Evaluation of Fbxw7 expression and its correlation with expression of SREBP-1 in a mouse model of NAFLD

KANGSHENG TU, XIN ZHENG, GUOZHI YIN, XIANFENG ZAN, YINGMIN YAO and QINGGUANG LIU

Department of Hepatobiliary Surgery, The First Affiliated Hospital of the Medical College of Xi'an Jiaotong University, Xi'an 710061, P.R. China

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Abstract. The problems of aging, obesity and a number of types of metabolic syndromes, including diabetes, are associated with a higher risk of non-alcoholic fatty liver disease (NAFLD). However, the mechanisms of occurrence and development of NAFLD in humans remain unclear. Sterol regulatory element binding protein (SREBP)-1 is a transcription factor that is important in the development of NAFLD, which regulates the expression of lipogenic genes. SREBP-1 might be degraded through an F-box and WD repeat domain-containing 7 (Fbxw7)-dependent degradation. However, whether or not there is a correlation between Fbxw7 and SREBP-1 in NAFLD remains to be determined. The aim of this study was to investigate whether there was a reduction of Fbxw7 in NAFLD and an induced accumulation of SREBP-1 in a mouse model of NAFLD. Forty C57BL/6J mice were divided into control and HF groups and maintained on normal or high-fat (HF) diets. Following 8 weeks of treatment, the mice were sacrificed and assays of blood biomarkers typical of human NAFLD were performed. Liver samples were processed for histological examination. Fbxw7 mRNA expression was detected by reverse transcription-polymerase chain reaction (RT-PCR) and the protein expression of Fbxw7 and SREBP-1 was evaluated by immunohistochemistry and western blot analysis in the mouse liver tissues in the control and HF groups. The mRNA and protein expression of Fbxw7 was significantly decreased in the HF group compared with the control group (P<0.05, for both). In the HF group, the Fbxw7 protein expression was negatively correlated with SREBP-1 (r=-0.584; P<0.05). Fbxw7 was decreased in NAFLD and negatively correlated with SREBP-1, indicating that the Fbxw7-SREBP-1 axis may play a key pathological role in the development of NAFLD.

Correspondence to: Dr Qingguang Liu, Department of Hepatobiliary Surgery, The First Affiliated Hospital of the Medical College of Xi'an Jiaotong University, Xi'an 710061, P.R. China
E-mail: tks0912@foxmail.com

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common type of chronic liver disease in Western Europe, America, Australia and Japan, and is secondary to the growing epidemic of metabolic syndrome (MetS) (1,2). Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD; this subset of the population exhibits hepatic steatosis accompanied by inflammation and fibrosis (3). NASH is the hepatic manifestation of MetS associated with obesity, non-insulin-dependent diabetes and hypertriglyceridemia (4,5).

To control the development of NAFLD, it is significant to determine the precise mechanism of regulation of lipid accumulation in the human liver. Nakamuta et al (6) investigated fatty acid synthesis in NAFLD and reported that the de novo synthesis of fatty acids, including acetyl-CoA carboxylase 1 (ACC1), ACC2, glycerol-3-phosphate acyltransferase (GPAT), stearoyl-Co A desaturase-1 (SCD) and fatty acid synthase (FAS), were upregulated in NAFLD patients. The expression of these lipogenic genes was regulated by sterol regulatory element binding protein-1 (SREBP-1) at the transcriptional level in response to insulin and glucose. SREBPs are transcription factors that belong to the basic helix-loop-helix leucine zipper family. SREBP-1, a SREBP isoform, is mostly expressed in the liver and regulates fatty acid synthesis (7,8). SREBP1 transgenic mice also manifest pronounced NASH (9). These findings indicate that SREBP1 is important in the development of NAFLD.

SREBP1 is degraded in a Fbxw7-dependent manner (10). Fbxw7 (also known as Fbw7, SEL-10, hCdc4 and hAgo) is a member of the F-box protein family which determines the substrate specificity of the SCF-type ubiquitin ligase (E3) complex. SCF\textsubscript{Fbw7} is a type of E3 that belongs to the ubiquitin-proteasome system (UPS). SCF\textsubscript{Fbw7} consists of the RING-finger protein ring-box 1 (Rbx1; also known as Roc1 and Hrt1), the scaffold protein cullin 1 (Cul1) and the adaptor protein S-phase kinase-associated protein 1 (Skp1), in addition to an F-box protein (11). Fbxw7 targets various mammalian proteins for degradation, including cyclin E, c-Myc, c-Jun, SREBPs, mammalian target of rapamycin (mTOR) and PPAR-γ coactivator-1α (PGC-1α) (11).

In light of the above findings, we hypothesized that there may be a reduction of Fbxw7 in NAFLD and an induced accumulation of SREBP-1. To investigate this hypothesis, we used a...
mouse model of NAFLD and initially detected the expression of Fbxw7 in the liver tissues by reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry and western blot analysis, and then evaluated its correlation with the expression of SREBP-1 at the protein level.

