약학석사학위논문

The AMPK activators, HL156A and HL176OUT04 reduce thioacetamide-induced hepatic fibrosis via the inhibition of hepatic stellate cell activation

AMPK activator 인 HL156A 와 HL176OUT04 의 간성상세포 활성화 억제를 통한 TAA 유발 간섬유화 감소 효과 연구

2016 년 8 월

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이 논문을 약학석사학위논문으로 제출함

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신 현 상의 석사학위논문을 인준함

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ABSTRACT

The AMPK activators, HL156A and HL176OUT04 reduce thioacetamide-induced hepatic fibrosis via the inhibition of hepatic stellate cell activation

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Chronic liver injury caused by many etiological factors, toxins, disturbance of the immune system leads to hepatic fibrosis.

Hepatic fibrosis bring about activation of hepatic stellate cells(HSCs), playing significant roles in the development of hepatic fibrosis and excessive accumulation of the extracellular matrix(ECM) proteins, such as collagens mediated by matrix metalloproteinase(MMPs) and tissue inhibitors of metalloproteinases(TIMPs).

In responses liver injury, hepatic stellate cells transforming from a quiescent form to a activated form. Activated hepatic stellate cells causes progression of the liver fibrosis and increase expression of the α-smooth muscle actin(α-SMA), a major marker of HSCs activation and liver fibrosis. HL156A and HL176OUT04, which are noble biguanide derivatives and based on metformin formula. HL156A and HL176OUT04 are widely known that activate AMP-activated protein kinase(AMPK). But function of thE HL156A and HL176OUT04 in liver are poorly understood. Therefore we investigated that anti-fibrotic effects of the HL156A and HL176OUT04 in liver. To investigate that antifibrotic effect on liver fibrosis of HL156A and HL176OUT04, we inject thioacetamide with HL156A and HL176OUT04 in mouse. As a result, we found that HL156A and HL176OUT04 reduce fibrosis marker, collagen, α-SMA in mouse liver. And we treat with HL156A and HL176OUT04 in hepatic stellate cells, whtich are activated by treated TGF-β. Then we found that HL156A and HL176OUT04 inhibit α-SMA expression in hepatic stellate cells.

These results suggest that HL156A and HL176OUT04 inhibit activation of hepatic stellate cells and reduce hepatic fibrosis.

Therefore HL156A and HL176OUT04 can be applied to the anti-fibrotic therapy.

Keywords : liver fibrosis, Hepatic stellate cells(HSCs),
α-smooth muscle actin(α-SMA),
HL156A, HL176OUT

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

1. Liver fibrosis and Hepatic stellate cells(HSCs)

Liver has a capability that adapted to injury through tissue repair system, which interact with multiple pathways and molecules. If the liver injury is repetitive or critical, its wound-healing response become dysregulated. Many etiological factors, toxins, distubance of immune system bring about liver fibrosis [2-4]. Liver fibrosis represents excessive accumulation of the extracellular matrix(ECM) proteins, such as collagens mediated by matrix metalloproteinases(MMPs) and tissue inhibitors of metalloproteinases(TIMPs) and is preceded innate and adaptive immune systems and proliferation and activation of extracellular matrix-produing myofibroblasts [5]. Above all, key mechanisms of fibrosis is activation of hepatic stellate cells(HSCs) (Fig. 1) [1].

Chronic liver injury activate hepatic stellate cells, which are characterized by storing vitamin A in lipid droplets. HSCs are pericytes of the liver and they located in space of Disse between hepatocytes and sinusoidal endothelial cells. When these cells activate, quiescent cells transdifferentate to myofibroblast-like cells, which lose lipid droplets. Activated HSCs upregulate collagen and α-SMA, which are mesenchymal

cell markers. As a rusults, extracellular matrix(ECM) synthesis and degradation are imbalanced and deposition of extracellular matrix(ECM) components [6-8].

