別記様式第 6 号（第 16 条第 3 項，第 25 条第 3 項関係）

論 文 審 査 の 結 果 の 要 旨

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学 位 授 与 の 条 件	学位規則第 4 条第①・2 項該当		

論 文 題 目
In Vitro Analysis of Hepatic Stellate Cell Activation influenced by Transmembrane 6 superfamily 2 Polymorphism
（TM6SF2 遺伝子多型に影響される肝星細胞活性化に関する基礎的解析）

論文審査担当者

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〔論文審査の結果の要旨〕

Non-alcoholic fatty liver disease (NAFLD), is becoming one of the most prevalent chronic liver diseases in modern countries, increasing rapidly as a result of recent upward trends in obesity and life-style changes. A subset of NAFLD patients go on to develop non-alcoholic steatohepatitis (NASH) by progression of steatosis and necro-inflammatory changes in the liver, leading to an increase in the incidence of cirrhosis and hepatocellular carcinoma. Mortality in NAFLD patients has been reported to be independently associated with the stage of liver fibrosis, and it is important to prevent the progression of liver fibrosis in NAFLD patients. Recently, several drugs have been developed and have entered phase 2 or 3 clinical trials, but no effective drugs against NAFLD are yet available. Therefore, it is important to clarify the mechanism of liver fibrosis in NAFLD in order to identify therapeutic targets. To identify clinical factors associated with the progression of liver fibrosis in NAFLD patients, several genome wide association studies have recently been performed worldwide. A single nucleotide polymorphism (SNP), rs738409 in patatin-like phospholipase domain containing 3 was identified as having strong associations with prevalence and disease progression in NAFLD and NASH. A SNP in transmembrane 6 superfamily 2 (TM6SF2) gene was also identified as a potential contributor to NAFLD pathogenesis. The SNP rs58542926 in TM6SF2 is significantly associated with incidence of NAFLD and with fibrosis stage. TM6SF2 protein is highly expressed in the small intestine and liver and plays a role in lipid synthesis and secretion of triglyceride-rich lipoproteins in the liver. TM6SF2 rs58542926 (C>T), a coding SNP that causes an amino acid substitution at codon 167 (E167K), is considered to lead to a loss of function and to accelerate hepatic steatosis. Hepatic stellate cells (HSCs) plays a main role in development of liver fibrosis. The influence of the coding SNP in TM6SF2 on the function of HSCs has not been clarified. HSCs are normally activated in response to stimulation by inflammatory cytokines, such as transforming growth factor beta 1 (TGF-β1). Activated HSCs transform into myofibroblasts and produce large amount

of alpha-smooth muscle actin (αSMA). Activation of HSCs leads to secretion of extra-cellular matrix proteins such as collagen type 1 into the sinusoids, resulting in collagen accumulation and progression of liver fibrosis. In the present study, this author aimed to explore the role of TM6SF2 SNP rs58542926 in liver fibrosis using an *in vitro* activated HSC model.

To create TM6SF2 overexpression model, human TM6SF2 mRNA was amplified from LX-2 cells and cloned into p3xFLAG-CMV-10 vector. The cloned plasmid containing the wild-type CC genotype at rs58542926 in TM6SF2 gene was designated as p3FLAG/TM6SF2-WT. Subsequently, a modified plasmid, designated as p3FLAG/TM6SF2-MT, was generated by introducing a C-to-T point mutation at rs58542926 in TM6SF2 to create an amino acid substitution E167K in the TM6SF2 gene. The activation of LX-2 cells was analyzed by overexpressing or knocking down TM6SF2 under TGF-β1 treatment. To knockdown of TM6SF2 in LX-2 cells, TM6SF2 siRNA was designed by siDirect using the TM6SF2 mRNA sequence (NM_001001524) as a reference. Prepared siRNAs were transfected into LX-2 cells by electroporation at 1,100 mV for 30 msec followed by 24-hour incubation with serum-free medium. Twenty-four hours after transfection, transfected cells were stimulated with 10 ng/ml of TGF-β1 for 48 hours and then the cells were harvested.

This author first analyzed the association between TM6SF2 and the activation of human HSCs. Intracellular αSMA mRNA expression in LX-2 cells was suppressed by TM6SF2 overexpression, and its expression was increased by knocking down TM6SF2. While αSMA expression in TM6SF2-knocked down cells was 1.5~2.0-fold elevated compared to control cells in both mRNA and protein levels. Since similar results were observed in the other experiments, these data suggest that TM6SF2 negatively regulates HSC activation.

Then I analyzed the impact of TM6SF2 on HSC activation via TGF-β1 signaling. Although intracellular αSMA expression in both control cells and TM6SF2 over-expressed cells was significantly up-regulated by TGF-β1 treatment, αSMA expression in TM6SF2 over-expressed LX-2 cells was significantly lower than that in control LX-2 cells after TGF-β1 treatment. Similar results were observed in TM6SF2 knock down cells. A similar tendency was also observed in intracellular collagen type 1 α1 expression measured using real time PCR. These results indicate that lower TM6SF2 expression could activate HSCs and that TGF-β1 could enhance this HSC activation.

Overexpression of wild type TM6SF2 in LX-2 cells resulted in greater intracellular αSMA decrease than mutant TM6SF2 overexpressed cells. Furthermore, αSMA expression in TM6SF2-mutant-overexpressed LX-2 cells increased to similar levels as control LX-2 cells after treatment with TGF-β1. Although the precise regulation mechanism of TM6SF2 in HSCs is yet to be determined, our results suggest that the TM6SF2 E167K isoform affects HSC sensitivity by enhancing the response to TGF-β1.

This paper greatly contributed for the understanding of progression of NASH fibrosis by clarifying the role of the TM6SF2 rs58542926 SNP on production of αSMA in HSCs. Therefore, all the committee members admitted that this paper is of sufficient value to confer the author in Medical Science.