Briefly, slides were dewaxed by xylene, rehydrated in gradient ethanol, and were processed in 3% H_2O_2 for 20 min to quench peroxidase. Antigen retrieval was performed using 0.01 M citric acid buffer in a microwave oven for 10 min. Normal goat serum (NGS) was used to block non-specific binding sites. Primary antibody (rabbit anti-rat α-SMA, 1:50) and secondary antibody (goat anti-rabbit IgG, 1:100) were sequentially added, followed by DAB substrates and PBS rinsing. Counter-staining and drying were performed. The positive of α-SMA was determined by brown granules in nucleus. The positive staining area was measured from 10 randomly selected high-magnification (400X) field from one sample slide.

Histopathology examination

Liver tissues were fixed in paraformaldehyde and were stained by HE and Masson methods [11]. In brief, paraffin slides were prepared, dewaxed and immersed in hematoxylin for 5 min. After rinsing in distilled water, 1% HCl-ethanol was used to treat slides, which were immersed in eosin for 8 min, followed by gradient ethanol (2 min each). Slides were dried in 60°C chamber, followed by transparency treatment and mounting slides. Masson staining was carried after routine de-waxed and incubation in Masson complex dye for 5 min. 1% phosphomolybdic acid buffer was used to incubate slides for 5 min. After drying, 1 mL toluidine blue was used to incubate slides for 5 min, followed by rinsing in distilled water. 1 mL 95% ethanol was used to differentiate slides for 30 sec, and were dehydrated for absolute ethanol. Xylene was used to treat slide, which was mounted with resin and coverslips. Light-field microscope was used to observe slides.

Statistical methods

SPSS19.0 software package was used to analyze all collected data, of which measurement data were presented as mean ± standard deviation. Between-group-comparison was done by student t-test. Comparison among three groups was performed by one-way analysis of variance