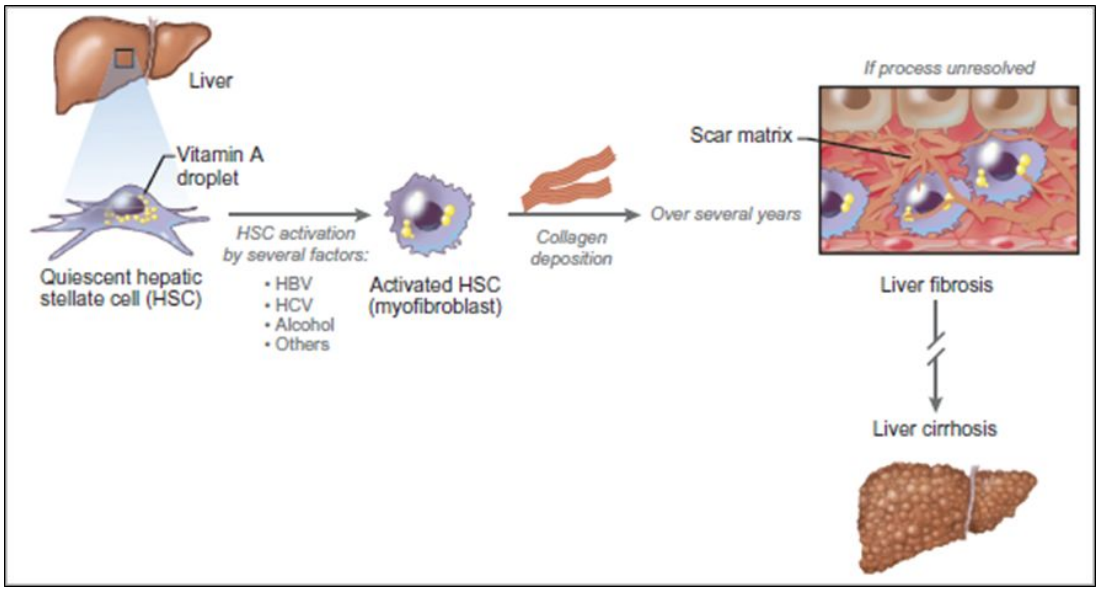

Figure 1. Liver fibrosis and Hepatic stellate cells(HSCs) [1].

2. HL156A and HL176OUT04

HL156A and HL176OUT04 are designed and synthesized by Hanall Biopharma Inc. (Fig. 2). HL156 and HL176OUT04 are derivative of phenyl bigunide that more potently than metformin. Especially, HL156A has been reported to possess anti-fibrotic activity in animal model of peritoneal fibrosis [18]. we hypothesized that HL156A and HL176OUT04 may has anti-fibrotic effects. Therefore we investigated the anti-fibrotic effects of HL156A and HL176OUT04 in mouse model of TAA-induced liver fibrosis in addition in cultured HSCs

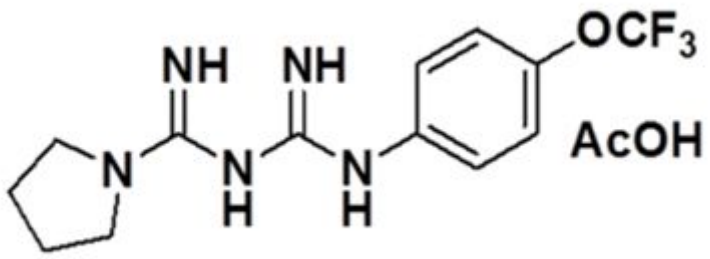

Figure 2. Structures of HL156A.

MATERIALS AND METHODS

1. Animal treatment

We purchased Male-8-week-old C57BL/6 mice from Orient BIO(Seoul, Korea). We assigned the mice to four groups. We injected saline into the vehicle group, and injected thioacetamide into the TAA group intraperitoneally three times a week for a total duration of 6 weeks. The first injection dose of TAA were 50 mg/kg, 100 mg/kg for the second injection and the dose of third to sixth injections were 150 mg/kg and rest of the injections were 300 mg/kg. We injected TAA and HL156A or HL176OUT04 into co-treatment groups. The doses of TAA were same as the TAA group and HL156A or HL176OUT04 were injected intraperitoneally on the alternative days of TAA injection at a dose of either 10 mg/kg of HL156A or 10 mg/kg of HL176OUT04. All our animal studies were approved by the Committee for Care and Use of Laboratory Animals at Seoul National University complying with the Guide for Animal Experiments revised by the Korean Academy for Medical Sciences.

