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

Received February 4, 2012; Accepted June 7, 2012

DOI: 10.3892/mmr.2012.953

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

Key words: non-alcoholic fatty liver disease, metabolic syndrome, ubiquitin ligase (E3), sterol regulatory element binding protein

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^{Fbxw7} is a type of E3 that belongs to the ubiquitin-proteasome system (UPS). SCF^{Fbxw7} 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'-CACAGGCCTTCAAGAGTGGC-3'; and antisense, 5'-TTGCATCATATGCTTCACTTGTGT-3'. The PCR product was ~117 bp. β-actin, a housekeeping gene, served as an internal control to ensure that an equal amount of mRNA was analyzed from each sample. The upstream primer sequence for β-actin was 5'-TGATGACATCAAGAAGGTGGTGAA-3' and the downstream sequence was 5'-TCCTTGGAGGCCAT GTAGGCCAT-3', which was predicted to produce a 239 bp PCR product.

The total RNA of the samples was extracted using the RNAfast 200 purification kit, according to the manufacturer's instructions. The optical density of RNA at A260/280 nm was between 1.7 and 1.9. The integrity of the RNA was confirmed by the presence of intact 18S and 28S bands on 2% agarose gels. The total RNA was then reverse-transcribed at 37˚C for 15 min, and the cDNA was incubated at 85˚C for 5 sec to inactivate the reverse transcriptase. For PCR amplification, the cDNA was used as the template to amplify specific PCR products of the Fbxw7 and GAPDH genes. The PCR reaction was performed in a 25 μl system, starting with denaturation for 5 min at 94˚C; then 35 cycles of denaturation for 30 sec at 94˚C; annealing at 62˚C for Fbxw7 and GAPDH for 30 sec; and extension for 30 sec at 72˚C, followed by a final extension for 7 min at 72˚C. The PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The electrophoresis bands were analyzed using the Gene Genius Gel Imaging System. The gene expression intensity (relative coefficient) was quantitatively analyzed by the ratio of the expression intensity of the electrophoresis bands to β-actin.

Immunohistochemical staining. The primary mouse anti-Fbxw7 antibody (ab74054) was purchased from Abcam (Hong Kong, China). The primary mouse anti-SREBP-1 antibody (2A4) was purchased from Lab Vision & Neomarkers (Fremont, CA, USA).

Immunohistochemistry was performed on paraformaldehyde-fixed paraffin sections. The sections were dewaxed, dehydrated, rehydrated and antigen retrieval was performed in citrate buffer. Endogenous peroxidase activity was blocked for 10 min using 3.0% hydrogen peroxide, the sections were blocked for 30 min using 10% goat plasma, and then separately incubated with the primary antibodies directed against Fbxw7 and SREBP-1 at 4˚C overnight. The primary antibody was detected using biotinylated secondary antibodies (Beijing Zhongshan Goldenbridge Biotechnology Ltd., Co., Beijing, China) according to the manufacturer's instructions. The staining of the sections was performed using streptavidin-HRP conjugates for Fbxw7 and SREBP-1 (SP method). The sections were visualized with diaminobenzidine, counterstained with hematoxylin, then dehydrated in alcohol and xylene and mounted onto glass slides.

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 NaF 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-

