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Curcumin interrupts leptin-regulated microRNA-122 in hepatic stellate cells *in vitro* and *in vivo*

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Abstract: The purpose of this study was to evaluate the effect of curcumin, an active polyphenol, on the leptin induced lowering of miR-122 in Hepatic stellate cells (HSCs) *in vivo* and an animal model. Gene expression was evaluated by transfection assay, real-time PCR, or Western blot analysis. The liver fibrosis model of leptin deficient mouse was used for *in vivo* experiment. As a result, curcumin showed inhibitory effect on leptin induced lowering of the miR-122 in HSCs. Curcumin suppressed leptin induced sonic hedgehog (Shh) expression and blocked leptin induced Shh signaling pathway, which was essential for curcumin inhibition of the negative role of leptin in miR-122 expression in HSCs. The influence of curcumin on the negative effect of leptin on miR-122 level was followed by the attenuation of liver fibrosis caused by leptin in leptin-deficient mouse model. In conclusion, curcumin could reduce the decrease of miR-122 level in HSCs induced by leptin and inhibit liver fibrosis induced by leptin. These data may have potential implications to treat with liver fibrosis by elevating the expression of leptin in humans especially obese patients.

Keywords: Leptin, hepatic stellate cell, microrna-122, curcumin, liver fibrosis, sonic hedgehog.

INTRODUCTION

Gene expressions were controlled at multiple levels by many mechanisms including microRNA (miRNA). The mechanisms for gene regulation by miRNA are classified into post transcriptional regulation. MicroRNA-122 (miR-122), defined as a liver-specific miRNA had been suggested as anti-inflammatory function in liver (Hsu *et al.,* 2012; Wen and Friedman, 2012). Almost 52% of the miRNA population in human liver were constituted by miR-122 (Bandiera *et al.,* 2015). Interestingly, miR-122 was down-expressed in hepatic fibrosis patients liver and showed a negative correlaion with hepatic fibrosis (Halász *et al.,* 2015; Trebicka *et al.,* 2013). Liver fibrosis is a result of the wound-healing response. Upon chronic damage of the liver, hepatic stellate cells (HSCs) are stimulated by multiple cytokines such as transforming growth factor beta-1 (TGFβ1), platelet-derived growth factor (Lee UE *et al.,* 2011) and leptin (Hernandez-Gea and Friedman, 2011), which cause the changes of gene expression profiles in quiescent HSCs and thus lead to the activation of HSCs, a key step in liver fibrogenesis (Mederacke I *et al.,* 2013).

It has been showed that, as compared with general population, liver fibrosis was apt to develop in obese patients (McCullough *et al.,* 1999; Ratziu *et al.,* 2000) who always suffered with hyperleptinemia (Stefanović *et al.,* 2008). Accumulating data have suggested that leptin, the adipocyte-derived hormone, promoted hepatic fibrosis (Aleffi *et al.,* 2005; Elinav *et al.,* 2009; Honda *et al.,* 2002; Qiao *et al.,* 2016;Saxena *et al.,* 2002; Zhai X *et al.,* 2013), especially in patients (Salman AA *et al.* 2020; Salman MA *et al.*, 2020; Rotundo L *et al.* 2018).

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Recently, we proved that the leptin-induced miR-122 decreased in HSCs (Zhai *et al.,* 2017), supporting the impact of leptin in HSCs activation as well as the liver fibrogenesis (Hernandez-Gea and Friedman, 2011). Furthermore, our results found that miR-122 suppressed leptin-induced thioacetamide (TAA)-associated hepatic fibrosis in a leptin-deficient animal model (Zhai *et al.,* 2017).

Curcumin is an active polyphenol and were confirmed against various diseases including liver diseases, cancer, metabolic disorders and cardiovascular injury (Gupta *et al.,* 2013). Curcumin reduced liver fibrogenesis (Tang *et al.,* 2015) and leptin-induced HSCs activation (Tang *et al.,* 2010). These results promote us to detect whether curcumin affects the impact of leptin in miR-122 expression in liver fibrosis.