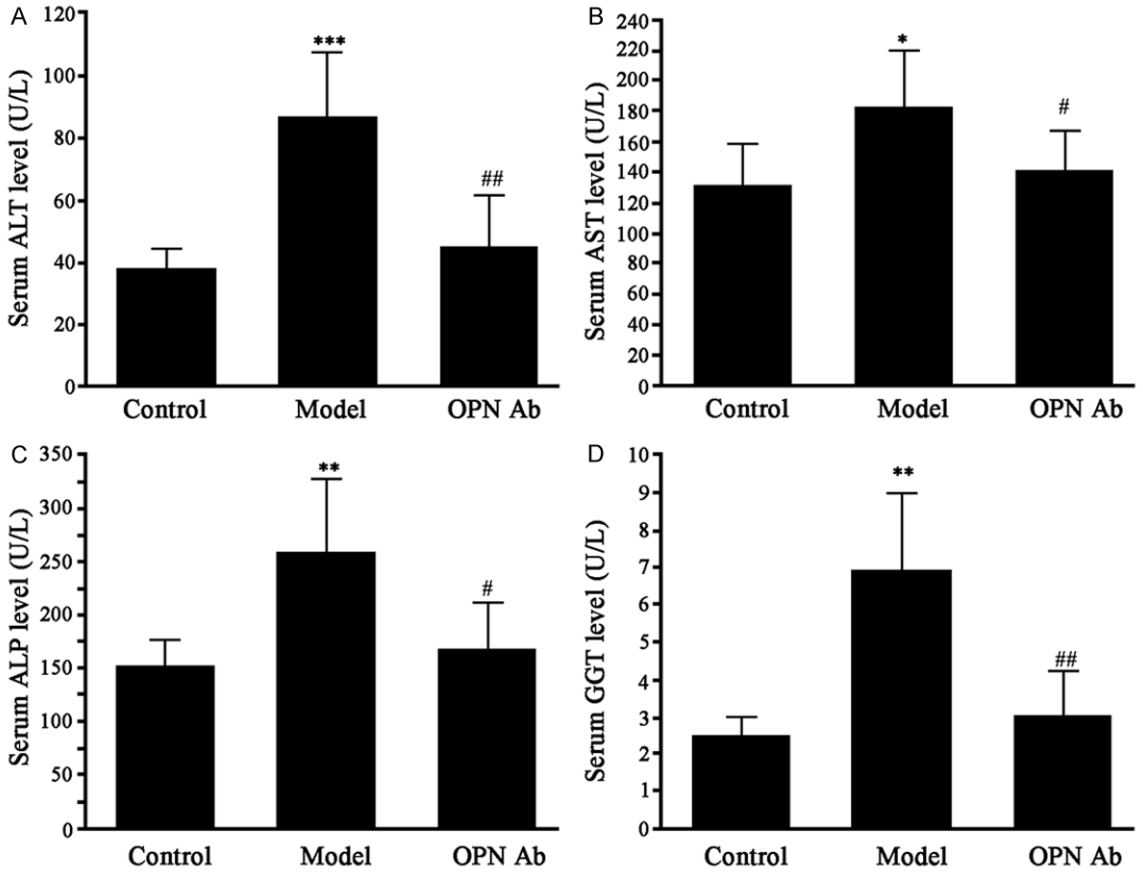

Figure 2. OPN antibody and rat liver function. *, P<0.05, **, P<0.01, ***, P<0.001 compared to control group. #, P<0.05, ##, P<0.01 compared to model group.

(ANOVA). A statistical significance was defined when P<0.05.

Results

OPN expression of model rat tissues

RT-PCR results showed that, with elongated time of model preparation, OPN gene expression level of NASH model rat was gradually increased, as expression level at 16 w was significantly higher than that in 12 w (P<0.001) but not with 20 w (**Figure 1A**). Similar results were obtained from Western blotting, as OPN protein level at 16 w after model generation was significantly higher than 12 w (P<0.01) but was indifferent from that at 20 w (**Figure 1B**). We thus selected rats at 16 w for further antibody intervention.

Effects of OPN antibody on liver function of model rat

16 weeks after generating model, ALT, AST, ALP and GGT levels were significantly elevated compared to control group (P<0.05 in all cases). After intervention using OPN antibody, liver function was significantly improved as all indexes were significantly depressed (P<0.05 in all cases, **Figure 2**).

Effect of OPN antibody on α-SMA in model rats

RT-PCR results showed that, after NASH model generation, the expression of α-SMA gene in rat liver tissues was significantly increased, and was significantly decreased by OPN antibody intervention (**Figure 3A**). Western blotting results showed significant elevation of α-SMA protein in model rats and down-regulation after OPN antibody intervention (**Figure 3B**). IHC staining also revealed elevation of α-SMA in model rat tissues and depression after OPN antibody treatment (**Figure 3C** and **3D**).

Effects of OPN antibody on histo-pathology of rat model liver tissues

HE staining showed normal and complete structure and morphology of liver tissues in normal