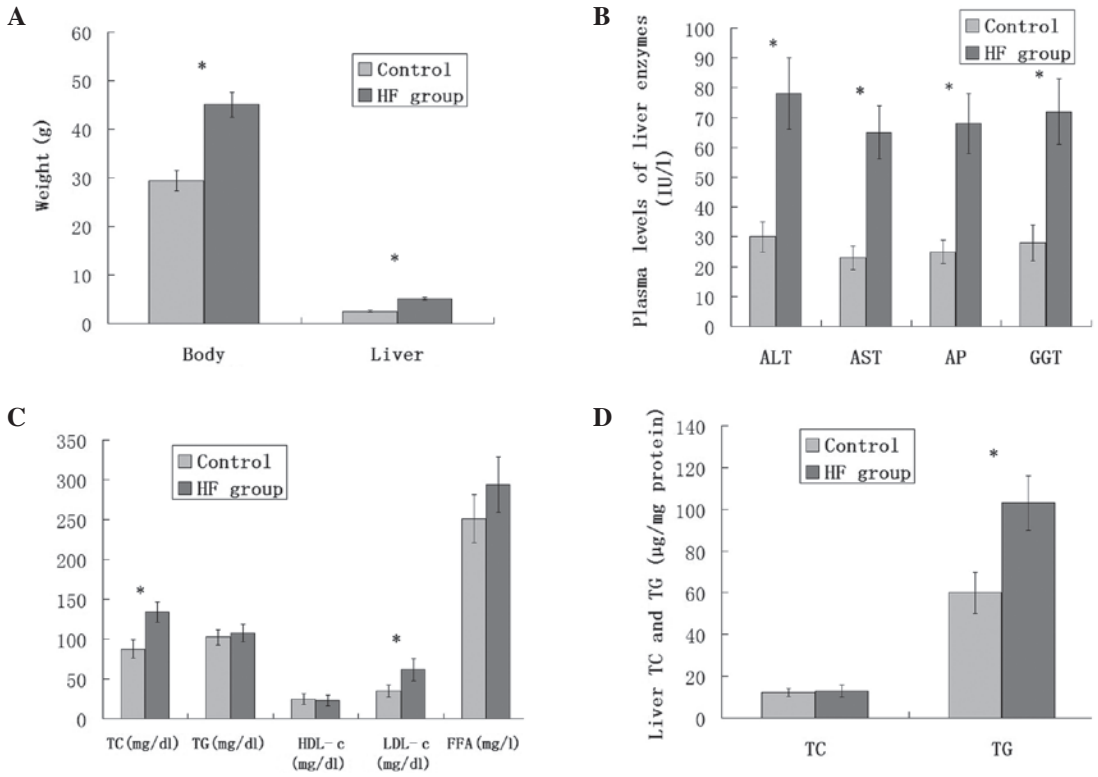

Figure 1. (A) Body and liver weight gain in C57BL/6J mice in the control or HF diet group fed for 8 weeks (n=20). (B) Plasma levels of the liver enzymes in the C57BL/6J mice in the control or HF diet groups fed for 8 weeks (n=20). (C) Plasma levels of TC, TG, HDL-c, LDL-c and FFA in the C57BL/6J mice in the control or HF diet groups fed for 8 weeks (n=20). (D) Triglyceride and total cholesterol concentrations in the livers of C57BL/6J mice fed the control or HF diet group fed for 8 weeks. Values are the mean ± standard error. *P<0.05. HF, high fat; TC, total cholesterol; TG, triglycerides; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; FFA, free fatty acids; ALT, alanine transaminase; AST, aspartate transaminase; AP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase.

acrylamide gels. Proteins from the gels were then transferred onto nitrocellulose membranes. Electroblots 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

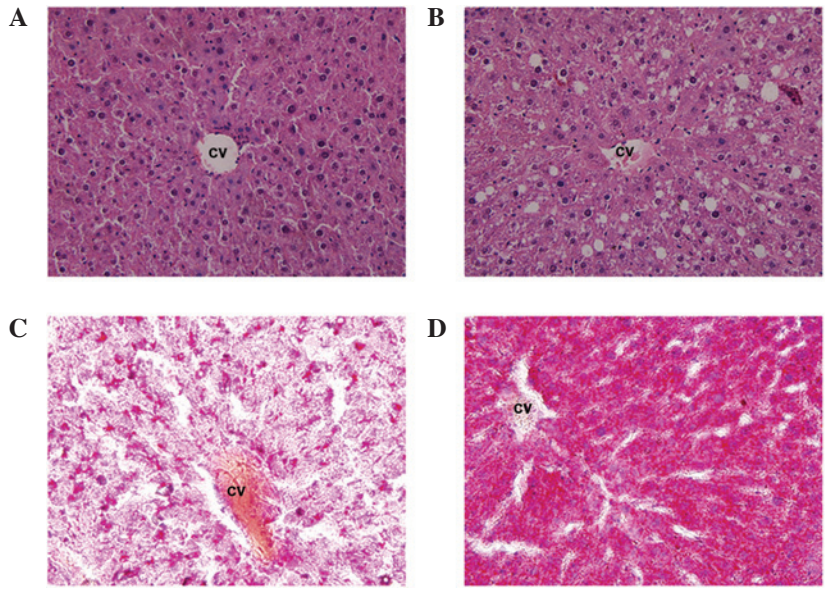

Figure 2. Histopathological findings in the control and HF groups. (A) In the control group, normal liver histology was observed. (B) In the HF group, macrovesicular and microvesicular steatosis was observed. (C) In the control group, normal liver histology was observed. (D) In the HF group, triglyceride molecules were observed in the cytoplasm during macrovesicular and microvesicular steatosis. A and B: hematoxylin and eosin staining; magnification, x40. C and D: Oil Red O staining; magnification, x40. HF, high fat.