MATERIALS AND METHODS

Materials

Thioacetamide (TAA, purity>98%), cyclopamine (purity>98%, a specific hedgehog (Hh) signaling pathway) and curcumin (purity>98%) were purchased from Sigma (St. Louis, MO, USA). Leptin (purity>95%) was stored in our laboratory (Zhai *et al.,* 2017).

HSCs isolation and cell culture

Isolated HSCs were obtained from mice (Animal Research Center of Nantong University, Nantong, China) by *in situ* digestion of the liver and by density gradient centrifugation according to our previous study (Cheng *et al.,* 2020) from the mice of Animal Research Center of our school. HSCs purity was calculated through an fluorescence microscope excited by ultraviolet and the purity was exceeded 95%. In

in vitro experiments, HSCs underwent 24h serum-starvation and following pretreated with curcumin at various doses or 5μM cyclopaminein in Dulbecco's modified Eagle's medium (DMEM) containing 0.4% FBS for 0.5h before leptin treatment (100ng/ml) (Saxena *et al.*, 2002; Zhai *et al.*, 2013) for following 24 h, unless otherwise stated.

Real-time PCR analysis

TRI-Reagent (Sigma, St. Louis, USA) was for total RNA extraction, followed by digesting with DNase I. Hairpin-it^{TM} MicroRNAs Quantitation PCR Kit (GenePharma, Shanghai, China) were bought for the miR-122 Real-time PCR analysis according to the recommended protocols of the manufacturers. U6 snRNA level was uesd to normalize the expression of miR-122. For detection of Sonichedgehog (Shh) mRNA levels, real-time PCR was performed with reaction mixes by using the conditions: 2 min at 95ºC for denaturation, followed by 35 cycles of segments of 95ºC for 5 s and 55ºC for 30s. The Ct normalized by GAPDH was analyzed by using the $2^{-\Delta\Delta Ct}$ method as our precious study (Xia Hu *et al.*, 2020). The primer sequences were showed as follow. Mouse miR-122: (F) 5′-GATGCTCTGGAGTG TGACAA TG-3′, (R) 5′-TATGGTTGTTCACGACTCCT TCAC-3′; Mouse U6 snRNA: (F) 5′-ATTGGAACGATA CAGAGA AGATT-3′, (R) 5′-GGAACGCTTCACGAAT TTG-3′; Mouse Shh: (F) 5'-ACT CCG AAC GAT TTA AGG AAC T -3', (R) 5'-TCT TTG CAC CTC TGA GTC ATC-3'; Mouse GAPDH: (F) 5'-TCACCA CCATGGAGA AGGC-3', (R) 5'-GCT AAG CAG TTG GTG GTG CA-3'.

Western blot assay

Western blot associated experiments were conducted as our earlier study (Zhou *et al.*, 2010). In brief, the whole proteins were underwent electrophoresis and then electro-transferred onto nitrocellulose membranes for detecting by primary antibody against Shh (SC-365112, 1:1000, Santa Cruz, CA, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, Santa Cruz, CA, USA) and the horseradish peroxidase(HRP) conjugated secondary antibodies (1:4000, Santa Cruz, CA, USA). The relative protein expression were calculated through a ImageJ Software.

Plasmid construction and transfection assay

The miR-122 promoter luciferase reporter plasmid pGL3miR122 (-1002) Luc contained the mouse miR-122 promoter fragment (between-1002 to+86) as we described previously (Zhai *et al.*, 2017). HSCs were cultured in 12-well plastic plates and were used for transiently transfection by using Lipofectamine 2000 reagent (Life Technologies, New York, USA) according to manufacturer's instructions. 1.6μg of pGL3miR122 (-1002)Luc and 10 ng of control vector expressing Renilla luciferase (pRL-TK; Promega, Madison, USA) were cotransfected into the HSCs and the cells were pre-incubated with cyclopamine or curcumn for 0.5h before addition of leptin for another 24h. Luciferase activities were quantified fluorimetrically by Dual-Luciferase Reporter Assay System (Promega, Madison, USA). The Photinus luciferase activity was normalized by the Renilla luciferase activity.