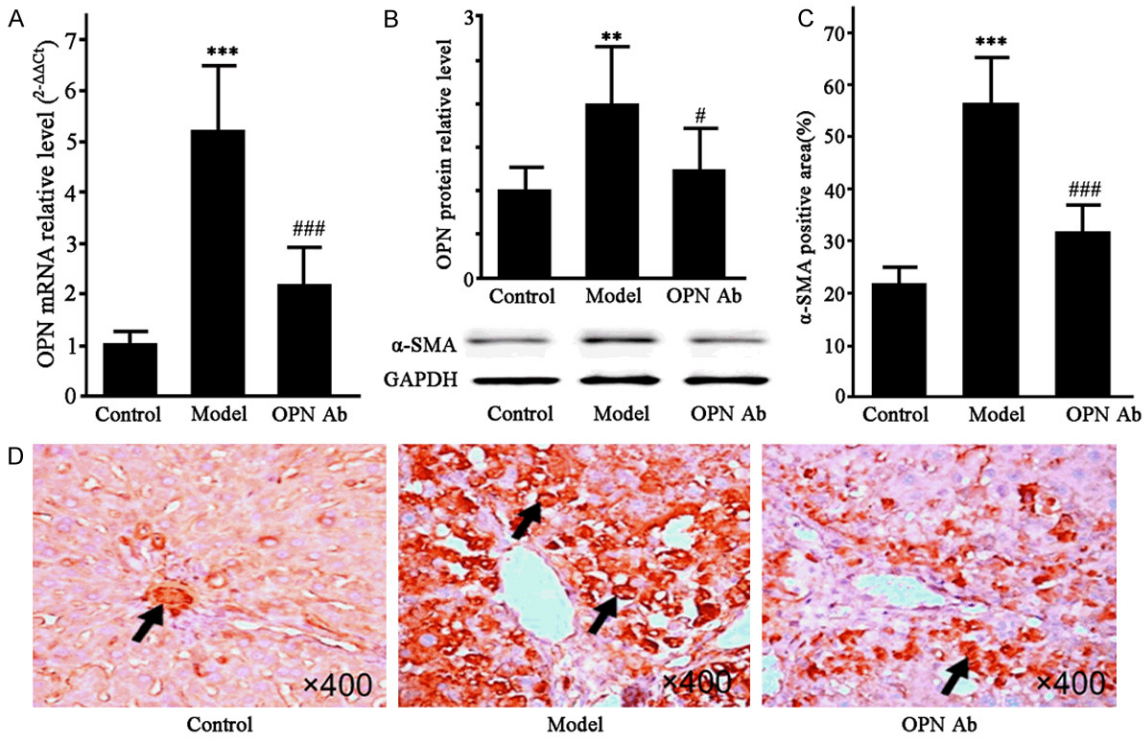

Figure 3. Effect of OPN antibody on α-SMA expression in model rats. **, P<0.01, ***, P<0.001 compared to control group. #, P<0.05, ##, P<0.01 compared to model group.

control group, without any inflammatory cell infiltration or fat denature. 16 weeks after generating model, the integrity of structure was disrupted with diffused denature of hepatocytes, which had vacuoles of adipocyte tissues. The point necrotic lesion is widely distributed especially in the central venous region, where there were significant infiltration and inflammation cells and less fibrous tissue hypertrophy. After OPN antibody intervention, the pathological area of liver tissues was decreased with less fibrous hypertrophy and depressed inflammatory cell infiltration (**Figure 4A**). Masson staining showed no abnormality in normal rat liver tissues, leaving only minor collagen fiber that was stained blue around central vein or liver portal peripheral regions. After NASH model preparation, there was a significant fibrous hypertrophy of liver tissues mainly in portal region and less in central venous area. The hypertrophy of collagen formed a network to separate liver solid tissues, and disrupted normal hepatic lobe structure. The treatment using OPN antibody significantly decreased fibrous tissue hypertrophy in central venous and portal regions, as the shortening and thinning of fibers around hepatic lobe, whose structure was partially restored (**Figure 4B**).

Discussion

Previous study believed that NASH was a benign disease. Recent studies, however, realized that the fatty denature of hepatic cells was a dynamic process, in which related factors could facilitate liver fibrosis, which further aggravate into liver cirrhosis. NASH therefore is one important step in the progression of NAFLD, and is one common pathological change caused by various chronic liver injuries [12]. Current study found that the activation and proliferation of HSCs are central step of NASH occurrence/progression, and thus play crucial roles in liver fibrosis. HSCs can differentiate into muscular fibroblasts under co-stimulus of various reactive oxygen free radicals and cytokines, to expressed larger amounts of α-SMA, and produce ECM predominantly composed of collagen to secrete various pro-fibrosis factors including transformation growth factor-β (TGF-β), platelet-derived growth factor (PDGF) to accelerate the occurrence and progression of liver fibrosis [13]. This study constructed NASH

