

Increased hepatic expression of dipeptidyl peptidase-4 in non-alcoholic fatty liver disease and its association with insulin resistance and glucose metabolism

MASAYUKI MIYAZAKI¹, MASAKI KATO¹, KOSUKE TANAKA¹, MASATAKE TANAKA¹,
MOTOYUKI KOHJIMA¹, KAZUHIKO NAKAMURA¹, MUNECHIKA ENJOJI²,
MAKOTO NAKAMUTA³, KAZUHIRO KOTOH¹ and RYOICHI TAKAYANAGI¹

¹Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582; ²Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-0180; ³Department of Gastroenterology, Kyushu Medical Center, National Hospital Organization, Fukuoka 810-8563, Japan

Received August 29, 2011; Accepted December 6, 2011

DOI: 10.3892/mmr.2011.707

Abstract. Dipeptidyl peptidase-4 (DPP4) is a serine protease that degrades glucagon-like peptide-1 (GLP-1), an incretin hormone that stimulates insulin secretion from pancreatic β -cells. DPP4 is also involved in the regulation of T cell-mediated inflammatory processes. These properties of DPP4 suggest that it may play a role in the progression of non-alcoholic fatty liver disease (NAFLD). Hepatic DPP4 mRNA expression levels were analyzed by real-time PCR using liver biopsy samples from 17 NAFLD patients and 10 healthy subjects. In NAFLD patients, we also examined correlations between DPP4 expression levels and metabolic factors, including homeostasis model assessment-insulin resistance (HOMA-IR), body mass index (BMI), and serum cholesterol and triglyceride levels. To examine the potential effects of nutritional factors, DPP4 expression levels were analyzed in HepG2 cells subjected to various culture conditions. Hepatic DPP4 mRNA expression was significantly greater in NAFLD patients than in control subjects. DPP4 expression levels were negatively correlated with HOMA-IR and positively correlated with serum cholesterol levels. In HepG2 cells, high glucose significantly enhanced DPP4 expression, whereas insulin, fatty acids and cholesterol did not. Increased hepatic expression of DPP4 in NAFLD may be associated with metabolic factors, including insulin resistance, and may adversely affect glucose metabolism in this liver disease.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological disorder characterized by the accumulation of triglycerides in hepatocytes. The incidence of NAFLD has increased markedly in recent years, accompanying the increased prevalence of obesity and type 2 diabetes mellitus (T2DM) (1). More than 10% of patients with NAFLD progress to non-alcoholic steatohepatitis (NASH), which is characterized by inflammatory cell infiltration and ballooning of hepatocytes in the liver. Liver cirrhosis and hepatocellular carcinoma occur in several patients with NASH (2). Obesity and insulin resistance (IR) are believed to be involved in the pathogenesis of NAFLD (3). Increased hepatic uptake of fatty acids as a result of elevated triglyceride degradation in adipose tissue causes hepatic fat accumulation. Reactive oxygen species (ROS) produced during lipid oxidation may induce hepatocyte death and inflammatory reactions. IR also reduces glucose uptake in muscles and diminishes insulin-dependent suppression of gluconeogenesis, ultimately progressing to T2DM (4).

Dipeptidyl peptidase-4 (DPP4) inhibitors were recently introduced for the treatment of T2DM (5). DPP4 degrades glucagon-like peptide-1 (GLP-1), which stimulates insulin secretion from pancreatic β -cells. DPP4 inhibitors enhance glucose-dependent insulin secretion by suppressing GLP-1 degradation. However, the physiological functions of DPP4 are not fully understood. In addition to its effect on GLP-1, multiple activities of DPP4 have been reported in various cellular processes. Of note, DPP4 seems to influence inflammation by regulating T cell proliferation and the cell cycle of NK cells (6,7). In addition, the enhanced tissue mRNA expression of DPP4 in diseases such as asthma or ulcerative colitis implies that DPP4 may play a role in the pathogenesis of several inflammatory diseases (8,9).

Prolonged intrahepatic inflammation as a result of elevated generation of ROS is a significant process underlying the progression of liver disease from NAFLD to NASH (10).

Correspondence to: Dr Masayuki Miyazaki, Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
E-mail: mmasayuki@med.kyushu-u.ac.jp

Key words: body mass index, cholesterol, dipeptidyl peptidase-4, fatty acids, insulin resistance, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis

Notably, IR is known to induce the release of tumor necrosis factor (TNF)- α and interleukins, which cause chronic activation of systemic low-grade inflammation (11). Therefore, the progression of NAFLD is accelerated by intrahepatic and systemic inflammatory processes, which are strongly associated with IR.