Materials and methods

Animals and diets. C57BL/6J mice (n=40; age, 5 weeks) purchased from the Centre of Laboratory Animals, The Medical College of Xi’an Jiao Tong University (Xi’an, China) were acclimatized to a vivarium (temperature, 23-25˚C; 12 h light/dark; 50-60% humidity) for 1 week. All animal protocols were approved by the Institutional Animal Care and Use Committee. Following acclimatization, animals were weighed and divided into two groups (n=20 each). The mice in the experimental group were fed HF diets (45% calories as fat), while the mice in the control group were maintained on a low-fat diet (10% calories as fat) for 8 weeks. The mice had access to food and water ad libitum. Mouse chow was purchased from Research Diets Inc. (New Brunswick, NJ, USA) (12).

Measurement of triglyceride and total cholesterol levels in the liver. The frozen liver tissue was homogenized, triglyceride and total cholesterol were extracted from the homogenate with chloroform/methanol (2:1, vol/vol), and then dried and resuspended in 2-propanol. The amounts of triglyceride and total cholesterol in the extract were measured using Lipidos liquid and Cholescolor liquid kits, respectively (Toyobo, Osaka, Japan).

Histopathological and biochemical analyses. Tissue samples excised from the liver were fixed with 4% paraformaldehyde before processing for histological analyses by conventional methods. Step sections (5 µm) of the liver were obtained and stained with hematoxylin and eosin (H&E). Frozen sections were stained with Oil Red O (Nakalai Tesque Inc., Kyoto, Japan), according to the manufacturer’s instructions, to examine the extent of lipid accumulation in the hepatocytes. Stained sections were evaluated (light microscopy; magnification, x40) by a pathologist (Department of Pathology, The First Affiliated Hospital of the Medical College of Xi’an Jiao Tong University, Xi’an, China) in a blinded analysis. Plasma levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), free fatty acids (FFA), low-density lipoprotein-cholesterol (LDL-c), high-density lipoprotein-cholesterol (HDL-c), triglycerides (TG) and total cholesterol (TC) were measured using a standard clinical autoanalyzer.

RT-PCR. The frozen specimens for RT-PCR were stored at -80˚C. The RNAfast 200 purification kit was obtained from Shanghai Fastagen Biotech Ltd. Co., Shanghai, China; the PrimeScript RT reagent kit was purchased from Takara Bio Inc., Shiga, Japan and the 2X Taq Master mix was obtained from Beijing CoWin Biotech., Beijing, China. The Fbxw7 primers for RT-PCR, designed by Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA), were: sense, 5’-TGATGACATCAAGAAGGTGGTGAA-3’; and antisense, 5’-TTGCATCATATGCTTCACTTGTG-3’. The PCR product was ~117 bp.

Protein expression revealed by western blot analysis. For western blot analysis, ~200 mg of liver samples were homogenized in 1 ml of lysis buffer (20 mM Tris, 145 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton-X, 0.5% Nonidet P-40, 100 M phenylmethylsulfonyl fluoride, 50 M Na F and 1 mM sodium orthovanadate). Lysates were centrifuged at 8850 x g at 4˚C for 10 min. The supernatant was collected, and the protein concentration was determined using the Bradford method; bovine serum albumin was used as a standard. Samples (1.5 g/mm gel thickness) were heated for 5 min under reducing conditions and loaded onto sodium dodecyl sulfate-poly-
acrylamide gels. Proteins from the gels were then transferred onto nitrocellulose membranes. Electrobots were blocked in Tris-buffered NaCl-Tween (TBST) containing 5% skimmed milk powder at room temperature. Western blot analysis was then conducted using specific antibodies for Fbxw7 and SREBP-1. Blots were incubated with antibodies of interest (in TBST buffer and 5% bovine serum albumin) and agitated overnight at 4°C. Following washing with TBST, the blots were incubated with horseradish peroxidase-labeled anti-rabbit antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) in skimmed milk for 1 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence. Bands were quantified by scanning densitometry and expressed as arbitrary units.

Statistical analysis. Data were shown as the mean ± standard deviation (SD) and were analyzed using the two-tailed Student's t-test. Additionally, the Spearman's rank test was used to analyze the correlation between the Fbxw7 protein expression and SREBP-1. P<0.05 was considered statistically significant. SPSS 13.0 software package was used for all statistical analyses (SPSS Inc., Chicago, IL, USA).

Results

Mouse model for NAFLD. The HF diet increased body weight and adiposity in C57BL/6J mice (Fig. 1A). Body weight and liver weight (wet) in the HF group were significantly higher than those of the control mice (45.10±2.56 vs. 29.40±2.10; 5.16±0.28 vs. 2.56±0.25, respectively; P<0.05) (Fig. 1A). Elevated plasma AST, ALT, AP and GGT blood biomarkers were associated with liver dysfunction in human NAFLD (Fig. 1B). The HF diet increased plasma FFA to an average of 39 mg/l in the HF group, which was higher than that in the control group, but not statistically significant (P=0.063). No significant changes were observed in plasma TG concentrations. However, clear hypercholesterolemia was observed in the HF group, mostly due to an increase in LDL-c, although no significant alterations occurred in HDL-c (Fig. 1C). TG levels were significantly increased in the livers of the HF group compared with those of the control group (P<0.05). The concentration of TC was not affected in the HF group (Fig. 1D).