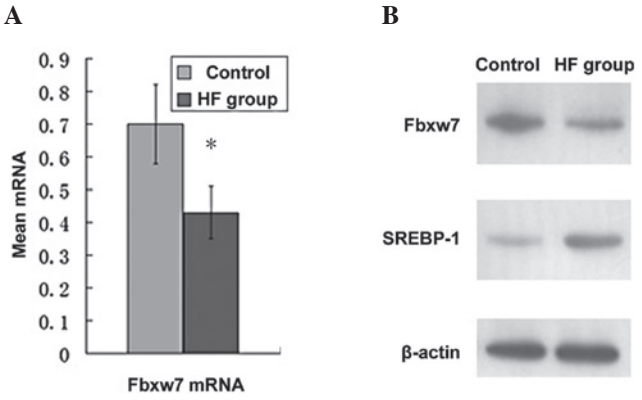

Figure 3. mRNA expression of Fbxw7 and the protein expression of Fbxw7 and SREBP-1 in the mouse livers of the control and HF groups. (A) mRNA expression of Fbxw7 in the mouse livers of the control and HF groups (n=20). (B) Fbxw7 and SREBP-1 expression in the mouse livers of the control and HF groups. Values are the mean ± standard error. *P<0.05. Fbxw7, F-box and WD repeat domain-containing 7; SREBP-1, sterol regulatory element binding protein; HF, high fat.

in the HF group 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 in the HF group (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

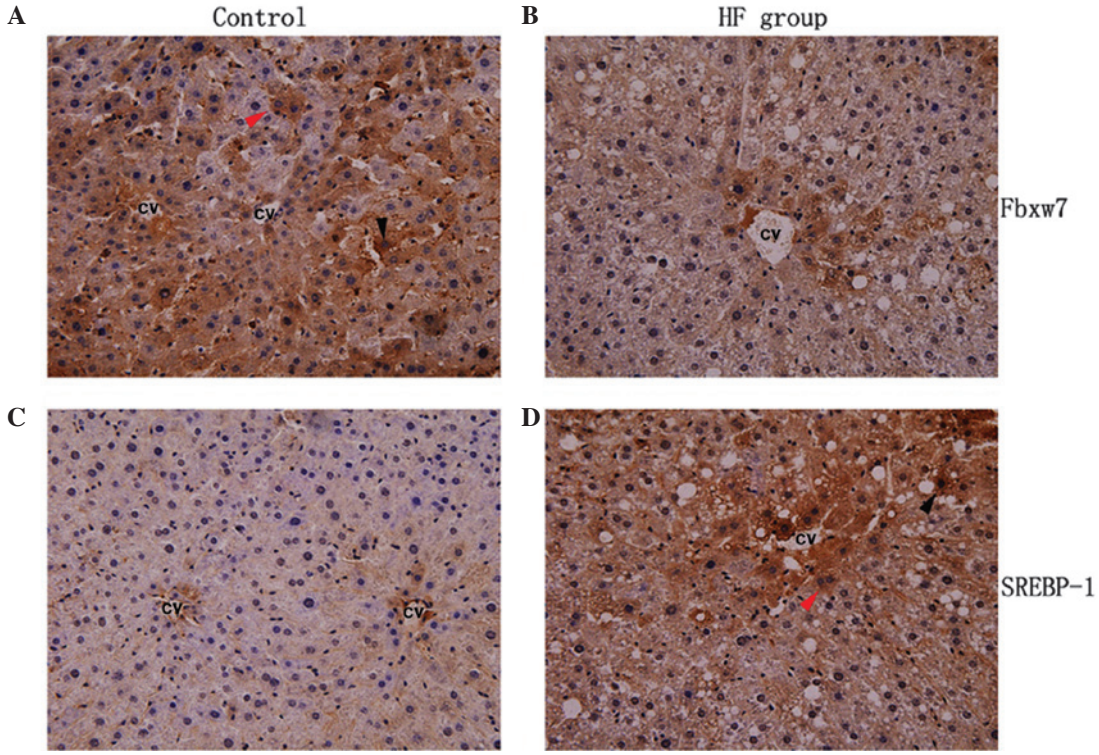

Figure 4. Immunohistochemical staining of Fbxw7 and SREBP-1 in the mouse livers of the control and HF groups. Immunohistochemical staining of Fbxw7 in the mouse livers of (A) the control group and (B) the HF group. Immunohistochemical staining of SREBP-1 in the mouse livers of (C) the control group and (D) the HF group. The Fbxw7 protein expression in (A) the control group was higher than that in (B) the HF group. The SREBP-1 protein expression in (C) the control group was lower than that in (D) the HF group. The majority of the liver cells demonstrated cytoplasmic staining (red arrowheads), whereas nuclear staining was identified in few cells (black arrowheads) (SP method; magnification, x40). Fbxw7, F-box and WD repeat domain-containing 7; SREBP-1, sterol regulatory element binding protein; HF, high fat.