DPP4 may be involved in the progression of NAFLD because of its glucose metabolism regulatory activity and its role in inflammatory processes. To investigate this possibility, we compared the mRNA expression levels of DPP4 in liver biopsy samples from NAFLD patients to those of control livers. We also determined correlations between DPP4 expression levels and metabolic factors. Finally, we examined the effects of nutritional factors, including glucose, insulin, fatty acids and cholesterol, on the expression of DPP4 in HepG2 cells.

Materials and methods

Patients and samples. Tissue samples were obtained by liver biopsy from 17 patients with histologically diagnosed NAFLD without T2DM who were admitted to the Kyushu University Hospital between 2004 and 2006. Liver tissue samples were also obtained from 10 healthy donors undergoing living donor liver transplantation. Written informed consent was obtained from all patients. Patient characteristics including gender, age, body mass index (BMI) and alanine aminotransferase (ALT), lactate dehydrogenase (LDH), total cholesterol, triglyceride and fasting plasma glucose levels were recorded.

Cell culture and treatment. HepG2 cells, a human hepatoblastoma-derived cell line, were cultured in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μ g/ml streptomycin. Cells were seeded at 10,000 cells/cm² on 6-well plates. To analyze the effects of glucose and insulin, HepG2 cells were cultured in four different media, comprising low insulin (0.001 ng/ml) plus low (100 mg/dl) or high (400 mg/dl) glucose, or in high insulin (1.0 ng/ml) plus low or high glucose. To determine the effects of lipids, 10 mM oleic acid or palmitic acid dissolved in ethanol, or 300 μ g of water-soluble cholesterol was added into the culture medium. The final concentrations of fatty acids and cholesterol in the media were 1.0 mM and 100 μ g/ml, respectively. The cells were incubated for 24 h and used for real-time PCR (RT-PCR).

RT-PCR. Total RNA was prepared with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 1.0 μ g RNA with GeneAmpTM RNA PCR (Applied Biosystems, Branchburg, NJ, USA). RT-PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Basel, Switzerland). To control for variations in the reactions, all PCR data were normalized against β -actin expression. The forward and reverse PCR primers for DPP4 were 5'-TGGAGGCATTCTACACAGCTTC-3' and 5'-CCATGTGACCCACTGTGTGTTG-3', respectively.

Statistical analysis. All results are expressed as the means \pm standard deviation. Significant differences between two groups were assessed using unpaired two-tailed t-tests. p-values <0.05 were considered to denote statistical significance.

Table I. Characteristics of the control subjects and NAFLD patients.

	Control (n=10)	NAFLD (n=17)	p-value
Male/female	5/5	9/8	
Age (years)	28.3 \pm 8.0	40.6 \pm 15.0	
BMI (kg/m ²)	20.2 \pm 1.3	27.2 \pm 5.2	<0.01
ALT (IU/l)	10.3 \pm 3.1	60.0 \pm 75.2	<0.01
LDH (IU/l)	125.8 \pm 15.5	219.9 \pm 80.1	<0.01
Total cholesterol (mg/dl)	164.6 \pm 23.1	193.9 \pm 47.8	
Triglyceride (mg/dl)	81.6 \pm 18.3	113.0 \pm 51.8	
Fasting plasma glucose (mg/dl)	87.2 \pm 5.7	110.1 \pm 36.7	

BMI, body mass index; ALT, alanine aminotransferase; LDH, lactate dehydrogenase. Continuous values are expressed as the means \pm SD.

Results

Patient characteristics. We obtained tissue samples from 17 NAFLD patients (9 males and 8 females) and 10 control subjects (5 males and 5 females) (Table I). The control group was younger than the NAFLD group. On the other hand, BMI, ALT and LDH were significantly higher in the NAFLD patients than in the control group. Nutritional parameters, including total cholesterol, triglyceride and fasting plasma glucose levels, were higher in the NAFLD patients than in the control group, although these differences were not statistically significant.