Animal studies

Researches had indicated that injected TAA plus leptin into C57BL/6J ob/ob mice for 4-week could promote HSCs activation and hepatic fibrosis (Zhou *et al.*, 2014). Thus, we chose 6 week old male ob/ob mice for *in vivo* experiments. Two groups (six mice/each group) were treated with TAA (200μg/g body weight, twice a week) plus vehicle or TAA plus leptin (1μg/g body weight, once per day) by intraperitoneal injection (i.p.) for 4-week as our precious study (Zhou *et al.*, 2014). The third group was administrated with 400 mg in PBS/kg body weight curcumin once per day (Fu *et al.*, 2008) throughout 4-week TAA plus leptin treatment. The mice involved in the study all took in humane care and were received free standard chow diet and water. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Nantong (2012-0031).

Sirius red staining

Collagen was stained by Sirius red to evaluate liver fibrosis. The paraformaldehyde (4%)-fixed liver slices were stained with picric acid-sirius red (Amresco, Solon, USA) for one hour. Light microscope was used for images capture and ImageJ software was used to quantify the staining area.

STATISTICAL ANALYSIS

The data were expressed as mean values ± standard deviation (S.D.). Differences between means were analysed using an unpaired two-sided Student's t-test whereas differences between more groups were evaluated by ANOVA with Tukey's post hoc test. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc.).

RESULTS

Curcumin reduces the influence of leptin on miR-122 in vitro

For examination of the role of curcumin on miR-122 *in vitro*, HSCs were stimulated with leptin and different concentration of curcumin. As shown in figure 1A, the leptin inhibiting effection miR-122 was reversed by different doses of curcumin. We also test the time course of 15μM of curcumin on miR-122 expression after the presence of leptin for 24h. Fig. 1B showed the promotional role of curcumin on miR-122 in the course of the time. Since leptin could affect miR-122 promoter activity (Zhai *et al.*, 2017), the impact of curcumin on leptin-induced

decline in miR-122 promoter activity was further detected. pGL3miR122 (-1002) Lucwere transient transfected into cells with leptin or curcumin stimulated. Results (fig. 1C) indicated that curcumin also negatively influenced the role of leptin in miR-122 promoter activity. Thus, these results prompted that curcumin could reduce the inhibitory effect of leptin on miR-122 expression *in vitro*.

Curcumin attenuates leptin-induced Shh expression in HSCs in vitro.

Our previous research confirmed that leptin could decrease miR-122 expression in HSCs through Shh pathway (Zhai *et al.,* 2017). Therefore, for detecting the mechanism underlying curcumin on leptin role in miR-122 expression, the influence of curcumin on Shh expression induced by leptin was tested. HSCs were treated with leptin or curcumin. The Shh were examined at protein and mRNA levels. As shown in fig. 2A and 2B, curcumin clearly reduced leptin-induced Shh protein level and mRNA level. These data implied that curcumin could reduce leptin-induced miR-122 level by interrupting leptin-induced Shh signaling *in vitro*.

The interruption of leptin-induced Shh pathway by curcumin contributes to its inhibition of leptin-induced down-regulationon miR-122 in vitro.

To test the role of curcumin interruption of Shh signaling on leptin-induced down-regulation in miR-122 level, cells were treated with leptin plus cyclopamine or/and curcumin as shown in fig. 3A. Results demonstrated that, as expected, cyclopamine or curcumin alleviated the inhibitory role of leptin in miR-122 expression. Notably, compared with the sample treated with leptin plus curcumin, pretreatment of HSCs with curcumin led to unconspicuous influence of cyclopamine on leptin-induced decline in miR-122. Furtherly, pGL3miR122 (-1002) Luc were also transfected into HSCs and treated with leptin plus cyclopamine or/and curcumin as shown in fig. 3B. Luciferas assay showed that cyclopamine also had no obvious effect on miR-122 level treated with leptin plus curcumin.