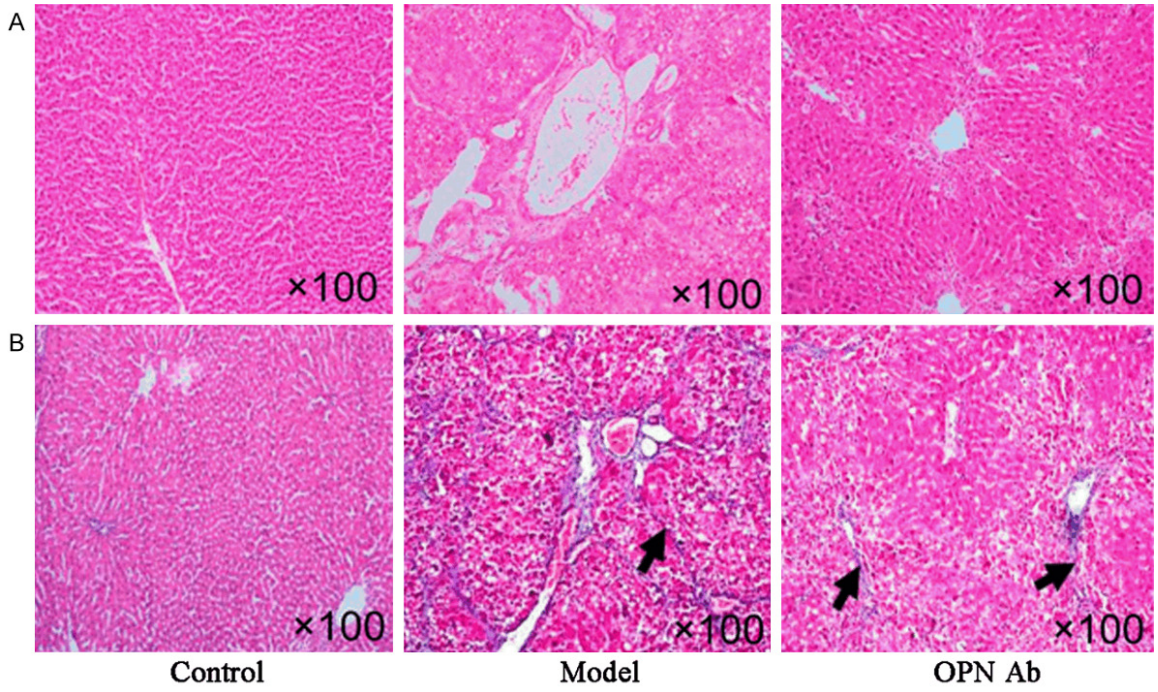

Figure 4. Effects of OPN antibody on rat liver morphology.

model on SD rats by high-fat diet feeding, and found significant elevation of serum ALT, AST, ALP and GGT levels 16 weeks later. RT-PCR and Western blotting methods confirmed such up-regulation of α-SMA gene, as consistent with IHC staining which showed higher α-SMA positive rates. Both HE and Masson staining found diffused denature of hepatocytes, with vacuole alternation in adipocytes with point necrotic lesion and significant inflammatory cell infiltration. The hypertrophy of collagen formed network fibers to destruct normal hepatic lobe structure, as consistent with previous reports [14], suggesting the reliability of model and providing basis for further OPN antibody intervention.