DPP4 expression is higher in NAFLD liver than in healthy liver. Hepatic mRNA levels of DPP4 were evaluated by RT-PCR in NAFLD patients and in the control group. DPP4 expression was 15-fold higher in the NAFLD liver than in the control liver (Fig. 1A). To determine possible associations with IR in NAFLD, DPP4 expression levels were compared among groups of patients stratified by homeostasis model assessment-insulin resistance (HOMA-IR) <2.5 and \geq 2.5. Notably, DPP4 expression levels were significantly lower in patients with HOMA-IR \geq 2.5 than in patients with HOMA-IR <2.5 (Fig. 1B).

Correlations between hepatic DPP4 expression levels and metabolic factors were also analyzed. Hepatic DPP4 expression levels were negatively correlated with HOMA-IR ($r=-0.68$, $p<0.001$) and BMI ($r=-0.50$, $p<0.01$) (Fig. 2A and B). Among biochemical parameters, DPP4 expression levels were positively correlated with total cholesterol levels ($r=0.61$, $p<0.005$), but not with triglyceride levels (Fig. 2C and D), or with other parameters, such as ALT, LDH, γ -glutamyl transpeptidase and platelet number (data not shown).

Effects of nutritional factors on the expression of DPP4 in HepG2 cells. Results regarding the NAFLD liver suggest that the hepatic expression of DPP4 may be affected by systemic metabolic status. To investigate this possibility, we

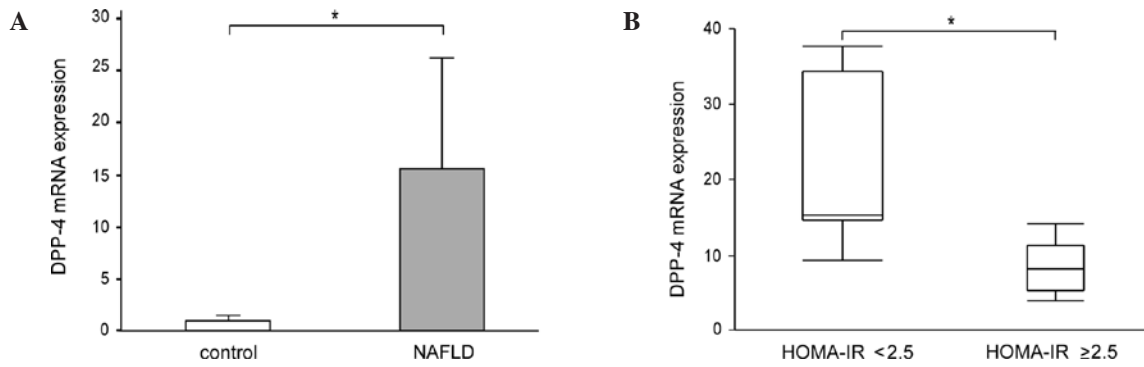


Figure 1. RT-PCR analysis of DPP4 mRNA expression in NAFLD liver. Total RNA was extracted from liver biopsy samples of NAFLD patients (n=17) and control subjects (n=10). Samples were analyzed by RT-PCR and normalized for β -actin expression. (A) Comparison between NAFLD livers and control livers. The mean expression in the control liver was set as 1. (B) DPP4 expression in NAFLD patients with homeostasis model assessment-insulin resistance (HOMA-IR) <2.5 (n=8) and in those with HOMA-IR \geq 2.5 (n=8). * p <0.05.