These results implied that the block of leptin-induced Shh signaling by curcumin was required for its inhibition of leptin-induced miR-122 down-regulation *in vitro*.

Curcumin reduces leptin-induced Shh level in HSCs in vivo, which together with the increase in miR-122 and by the decrease in liver fibrosis

To detect the influence of curcumin on leptin-induced miR-122 down-regulation *in vivo*, ob/ob mice were divided randomly and treatment as described in *Materials and Methods.* Livers HSC RNA were extracted for real-time PCR. As expected, leptin down-regulated miR-122 expression in HSCs, which was neutralized by curcumin (fig. 4A, the upper panel). Contrarily, the transcription of Shh was enhanced by leptin, which was also counteracted by curcumin (fig. 4A, the bottom panel). As Shh mediated leptin increased miR-122 level in HSCs (Zhai *et al.,* 2017), the results in fig. 4A indicated that curcumin reduced leptin-induced Shh expression, leading to the decline in miR-122 *in vivo*. Moreover, we evaluated fibrosis degree by Sirius red staining. Results showed that, in TAA-induced ob/ob mouse model, leptin promoted liver fibrosis by comparing with the samples received TAA alone, which were reduced by curcumin treatment (fig. 4B).

DISCUSSION

Leptin decreased miR-122 level in HSCs (Zhai *et al*., 2017). In this studies, we detected the effect of curcumin on leptin-induced reduction of miR-122 levels. As a result, curcumin lowered the role of leptin in miR-12 expression in HSCs. The influence of curcumin on leptin-induced reduction in miR-122 was related to its interruption of leptin-induced Shh signal pathway. The impact of curcumin on the effect of leptin in miR-122 expression was followed by lowering hepatic fibrosis caused by leptin in a *vivo* mouse model.

In our previous researches, the results demonstrated that leptin might had roles in reducing miR-122 level in HSCs (Zhai *et al*., 2017), contributing to leptin-induced hepatic fibrosis (Zhai *et al.,* 2017). Curcumin worked as an active polyphenol has been demonstrated against different diseases including liver fibrogenesis (Tang, 2015). Curcumin can ameliorate CCl_4-induced liver fibrosis by inhibiting transforming growth factor-β1 (TGFβ1) signaling (Yao *et al*., 2012). Anti-inflammatory role of curcumin also contributes to its anti-fibrotic action (Huang *et al.,* 2016). It should be noted that curcumin exerted a suppressive action on leptin-induced HSCs activation (Tang and Chen, 2010), thus inhibiting leptin-induced liver fibrosis and the underlying mechanisms was associated with the promotion role of curcumin in the accumulation of intracellular lipids in HSCs (Tang and Chen, 2010). Our present study provided new mechanism for curcumin's inhibition of leptin-induced liver fibrosis. Namely, curcumin inhibited leptin-induced decrease of miR-122 expression and thus attenuated the promotion effect of leptin on HSCs activation and hepatic fibrosis. We also clarified that curcumin could counteract leptin-induced decline in miR-122promoter activity, which suggested that the impact of curcumin on the role of leptin in miR-122 was, at least in part, through its positive action on activity of miR-122 promoter.

As one of the important transcription factors of adiponectin (Rangwala and Lazar, 2000), Sterol regulatory element binding protein 1c (SREBP-1c) played an important role in HSCs activation inhibition and had the mutual promotional roles between miR-122and SREBP-1c (Zhai *et al.,* 2017).

Thus curcumin-induced accumulation of intracellular lipids in HSCs (Tang and Chen, 2010) might be related to the promotion of curcumin on miR-122 in HSCs.