Liver histology of the control and HF groups was evaluated to investigate the occurrence of NAFLD. Representative images of liver H&E- and Oil Red O-stained sections from the control and HF diet groups were captured. Fig. 2A shows sections of the liver from the control animals demonstrating no steatosis, inflammation or fibrosis. Macrovesicular and microvesicular steatosis was observed in the HF group (Fig. 2B). Oil Red O staining demonstrated an accumulation of triglyceride molecules in the HF group (Fig. 2D), which was significantly higher than that in the control group (Fig. 2C).

Expression of Fbxw7 in the control and HF groups. Following analysis of the RT-PCR results, the expression of Fbxw7 mRNA...
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In the HF group, the expression of SREBP-1 was found to be significantly lower than that in the control group (0.43±0.12 vs. 0.70±0.18; P<0.05) (Fig. 3A). Immunohistochemistry results revealed that the majority of the positive liver cells demonstrated diffuse cytoplasmic staining of Fbxw7, while nuclear staining was identified in few cells (Fig. 4). Results of the western blot analysis indicated that the expression of the Fbxw7 protein was also significantly lower in the HF group compared to the control group (0.44±0.04 vs. 0.82±0.08; P<0.05) (Fig. 3B). Additionally, the results were in accordance with those at the mRNA level.

Expression of SREBP-1 in the control and HF groups. The protein expression of SREBP-1 was assessed by immunohistochemical and western blot analysis. SREBP-1 protein was localized in the cytoplasm and nucleus (Fig. 4) and was significantly elevated in the HF group (P<0.05) (Fig. 3B).

Correlation between Fbxw7 and SREBP-1 protein expression in the HF group. As mentioned above, the Fbxw7 protein expression in the HF group was significantly lower compared to the control group. However, the results showed SREBP-1 protein to be higher in the mouse liver tissues in the HF group compared to in the control group. Spearman's rank test analysis revealed a significant negative correlation between Fbxw7 and SREBP-1 at the protein level (r=-0.584, P<0.05).

Discussion

The abundance of cell proteins is regulated in a coordinated manner at the levels of synthesis and degradation (13). In particular, intracellular proteolysis is important to cell survival and self-repair. The UPS is responsible for the specific degradation of proteins and contains 3 enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and E3, and a 26S proteasome (14). Ubiquitin is first bound and activated via the C-terminal adenylation by E1. E1 catalyzes the transfer of charged ubiquitin to a cysteine residue of E2, E3 recognizes the substrate of the UPS, and then the ubiquitylated substrates are recognized and degraded by the 26S proteasome (15-16). UPS regulates a number of significant cell processes, including differentiation, apoptosis and proliferation (17).

F-box proteins determine the substrate specificity of the SCF-type E3 complex (11). Fbxw7 is a member of the F-box family, and was first identified as a negative regulator of Notch-mediated (LIN-12-mediated) signaling in Caenorhabditis elegans by genetic analysis (18). The Fbxw7 target proteins contain conservative phosphorylation amino-acid residues, known as CDC4 phosphodegrons
(CPDs) (19). Phosphorylated CPDs bind to the WD40 repeat domains of Fbxw7 and target proteins are recognized and degraded in a sequential manner. Results of our study showed that the Fbxw7 mRNA and protein expression in the HF group was lower than that in the control group (P<0.05), indicating that Fbxw7 may be closely correlated with the pathogenesis of NAFLD.

SREBPs, specific substrates of Fbxw7, are significant for the onset and development of NASH (20). SREBPs have 3 isozymes: SREBP-1 (SREBP-1a and SREBP-1c) and SREBP-2. SREBP-1 mainly regulates enzymes involving triglycerides and fatty acid synthesis, including ACC-1, ACC-2, GPAT, SCD1 and FAS. SREBP-2 regulates cholesterol synthesis enzymes, including hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) and HMG-CoA reducing enzymes. Our results indicated that SREBP-1 was accumulated in the HF group, and was also negatively correlated with the expression of Fbxw7.

Recently, Onoyama et al (13) demonstrated that mice with the liver-specific ablation of Fbxw7 developed clinicopathological features similar to those of NAFLD or NASH in humans. These authors also found that an abundance of nuclear SREBP1 was increased in mice with liver-specific null mutations of Fbxw7. Our results combined with the above findings suggest that the Fbxw7-SREBP-1 axis plays a key physiological role in the regulation of lipid metabolism in the liver as well as a pathological role in the development of NAFLD. One possible mechanism is that Fbxw7 expression is reduced during the development of NAFLD, which prevents UPS from recognizing and degrading SREBP-1, leading to the accumulation of SREBP-1 which could sequentially upregulate lipogenic gene transcription and induce NAFLD. However, due to the complex pathogenesis of NAFLD, more studies are required to precisely identify the potential mechanisms of occurrence and development of NAFLD.

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References