Osteopontin (OPN) is one phosphorylated glycoprotein that includes Arg-Gly-Asp integrin binding domain and can be synthesized and secreted by various cells [15]. It is widely distributed in multiple tissues including cytoplasm, serum and urine [16], and can participate in various pathological and physiological processes [17]. OPN mainly binds with cell surface adhesion molecules such as integrin and CD44, to participate in cell immunity, migration, inflammation infiltration and tissue repair processes [18]. Previous study has shown that OPN was closely related with fibrosis. For example, OPN could facilitate fibrosis and heart failure via accelerating differentiation of myocardial fibroblast and depressing collagen degradation. *In vitro* study has shown OPN was the major component of non-collagen glycoprotein in extra cellular matrix (ECM), and could facilitate the expression of TGF-β receptor II (TGF-βRII) for inducing HSC secreting collagen. Some studies found that serum OPN level in NAFLD patients was significantly higher than control group, and was positively correlated with AST, ALT and liver fibrosis staging [19]. This study constructed NASH model rats and found the possible involvement of OPN in liver fibrosis. OPN gene was expressed in control group at low levels, and was gradually up-regulated with aggravation of liver fibrosis in NASH rats. Previous studies found the over-expression of OPN in CCl_4-induced liver fibrosis and *in vitro* cultured activated HSCs. cDNA microarray suggested the critical role of OPN expression in HSC activation as elevation of OPN expression with the progression in HSC activation [20]. Another study also found that with elongated injection time of CCl_4, liver fibrosis is aggravated, accompanied with elevated OPN expression [21]. Such synchronized up-regulation with α-SMA in liver tissues was significantly inter-

correlated. This study applied OPN antibody on NASH model rats and found significant down-regulation of liver indexes including ALT, AST, ALP and GGT, along with decreased α-SMA gene expression and IHC positive rates. Both HE and Masson staining suggested significant improvement of liver tissue histo-pathology. Another study by Kiefer et al found the decreased triglyceride in OPN gene knockout mice to improve the glycogenesis ability and prevent obesity-induced NASH [22].

In summary, this study observed the expression of OPN in NASH model rats and its correlation with HSC activation, and found the significant positive correlation. The intervention of OPN antibody could significantly inhibit HSC activation, alleviate liver tissue inflammation and fibrosis. Moreover, we also found higher safety using OPN antibody to treat NASH, as no mortality has been occurred in OPN antibody-injected rats, which had smooth fur and normal diet. Therefore, the inhibition of OPN expression could alleviate liver fibrosis via inhibiting HSC activation. OPN antibody thus may be promoted in clinics.

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Disclosure of conflict of interest

None.

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References

[1] Ibrahim MA, Kelleni M and Geddawy A. Nonalcoholic fatty liver disease: current and potential therapies. Life Sci 2013; 92: 114-8.

[2] White DL, Kanwal F and El-Serag HB. Association between nonalcoholic fatty liver disease and risk for hepatocellular cancer, based on systematic review. Clin Gastroenterol Hepatol 2012; 10: 1342-1359.e2.

[3] Tamaki Y, Nakade Y, Yamauchi T, Makino Y, Yokohama S, Okada M, Aso K, Kanamori H, Ohashi T, Sato K, Nakao H, Haneda M, Yoneda M. Angiotensin II type 1 receptor antagonist prevents hepatic carcinoma in rats with nonalcoholic steatohepatitis. J Gastroenterol 2013; 48: 491-503.

[4] Raziel A, Sakran N, Szold A, Goitein D. Current solutions for obesity-related liver disorders: non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Isr Med Assoc J 2015; 17: 234-8.

[5] Eslami L, Merat S, Malekzadeh R, Nasseri-Moghaddam S, Aramin H. Statins for non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Cochrane Database Syst Rev 2013; 12: Cd008623.

[6] Kaleta B. Role of osteopontin in systemic lupus erythematosus. Arch Immunol Ther Exp (Warsz) 2014; 62: 475-82.

[7] Lopez B, González A, Lindner D, Westermann D, Ravassa S, Beaumont J, Gallego I, Zudaire A, Brugnolaro C, Querejeta R, Larman M, Tschöpe C, Díez J. Osteopontin-mediated myocardial fibrosis in heart failure: a role for lysyl oxidase? Cardiovasc Res 2013; 99: 111-20.

[8] Xiao X, Gang Y, Gu Y, Zhao L, Chu J, Zhou J, Cai X, Zhang H, Xu L, Nie Y, Wu K, Liu Z, Fan D. Osteopontin contributes to TGF-beta1 mediated hepatic stellate cell activation. Dig Dis Sci 2012; 57: 2883-91.