2. Histological analysis and Immunohistochemistry

We performed immunohistochemistry by paraffin sections to investigate expression of collagens. Paraffin sections were de-paraffinized and stained with Sirius Red(Abcam, Cambridge, MA, USA). Also We investigate expression of α-SMA by performing immunohistochemistry by paraffin sections. We performed antigen retrieval with paraffin sections by inucubating in Tris-EDTA buffer(10mM Tris Base, 1mM EDTA, 0.05% Tween 20, pH 9.0) for 40 min at 95℃. The sections were subjected to blocking with 5% normal donkey serum and incubated overnight at 4 ℃ with primary antibodies for alpha smooth muscle actin(α-SMA)(1:200, Dako, San Diego, CA, USA). And The sections were washed in PBS/0.1% tween 20 solution and treated with biotinylated secondary antibody IgG and 3,3'-Diaminobenzidine followed by counter staining with Hochest(sigma). We investigate expression of desmin by performing immunohistochemistry by frozen sections. The sections were fixed by 100% cold methanol for 10 min at 4℃. And the sections were subjected to permeabilization by PBS/0.1% tween 20 solution The sections were subjected to blocking with 5% normal donkey serum and incubated overnight at 4 ℃ with primary antibodies for desmin(1:200, Abcam). After washing with PBS/0.1% tween 20 solution and treated with Alexa-546-conjugated secondary antibodies(1:750, Invitrogen, Carlsbad, CA, USA) followed by counter staining with Hochest(sigma).

We take under fluorescent images by confocal microscope(Carl Zeiss AG, Oberkochen, Germany) and quantify positive areas by Image J software.

3. Cell culture

HSC-T6 cells were cultured in Dulbecco's modified Eagle's medium(DMEM;GeDEPOT, Barker, TX, USA) containing 10% fetal bovine serum(FBS, GenDEPOT). To investigate anti fibrotic effects of HL156 and HL176OUT04 on HCS activation, we took treating with HSC-T6 in dose of TGF-β 10ng/ml. and we also took treating with each 2 drugs at different dose. And we checked effect of expression of α-SMA classed by it's dose.

4. Western blot

We lysed HSC-t6 cell by radioimmunoprecipitation assay(RIPA) buffer containing 25mM Tris pH 7.4, 150mM NaCl, 5mM $MgCl_2$, 0.5% NP-40, phosphatase inhibitor cocktail(sigma) and proteinase inhibitor cocktail(calbiochem, Billerica, MA, USA). We measured protein concentrations by using bicinchoninic acid(BCA) Assay Kit(Thermo). We used antibodies, mouse anti-α-SMA(DAKO, Glostrup, Denmark).

5. MTS assay

I assessed cell viability with nonradioactive cell proliferation MTS assay using cellTiter94Aqueous One Solution Reagent(Promega, Madison, WI, USA). Shortly, 100㎕ cell sespension and 20 ㎕ CellTiter96AQueousOne Solution Reagent were incubated in 96-well pates for 1 hour at 37 ℃, 5% CO_2, absorbance was measured at 570 nm on a ELISA.

RESULTS

1. HL156A, HL176OUT04 reduces TAA-induced liver fibrosis in mice

TAA is one of the common hepatotoxins used in experimental liver fibrosis that causes centrilobular firosis [10]. Since TAA-induced liver fibrosis is reversible by the withdrawl of TAA exposure, mice were treated with HL156A, HL176OUT04 and TAA together to investigate two drugs' s anti-fibrotic effect in liver. Repeated TAA injection for a total duration of 6 weeks resulted in inflammation and alteration of liver histology. Masscive ECM deposition was also obvious in TAA group as revealed by Sirius Red staining. Co-treatment with HL156, HL176OUT04 significantly reduced TAA-induced ECM deposition compared to TAA group (Fig. 3).