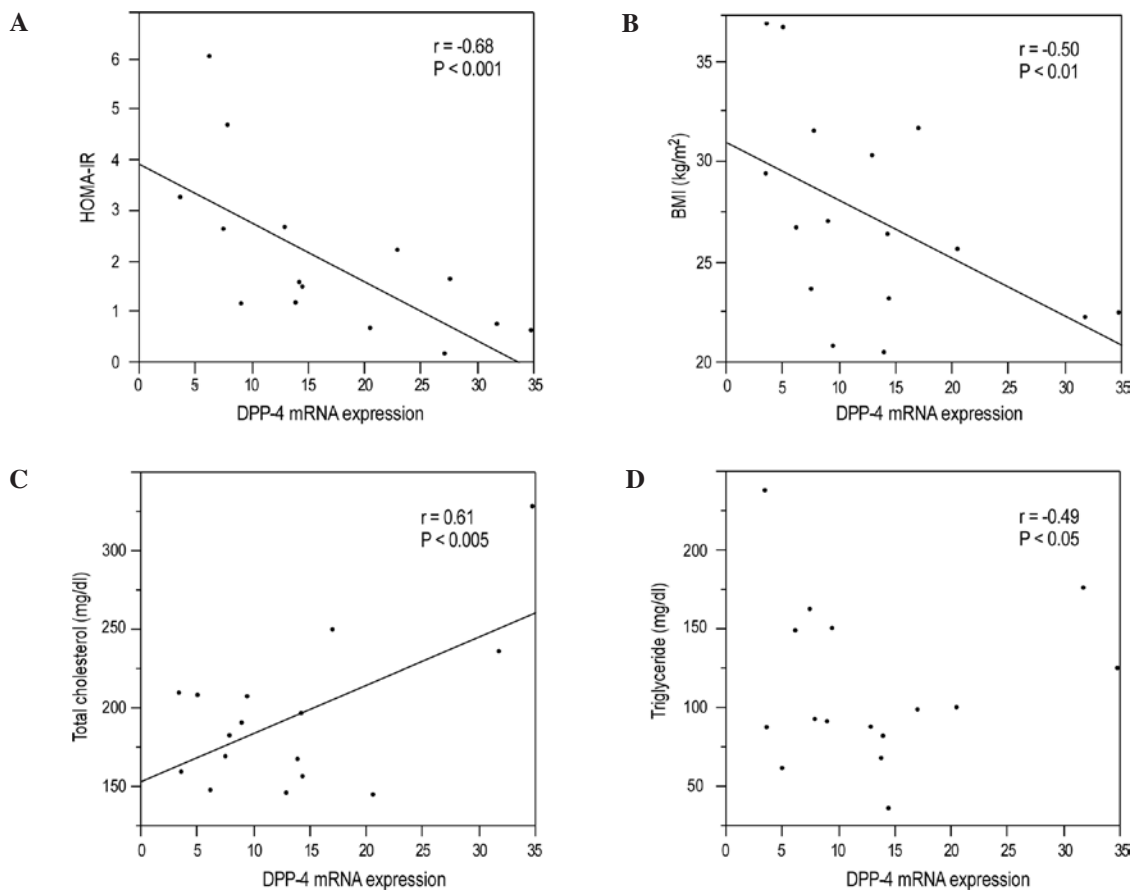


Figure 2. Correlations between hepatic DPP4 mRNA expression and metabolic factors in patients with NAFLD. (A) Homeostasis model assessment-insulin resistance (HOMA-IR), (B) body mass index (BMI), (C) serum total cholesterol levels and (D) serum triglyceride levels.

determined the expression levels of DPP4 in HepG2 cells cultured in various nutritional conditions, including high/low glucose, high/low insulin, fatty acids and cholesterol. Fig. 3A shows the effects of glucose (100 and 400 mg/dl) and insulin (0.001 and 1.0 ng/ml) concentrations on DPP4 expression. Unlike the results for NAFLD patients in which hepatic DPP4 expression was lower in patients with HOMA-IR \geq 2.5 than in patients with HOMA-IR <2.5, the high concentration of insulin did not suppress DPP4 expression. However, DPP4 expression was significantly increased by high glucose. Palmitic acid and oleic acid are fatty acids

known to induce triglyceride accumulation and form lipid droplets in HepG2 cells (12). Unexpectedly, neither of the fatty acids up-regulated, but rather suppressed DPP4 expression in HepG2 cells (Fig. 3B).

We next examined the effect of water-soluble cholesterol, as cholesterol is involved in the pathogenesis of NAFLD (13,14). In addition, its oxidized derivative, oxysterol, is an agonist of liver X receptor (LXR), which transactivates lipogenic transcriptional factors, including sterol regulatory element-binding protein (SREBP)-1c and carbohydrate response element-binding protein (ChREBP) (15). However,