(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.

Acknowledgements

This study was supported by a grant from the National Natural Science Foundation of China (No. 81071897).

References

1. Hamaguchi E, Takamura T, Sakurai M, *et al*: Histological course of nonalcoholic fatty liver disease in Japanese patients: tight glycemic control, rather than weight reduction, ameliorates liver fibrosis. Diabetes Care 33: 284-286, 2010.
2. Vanni E, Bugianesi E, Kotronen A, De Minicis S, Yki-Järvinen H and Svegliati-Baroni G: From the metabolic syndrome to NAFLD or vice versa? Dig Liver Dis 42: 320-330, 2010.
3. Ogawa T, Fujii H, Yoshizato K and Kawada N: A human-type nonalcoholic steatohepatitis model with advanced fibrosis in rabbits. Am J Pathol 177: 153-165, 2010.
4. Ota T, Takamura T, Kurita S, Matsuzawa N, Kita Y and Uno M: Insulin resistance accelerates a dietary rat model of nonalcoholic steatohepatitis. Gastroenterology 132: 282-293, 2007.
5. Bugianesi E, Moscatiello S, Ciaravella MF and Marchesini G: Insulin resistance in nonalcoholic fatty liver disease. Curr Pharm Des 16: 1941-1951, 2010.

6. Nakamuta M, Kohjima M, Morizono S, *et al*: Evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. Int J Mol Med 16: 631-635, 2005.
7. Foufelle F and Ferré P: New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. Biochem J 366: 377-391, 2002.
8. Hagen RM, Rodriguez-Cuenca S and Vidal-Puig A: An allostatic control of membrane lipid composition by SREBP1. FEBS Lett 584: 2689-2698, 2010.
9. Nakayama H, Otabe S, Ueno T, *et al*: Transgenic mice expressing nuclear sterol regulatory element-binding protein 1c in adipose tissue exhibit liver histology similar to nonalcoholic steatohepatitis. Metabolism 56: 470-475, 2007.
10. Sundqvist A, Bengoechea-Alonso MT, Ye X, *et al*: Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF^{Fbw7}. Cell Metab 1: 379-391, 2005.
11. Welcker M and Clurman BE: FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. Nat Rev Cancer 8: 83-93, 2008.
12. Kirpich IA, Gobejishvili LN, Bon Homme M, *et al*: Integrated hepatic transcriptome and proteome analysis of mice with high-fat diet-induced nonalcoholic fatty liver disease. J Nutr Biochem 22: 38-45, 2011.
13. Onoyama I, Suzuki A, Matsumoto A, *et al*: Fbxw7 regulates lipid metabolism and cell fate decisions in the mouse liver. J Clin Invest 121: 342-354, 2010.
14. Livnat-Levanon N and Glickman MH: Ubiquitin-proteasome system and mitochondria-reciprocity. Biochim Biophys Acta 1809: 80-87, 2011.
15. Chan NC, Salazar AM, Pham AH, *et al*: Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. Hum Mol Genet 20: 1726-1737, 2011.
16. Willis MS, Townley-Tilson WH, Kang EY, Homeister JW and Patterson C: Sent to destroy: the ubiquitin proteasome system regulates cell signaling and protein quality control in cardiovascular development and disease. Circ Res 106: 463-478, 2010.
17. Bashir T and Pagano M: Aberrant ubiquitin-mediated proteolysis of cell cycle regulatory proteins and oncogenesis. Adv Cancer Res 88: 101-144, 2003.
18. Sundaram M and Greenwald I: Suppressors of a lin-12 hypomorph define genes that interact with both lin-12 and glp-1 in *Caenorhabditis elegans*. Genetics 135: 765-783, 1993.
19. Nash P, Tang X, Orlicky S, *et al*: Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature 414: 514-521, 2001.
20. Bengoechea-Alonso MT and Ericsson J: SREBP in signal transduction: cholesterol metabolism and beyond. Curr Opin Cell Biol 19: 215-222, 2007.