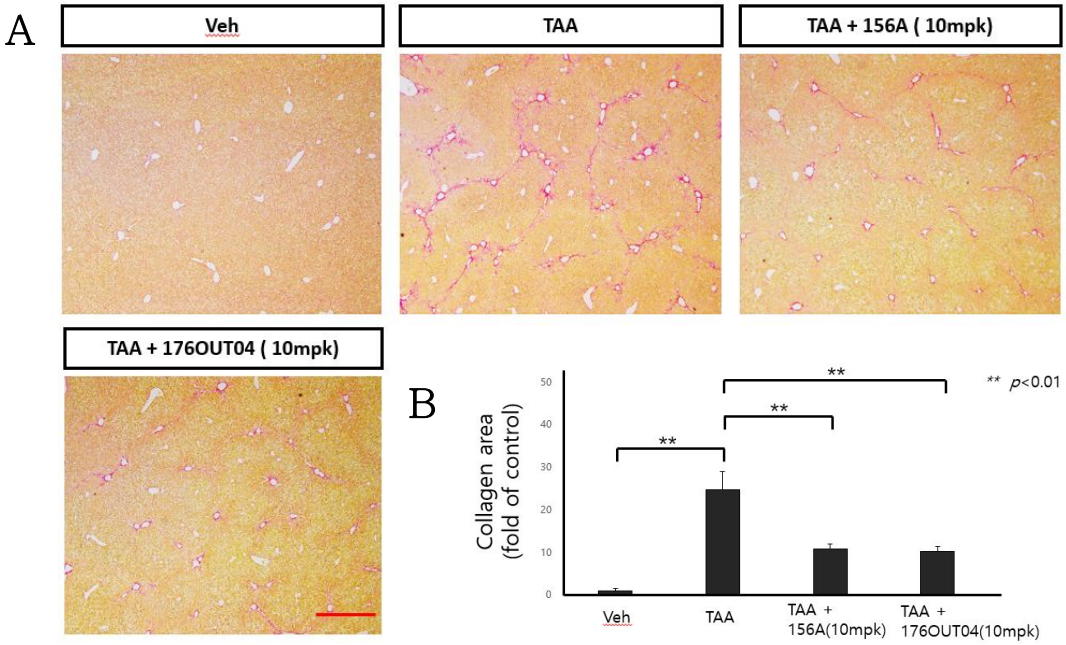

Figure 3. HL156A, HL176OUT04 reduce expression of collagen.

(A) Sirius Red images of mouse liver section o four experimental mice groups. Veh, vehicle; TAA, TAA alone; TAA+156A(10 mpk), co-administration of TAA and HL156A at dose of 2 mg/kg; TAA+176OUT04(10 mpk), co-administration of TAA and 176OUT04 at a dose of 10 mg/kg. Scale bar represents 100 pixel. (B) Bar graph represents relative Sirius Red-positive area of each group. Error bars showing S.E.M, $**p < 0.01$.

2. TAA-induced HSC activation and endothelial capillarization were reduced by HL156A, HL176OUT04

The activation of hepatic stellate cells is one major feature of hepatic fibrosis, which is characterized by the expression of myofibroblast markers such as α-SMA and desmin. To investigate that antifibrotic effect on liver fibrosis of HL156A and HL176OUT04, we analyzed liver histology using myofibroblast markers. As shown earlier, TAA administration caused up-regulation of α-SMA and desmin-positive cells (Fig. 4A, B). HL156A and HL176OUT04 significantly reduced expression of α-SMA and desmin (Fig. 4A, B). By these results, We supposed that HL156A, HL176OUT04 inhibits HSC activation, which is a crucial step in hepatic fibrogenesis.