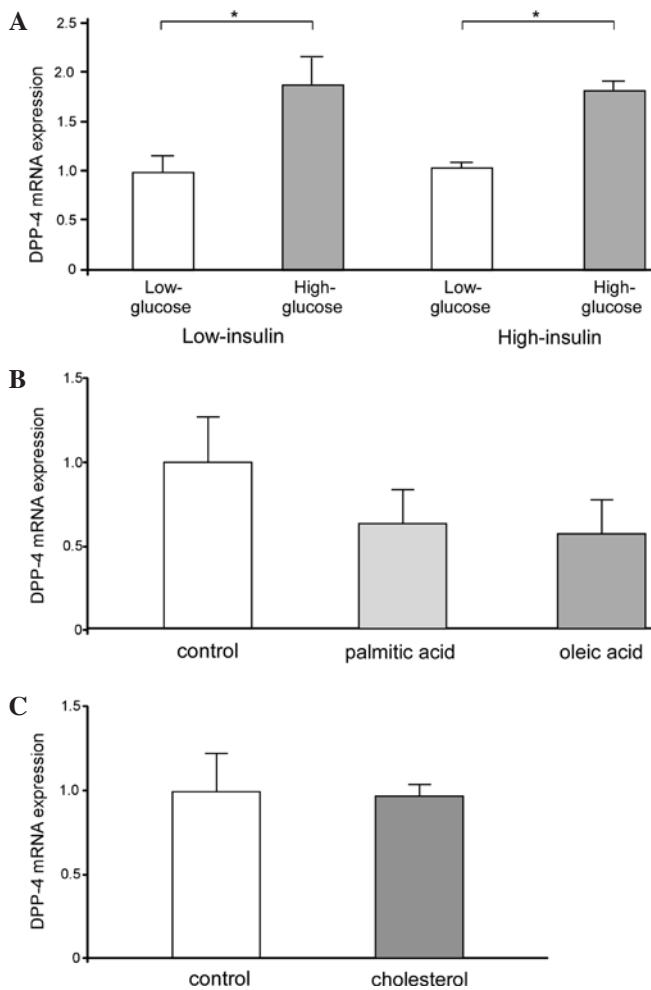


Figure 3. Effects of nutritional factors on DPP4 mRNA expression in HepG2 cells. HepG2 cells were cultured in the indicated nutritional conditions for 24 h and DPP4 mRNA expression levels were determined by RT-PCR. All PCR reactions were normalized for β -actin expression (n=6). Effects of (A) insulin (0.001 or 1 ng/ml) and glucose (100 or 400 mg/dl), (B) fatty acids (1.0 mM palmitic acid or oleic acid) and (C) water-soluble cholesterol (100 μ g/ml) on DPP4 mRNA expression. *p<0.05.

the addition of cholesterol did not affect DPP4 expression levels in HepG2 cells (Fig. 3C).

Discussion

The mRNA expression levels of DPP4 were significantly increased in NAFLD livers compared to that in control livers. In NAFLD patients, DPP4 expression levels were negatively correlated with HOMA-IR and BMI, and positively correlated with total cholesterol levels, but not with ALT, LDH or triglyceride levels. In HepG2 cells, DPP4 expression was increased in the high glucose condition. Other nutritional conditions, including high insulin, or the presence of fatty acids and cholesterol, did not significantly affect DPP4 expression. These observations suggest that enhanced DPP4 expression in NAFLD liver may be associated with IR, and may promote the progression of liver disease via subsequent deteriorations in glucose metabolism.

Increased serum activity and/or hepatic expression of DPP4 have been reported in various hepatic diseases. Serum DPP4 activity was significantly higher in patients with chronic hepa-

titis C virus (HCV) infection and primary biliary cirrhosis than in healthy controls (16,17). Increased DPP4 protein was also detected in the ileum and liver in HCV-infected patients (18). In animal models, elevated serum DPP4 activity was observed in rat cirrhosis induced by diethyl-nitrosamine, phenobarbital and carbon tetrachloride, and was positively correlated with serum transaminase levels (19). These observations suggest that destruction of liver cells may increase the serum activity and hepatic expression of DPP4.

DPP4 also appears to be involved in liver diseases originating from hepatic steatosis. Indeed, DPP4 activity was greater in NAFLD patients than in control subjects and patients with T2DM, and DPP4 activity was correlated with HOMA2-IR (20). Serum DPP4 activity was significantly higher in NASH patients than in control subjects and was correlated with the histopathological grade of liver disease. Furthermore, the intensity of hepatic DPP4 immunostaining was correlated with the extent of hepatic steatosis (21). These observations support the hypothesis that IR, which is thought to promote the progression of NAFLD and NASH, is associated with the serum activity and hepatic expression of DPP4.