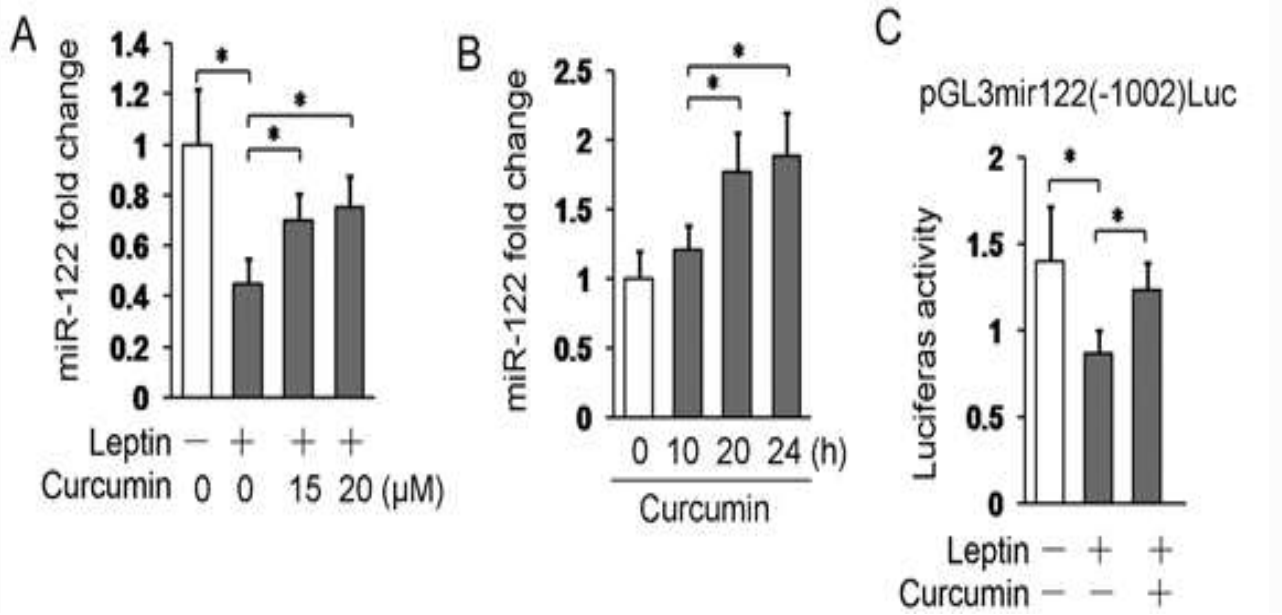

Fig. 1: Curcumin reduces the effect of leptin on miR-122 level in HSCs *in vitro*. a, b Real-time PCR analysis of miR-122 level (n=3). The serum-starved HSCs were pre-incubated with different doses of curcumin for 30 min before treatment with or without 100ng/ml of leptin for another 24h (A), or HSCs were incubated with 100ng/ml of leptin for 24h before addition of 15μM of curcumin for different time periods (B). **P*<0.05. c Analysis of miR-122 promoter activity by luciferase assay (n=3). HSCs were cotransfected with 1.6μg of pGL3miR-122(-1002) Luc and 10ng of control vector pRL-TK. HSCs were pre-incubated with 15μM of curcumin and then treated with 100 ng/ml of leptin for 24h. **P*< 0.05.