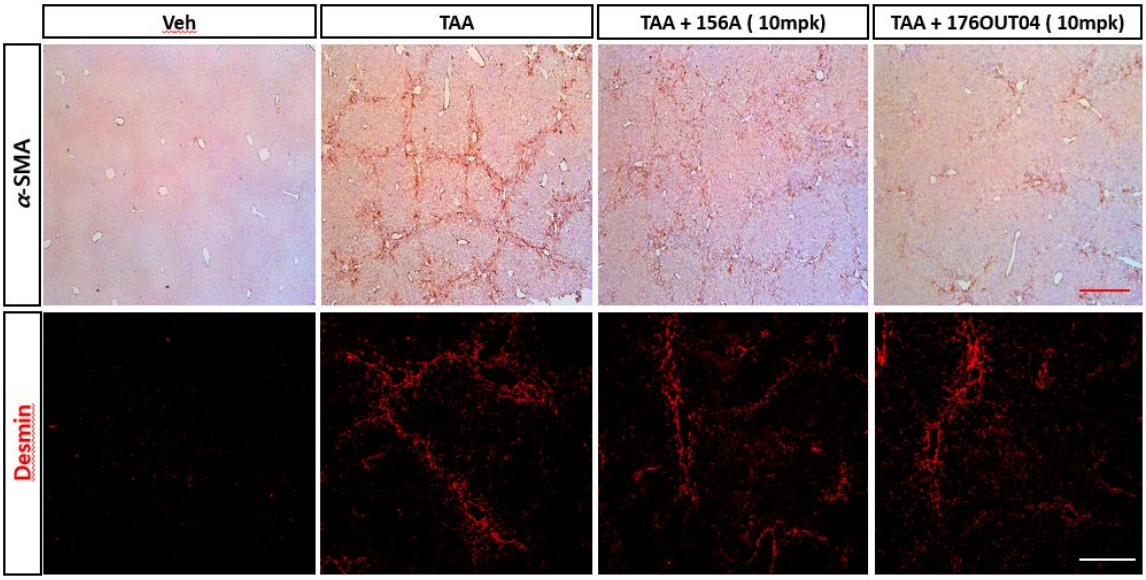

Figure 4. HL156A, HL176OUT04 decrease α-SMA and desmin.

Representative fluorescence image for α-SMA(upper row) and desmin(lower row) staining of liver sections. Scale bar represents 100 pixel.

3. HL156A, HL176OUT attenuates TGF-β1-mediated HSC activation.

The activation of hepatic stellate cells is major feature of hepatic fibrosis. The activation of hepatic stellate cells is characterized by the expression of myofibroblast markers. To further study the anti-fibrotic effect of HL156A in vitro, we took advantage of the rat HSC cell line, HSC-T6 cells. MTS assay was carried out to determine proper dose of HL156, HL176OUT04 without cytotoxicity. HSC-T6 cells were treated with serial doses of HL156A ranging from 1 μM to 100 μM for 24 h and relative cell viabilities were measured. No significant cytotoxicity was observed up to 20 μM while HL156A concentrations over 50 μM showed some cytotoxicity (Fig. 5). HL176OUT04 was no obvious cytotoxic in every tested dose(Fig. 5). As shown earlier, TAA administration upregulate expression of α-SMA and hepatic stellate cells, which is desmin-positive cell. The treatment of HSC-T6 cells with TGF-β1 resulted in induction of α-SMA; simutaneous tratment with HL156A attenuated TGF-β1-mediated α-SMA induction (Fig. 6). By these results, We found that HL156A and HL176OUT04 inhibits HSC activation, which is a liver fibrogenesis.

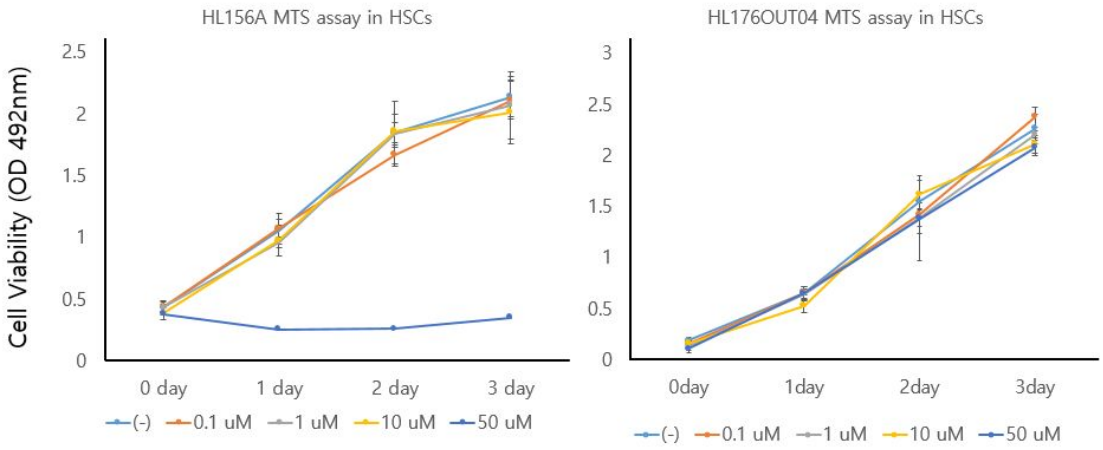

Figure 5. Effects of HL156A, HL176OUT04 on cell viability of HSCs.