[9] Luo WZ. Effect of Monascus on Improving Hepatic Tissue and Biochemistry in Rat Models of Non-alcoholic Fatty Liver Disease. Chinese Journal of General Practice 2011.

[10] Zhang X, Yang J, Guo Y, Ye H, Yu C, Xu C, Xu L, Wu S, Sun W, Wei H, Gao X, Zhu Y, Qian X, Jiang Y, Li Y, He F. Functional proteomic analysis of nonalcoholic fatty liver disease in rat models: enoyl-coenzyme a hydratase down-regulation exacerbates hepatic steatosis. Hepatology 2010; 51: 1190-9.

[11] Nakagami H, Shimamura M, Miyake T, Shimosato T, Minobe N, Moritani T, Kiomy Osako M, Nakagami F, Koriyama H, Kyutoku M, Shimizu H, Katsuya T, Morishita R. Nifedipine prevents hepatic fibrosis in a non-alcoholic steatohepatitis model induced by an L-methionine- and choline-deficient diet. Mol Med Rep 2012; 5: 37-40.

[12] Wree A, Broderick L, Canbay A, Hoffman HM, Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. Nat Rev Gastroenterol Hepatol 2013; 10: 627-36.

[13] Presser LD, McRae S and Waris G. Activation of TGF-beta1 promoter by hepatitis C virus-induced AP-1 and Sp1: role of TGF-beta1 in hepatic stellate cell activation and invasion. PLoS One 2013; 8: e56367.

[14] Xiao J, Ho CT, Liong EC, Nanji AA, Leung TM, Lau TY, Fung ML, Tipoe GL. Epigallocatechin

gallate attenuates fibrosis, oxidative stress, and inflammation in non-alcoholic fatty liver disease rat model through TGF/SMAD, PI3 K/Akt/FoxO1, and NF-kappa B pathways. Eur J Nutr 2014; 53: 187-99.

[15] Teixeira LN, Castro-Raucci L, Coletta R. Effects of secreted osteopontin from human osteoblastic cells on adhesion and migration of an oral squamous cell carcinoma cell line. Oral Surgery Oral Medicine Oral Pathology & Oral Radiology 2014; 118: e196-e197.

[16] Wang J, Tang Q, Qiu Y, Xu M, Rong R, Zhu T. Osteopontin level correlates with acute cellular renal allograft rejection. J Surg Res 2013; 182: 161-5.

[17] Chiodoni C, Sangaletti S, Tripodo C, Colombo MP. The ins and outs of osteopontin. Oncoimmunology 2015; 4: e978711.

[18] Kahles F, Findeisen HM and Bruemmer D. Osteopontin: A novel regulator at the cross roads of inflammation, obesity and diabetes. Mol Metab 2014; 3: 384-93.

[19] Yilmaz Y, Ozturk O, Alahdab YO, Senates E, Colak Y, Doganay HL, Coskunpinar E, Oltulu YM, Eren F, Atug O, Tuncer I, Imeryuz N. Serum osteopontin levels as a predictor of portal inflammation in patients with nonalcoholic fatty liver disease. Dig Liver Dis 2013; 45: 58-62.

[20] Lenga Y, Koh A, Perera AS, McCulloch CA, Sodek J, Zohar R. Osteopontin expression is required for myofibroblast differentiation. Circ Res 2008; 102: 319-27.

[21] Dai WL, Huang HF and Lu CH. Correlation between expression of osteopontin and activation of hepatic stellate cells in hepatic fibrosis. World Chinese Journal of Digestology 2013; 21.

[22] Kiefer FW, Neschen S, Pfau B, Legerer B, Neuhofer A, Kahle M, Hrabé de Angelis M, Schlederer M, Mair M, Kenner L, Plutzky J, Zeyda M, Stulnig TM. Osteopontin deficiency protects against obesity-induced hepatic steatosis and attenuates glucose production in mice. Diabetologia 2011; 54: 2132-42.