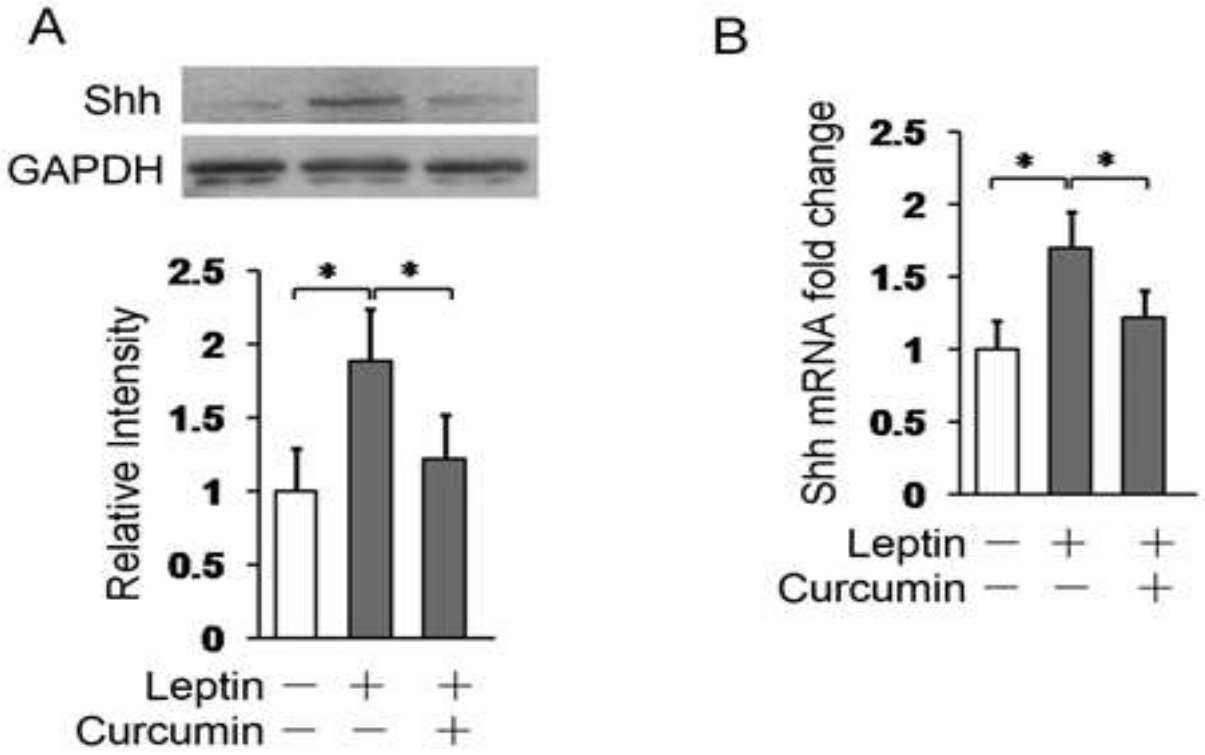

Fig. 2: Curcumin attenuates leptin-induced Shh level in HSCs *in vitro*. Serum-starved HSCs were pretreated with or without 15μM of curcumin for 30 min and then treated with or without 100ng/ml of leptin for 24h. Western blot (a) and Real-time PCR (b) analyses were performed (n=3). **P*< 0.05.