MTS assay to check cytotoxicity of HL156A and HL176OUT04. The result was that there was no obvious cytotoxic except 50uM in HL156A(left). In HL176OUT04, there was no obvious cytotoxic in every tested doseI(right).

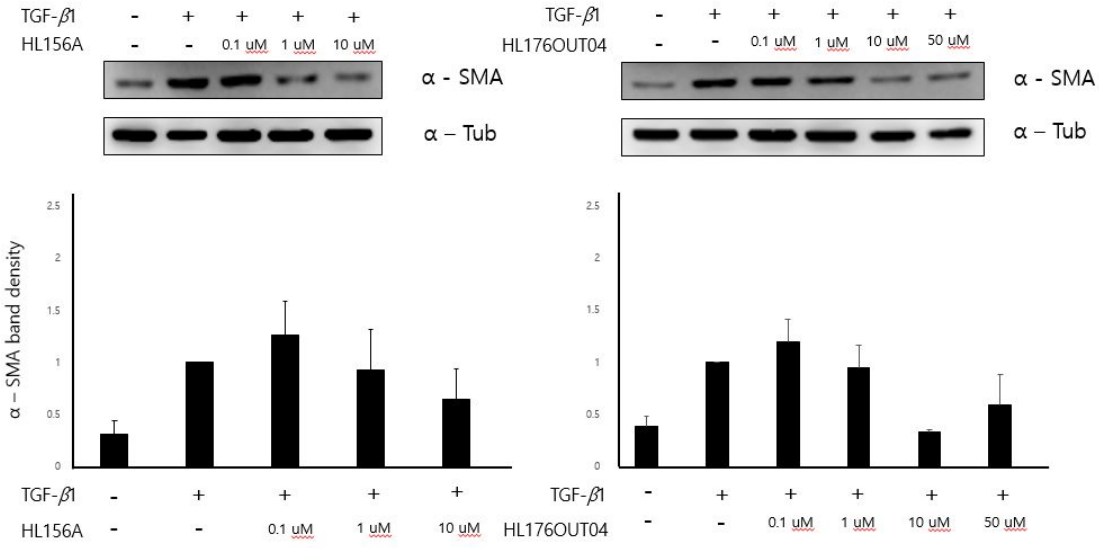

Figure 6. HL156A, HL176OUT04 inhibits α-SMA expression in HSCs.

HSC-T6 cells were treated with TGF-β1 (10 ng/ml) alone or in co-treatment with HL156A and HL176OUT04 at indicated dose. TGF-β1 upregulated expression of α-SMA. But western blot shows that HL156A and HL176OUT04 reduce TGF-β1-induced α-SMA expression.

DISCUSSION

Liver cirrhosis a lift-threatening disease which is closely associated with liver cancers, however, there are no approved agents as anti-fibrotic drugs to date. We demonstrate that the potential anti-fibrotic effect of a AMPK activator, HL156A and HL1760UT04. It reduced collagen, desmin and α-SMA, which are induced by TAA administration. We found that HL156, HL176OU04 diminish activation of HSCs by Histological analysis. Also *In vitro* experiments using rat HSC cell line further proved the anti-fibrotic effect of HL156A, HL1760UT04 on TGF-β1-induced HSC activation. However, further studies would be needed to find out the exact mechanism in *in vivo* settings.

As Fibrosis is a wound healing response, multiple organs commonly bring out fibrosis upon chronic tissue damages [11]. Fibrosis of various organs are involved in α_V integrin and TGF-β signaling pathways [12-13]. Many human cancers are caused by Metabolic derangement. Therefore AMPK activators could be a potentially effective agent for cancer therapy [14]. As the importance of metabolic dysfuction in liver cancer, It has been studied significantly in recent years. There are AMPK signaling pathway, which play a crucial role in hepatocellular carcinogenesis [15-17]. Liver is the most closely involved in cancer among organs that progress fibrosis [18]. Actually, approximately 90% of hepatocellular carcinoma (HCC) arise from

cirrhotic livers [19]. Through these research results, targeting liver fibrosis and HCC simultaneously could be an efficient anti-fibrotic therapeutic agent.

REFERENCES

1. Mona H Ismil, Massimo Pizani: Reversal of hepatic fibrosis: pathophysiological basis of antifibrotic therapies. Hepatic Medicine: Evidence and Research 3: 69-80, 2011.

2. Lee SJ, Kim KH and Park KK: Mechanisms of fibrogenesis in liver cirrhosis: The molecular aspects of epithelial-mesenchymal transition. *World J Hepatol* 6: 207-216, 2014.

3. Seki E and Schwabe RF: Hepatic inflammation and fibrosis: functional links and key pathways. *Hepatology* 61: 1066-1079, 2015.

4. Pellicoro A, Ramachandran P, Iredale JP and Fallowfield JA: Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat Rev Immunol* 14: 181-194, 2014.

5. Friedman SL: Mechanisms of hepatic fibrogenesis. *Gastroenterology* 134: 1655-1669, 2008.

6. Iwakiri Y, Shah V and Rockey DC: Vascular pathobiology in chronic liver disease and cirrhosis - current status and future directions. *J Hepatol* 61: 912-924, 2014.

7. Langer DA, Das A, Semela D, *et al*: Nitric oxide promotes caspase-independent hepatic stellate cell apoptosis through the generation of reactive oxygen species. *Hepatology* 47: 1983-1993, 2008.

8. Deleve LD, Wang X and Guo Y:Sinusoidal endothelial cells prevent rat stellate cell activation and promote reversion to quiescence. *Hepatology* 48: 920-930, 2008.

9. Ju KD, Kim HJ, Tsogbadrakh B, *et al*: HL156A, a novel AMP-activated protein kinase activator, is protective against peritoneal fibrosis in an in vivo an in vitro model of peritoneal fibrosis. *AM J Physiol Renal Physiol* 310: F342-350, 2016.

10. Liu Y, Meyer C, Xu C, *et al*: Animal models of chronic liver diseases. *Am J Physiol Gastrointest Liver Physiol* 304: G449-468, 2013.

11. Rockey DC: Current and future anti-fibrotic therapies for chronic liver disease. *Clin Liver Dis* 12: 939-962, xi, 2008.

12. Henderson NC, Arnold TD, Katamura Y, *et al*: Targeting of alphav integrin identifies a core molecular pathway that regualtes fibrosis in several organs. *Nat Med* 19: 1617-1624, 2013.

13. Munger JS, Huang X, Kawakatsu H, *et al*: The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmunary inflammation and fibrosis. *Cell* 96: 319-328, 1999.

14. Kuhajda FP: AMP-activated protein kinase and hyman cancer: cancer metabolism revisited. *Int J Obes (Lond)* 32 Suppl 4: S36-41, 2008.

15. Ferretti AC, Tonucci FM, Hidalgo F, Almada E, Larocca MC and Favre C: AMPK and PKA interaction in the regulation of survival of liver cancer cells subjected to glucose starvation. *Oncotarget* 2016.

16. Park SY, Lee YK, Kim HJ, Park OJ and Kim YM: AMPK interacts with beta-catenin in the regulation of hepatocellular carcinoma cell proliferation and survival with selenium treatment. *Oncol Rep* 35: 1566-1572, 2016.

17. Yang CC, Chang SF, Chao JK, *et al*: Activation of AMP-activated protein kinase attenuates hepatocellular carcinoma cell adhesion stimulated by adipokine resistin. *BMC Cancer* 14: 112, 2014.

18. Zhang DY and Friedman SL: Fibrosis-dependent mechanisms of hepatocarcinogenesis. *Hepatology* 56: 769-775, 2012.

19. Seitz HK and Stickel F: Risk factors and mechanism of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. *Biol Chem* 387: 349-360, 2006.