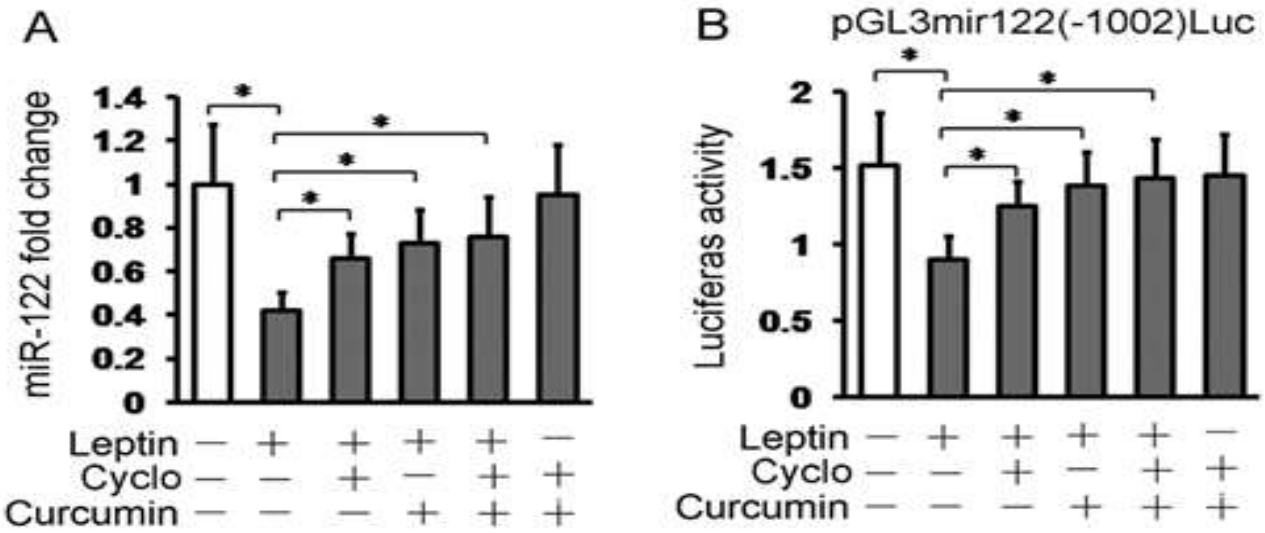

Fig. 3: The interruption of leptin-induced Shh signaling by curcumin contributes to its inhibition of leptin-induced decline in miR-122 level in HSCs *in vitro*. a Real-time PCR analysis of miR-122 level (n=3). Serum-starved HSCs were pretreated with or without 5μM of cyclopamine (Cyclo) or 15μM of curcumin for 30 min and followed by treatment with 100ng/ml of leptin for another 24h. **P*<0.05. b Analysis of miR-122 promoter activity by luciferase assay (n=3). After HSCs were cotransfected with 1.6μg of pGL3miR-122(-1002)Luc and 10ng of control vector, the HSCs were pretreated with or without 5μM of cyclo or 15μM of curcumin for 30 min, followed by treatment with 100ng/ml of leptin for another 24h. **P*<0.05.