20. Fattovich G, Stroffolini T, Zagni I and Donate F: Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 127: S35-50, 2004.

21. Grahame Hardie D: Regulation of AMP-activated protein kinase by natural and synthetic activators. *Acta Pharm Sin B* 6: 1-19, 2016.

22. Vallianou NG, Evangelopoulos A and Kazazis C: Metformin and cancer. *Rev Diabet Stud* 10: 228-235, 2013.

23. Salt IP and Palmer TM: Exploiting the anti-inflammatory effects of AMP-activated protein kinase activation. E*xpert Opin Investing Drugs* 21: 1155-1167, 2012.

24. Salminen A, Kaarniranta K, Haapasalo A, Soininen H and Hiltunen M: AMP-activated protein kinase: a potential player in Alzheimer's disease. *J Neurochem* 118: 460-474, 2011.

25. Li J, Pan Y, Kan M, *et al*: Hepatoprotective effects of berberine on liver fibrosis via activation of AMP-activated protein kinase. *Life Sci* 98: 24-30, 2014.

26. Tripathi DM, Erice E, Lafoz E, *et al*: Metformin reduces hepatic resistance and portal pressure in cirrhotic rats. AM J Physiol Gastrointest Liver Physiol 309: G301-309, 2015.

27. Lim JY, Oh MA, Kim WH, Sohn HY and Park SI: AMP-activated protein kinase inhibits TGF-beta-induced fibrogenic responses of hepatic stellate cells by targeting transcriptional coactivator p300. *J cell Physiol* 227: 1081-1089, 2012.

ABSTRACT IN KOREAN
(국 문 초 록)

독성물질, 면역계의 이상과 같은 병인적 요인들로 인해 야기된 만성적인 간 손상은 간 섬유화로 이어진다. 간 섬유화는 간 섬유화 진행에 중요한 역할을 하는 간성상 세포(hepatic stellate cell)를 촉진을 발생시킨다. 간성상 세포는 또한 metalloproteinase(MMPs)와 tissue inhibitors of metalloproteinases(TIMPs)에 의해 일어나는 콜라겐과 같은 세포외기질 단백질의 과도한 축적에도 중요한 역할을 한다. 간의 손상에 대한 반응으로 간성상세포는 휴지기 형태에서 활성화된 형태로 변화한다. 촉진된 간성상세포는 간섬유화 진행을 야기하고 간성상 세포의 촉진과 간섬유화의 주요한 지표인 α-smooth muscle actin(α-SMA)의 발현을 증가시킨다. HL156A와 HL176OUT04는 메트포린에 기반을 둔 효과가 좋은 비구아니드 유도체이다. HL156A와 HL176OUT04는 AMP-activated protein kinase(AMPK)을 촉진하는 물질로 잘 알려져 있다. 그렇지만 HL156A와 HL176OUT04의 간에서의 기능은 잘 알려져 있지 않다. 그래서 우리는 간에서의 HL156A와 HL176OUT04의 항섬유화 효과를

연구하였다. 간섬유화에서 HL156A와 HL176OUT04의 항섬유화 효과를 연구하기 위하여 쥐에 TAA를 HL156A와 HL176OUT04와 함께 주입하였다. 그 결과 우리는 HL156A와 HL176OUT04가 쥐의 간에서 간섬유화의 지표인 콜라겐, α-SMA를 감소시킨다는 것을 발견하였다. 그리고 우리는 HL156A와 HL176OUT04를 간성상세포를 촉진시키는 TGF-β와 함께 간성상에 주입하였다. 그리고 나서 우리는 HL156A와 HL176OUT04가 간성상세포에서 α-SMA의 발편을 감소시키는 것을 발견하였다. 이 결과들을 바탕으로 우리는 HL156A와 HL176OUT04가 간성상세포의 촉진을 억제하고 간섬유화를 감소시킬 것이라고 추측하였다. 그러므로 HL156A와 HL176OUT04는 항섬유화 치료제로 쓰일 수 있을 것이다.

주요어 : 간섬유화, 간성상세포,
α-smooth muscle actin(α-SMA), HL156A, HL176OUT

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