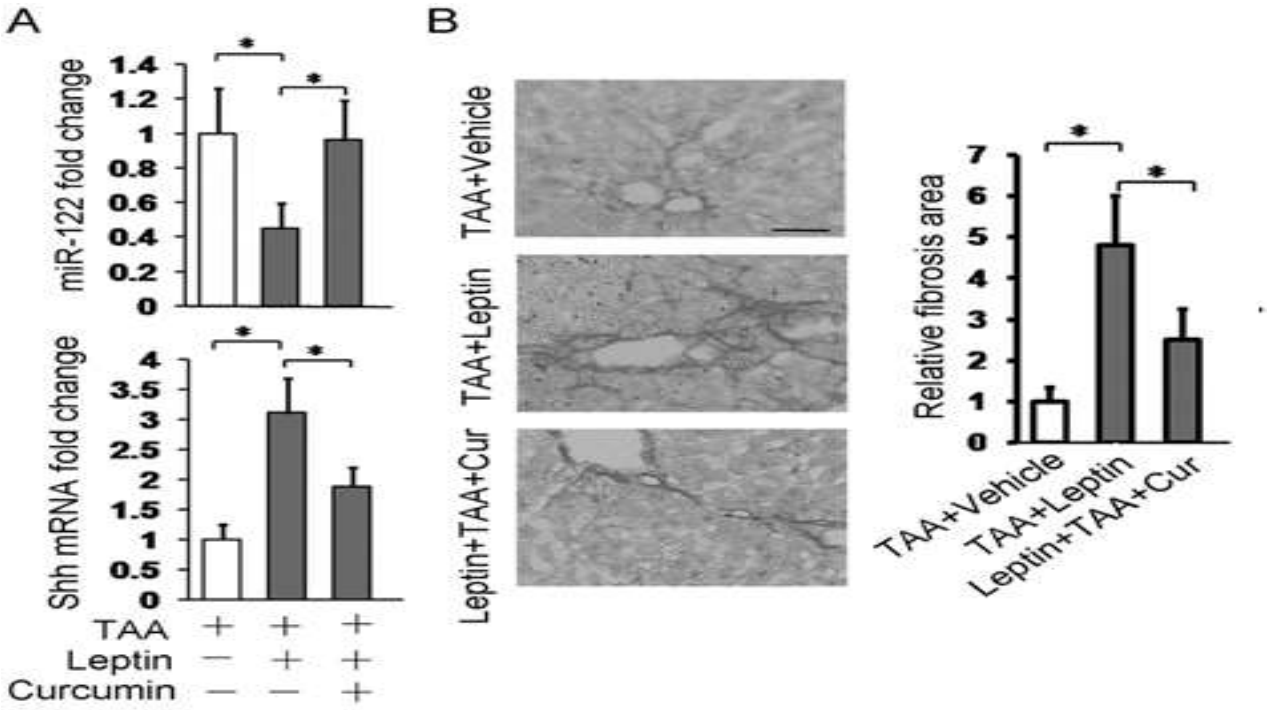

Fig. 4: Curcumin reduces leptin-induced Shh level in HSCs *in vivo*, which was accompanied by the increase in miR-122 and the decrease in liver fibrosis. Three groups (six mice/each group) of C57BL/6J ob/ob mice were received leptin (1μg/g body weight, once per day, by intraperitoneal injection) or curcumin (Cur, 400 mg suspended in PBS/kg body weight, once per day, by gavage) or Vehicle throughout 4-week treatment with TAA (200μg/g body weight, twice a week, by intraperitoneal injection). HSCs were isolated from the livers for real-time PCR analyses of the levels of miR-122 and Shh (a). Parts of the livers were used for evaluating liver fibrosis by Sirius red staining (b). Scale bar: 50μm. **P*< 0.05.

Hh is a classic morphogen with important roles in embryogenesis. Research suggested that the Hh pathway is a crucial regulator of adult liver repair and thus acts as a potential diagnostic and therapeutic target in liver cirrhosis (Machado *et al.,* 2018). Various cytokines stimulate Hh signaling pathway. Platelet-derived growth factor or leptin can induce Hh signaling pathway in HSCs (Choi *et al.*, 2010; Yang*et al.,* 2008), promoting liver fibrogenesis. Curcumin was demonstrated to suppress Shh pathway, thus inhibiting bladder cancer stem cells (Wang *et al.,* 2017), cisplatin-induced renal fibrosis (Maghmomeh *et al.,* 2020) and HSCs activation [34]. Curcumin can also reduce the levels of the protein which are required for HSC proliferation (such as cyclin D1) and HSC apoptosis (such as bcl-2) through Hh pathway, influencing CCl4-induced hepatic fibrosis (Lian *et al.,* 2015). We demonstrated that curcumin exerted an inhibiting effect on leptin-induced Shh expression in HSCs, which led to the increase in miR-122 level followed by the decrease in liver fibrosis. These data also strengthened the notion that Hh might be an important target for the inhibitory effect of curcumin on liver fibrogenesis.

In conclusion, the study revealed that curcumin exerted suppressive effect on leptin-induced decrease in miR-122 *in vitro* cell model. More importantly, the influence of curcumin on leptin-induced decrease in miR-122 was required for its inhibiting Shh pathway, which was followed by alleviating leptin-induced liver fibrosis *in vivo* mouse model. Many results revealed the positive relationship between leptin and liver fibrosis and recent research also suggested that serum leptin level might be associated with the rate of fibrosis progression in nondiabetic patients with chronic HBV infection (Mousa *et al.,* 2018). Therefore, our results might have potential implications for treating liver fibrosis in obese patients.

CONCLUSION

Curcumin reduced leptin-induced decline in miR-122 level in HSCs, inhibiting leptin-caused liver fibrosis. These data might have potential implications for treating liver fibrosis in obese patients.

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