The physiological roles of DPP4 in IR or T2DM have not been established. Ryskjaer *et al* reported that the plasma DPP4 activity was significantly elevated in patients with T2DM, and was correlated with fasting glucose and HbA1c levels (22). However, conflicting results have been reported. For example, in one study, serum DPP4 activity was reduced in patients with T2DM, and DPP4 activity was negatively correlated with glucose and HbA1c levels (23). Researchers speculated that these discrepancies in diabetic patients may be due to factors such as disease duration, patient age and glycemic control. In patients with NAFLD, we found that hepatic DPP4 expression was negatively correlated with HOMA-IR, which was inconsistent with that of a previous report showing a positive correlation between serum DPP4 activity and HOMA2-IR in NAFLD patients (20). Firneisz *et al* (20) also showed that DPP4 activity in NAFLD patients with glucose intolerance was lower than that in NAFLD patients with normal glucose tolerance, complicating the role of IR in DPP4 activity in NAFLD. In our study using HepG2 cells, DPP4 expression was enhanced by high glucose, but not by high insulin. Therefore, we speculate that high concentration of glucose may induce the hepatic DPP4 expression, but insulin itself may not affect the transcription of DPP4. This hypothesis is consistent with a previous report describing increased DPP4 activities and hepatic expression of DPP4 in streptozotocin-treated animals with type 1 diabetes mellitus (24).

We also investigated the effects of lipids on DPP4 expression in HepG2 cells. The addition of oleic acid or palmitic acid to the culture medium did not increase DPP4 expression. Therefore, triglyceride accumulation may not be involved in the induction of hepatocyte DPP4 expression. We found that serum cholesterol levels were positively correlated with hepatic DPP4 expression levels in NAFLD patients. Previous reports have suggested that cholesterol plays a role in the pathogenesis of NAFLD. The daily intake of cholesterol was reported to be significantly greater in non-obese NAFLD patients than in control subjects, even though total calorie and carbohydrate intake did not differ (14). Meanwhile, the NPC1L1 inhibitor ezetimibe, which inhibits intestinal choles-

terol absorption, has been shown to improve the clinical parameters of NAFLD (13). However, the culturing of HepG2 cells with cholesterol did not enhance DPP4 expression, suggesting that cholesterol does not affect the transcriptional regulation of DPP4 in the liver.

Taken together, we hypothesize that hepatic DPP4 is involved in the progression of NAFLD in the following ways: i) when NAFLD is induced by nutritional overload, hepatic inflammation enhances hepatic DPP4 expression; ii) accelerated degradation of GLP-1 by DPP4 inhibits insulin secretion and causes hyperglycemia; and iii) hyperglycemia further enhances DPP4 expression, with further worsening in glucose metabolism.

The increased hepatic expression of DPP4 in NAFLD patients suggests that DPP4 may be involved in the onset and/or progression of NAFLD. Hepatic inflammation may induce this phenomenon, although DPP4 causes deteriorations in systemic glucose metabolism. Further studies are required to examine the physiological role of DPP4 in the NAFLD liver and to determine whether DPP4 may offer a new treatment target to suppress the progression of liver disease.

References

- Browning JD and Horton JD: Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* 114: 147-152, 2004.
- Angulo P: Nonalcoholic fatty liver disease. *N Engl J Med* 346: 1221-1231, 2002.
- Zafrani ES: Non-alcoholic fatty liver disease: an emerging pathological spectrum. *Virchows Arch* 444: 3-12, 2004.
- Mlinar B, Marc J, Janez A and Pfeifer M: Molecular mechanisms of insulin resistance and associated diseases. *Clin Chim Acta* 375: 20-35, 2007.
- Neumiller JJ, Wood L and Campbell RK: Dipeptidyl peptidase-4 inhibitors for the treatment of type 2 diabetes mellitus. *Pharmacotherapy* 30: 463-484, 2010.
- Ohnuma K, Munakata Y, Ishii T, *et al*: Soluble CD26/dipeptidyl peptidase IV induces T cell proliferation through CD86 up-regulation on APCs. *J Immunol* 167: 6745-6755, 2001.
- Buhling F, Kunz D, Reinhold D, *et al*: Expression and functional role of dipeptidyl peptidase IV (CD26) on human natural killer cells. *Nat Immun* 13: 270-279, 1994.
- Schmiedl A, Krainski J, Schwichtenhovel F, *et al*: Reduced airway inflammation in CD26/DPP4-deficient F344 rats is associated with altered recruitment patterns of regulatory T cells and expression of pulmonary surfactant proteins. *Clin Exp Allergy* 40: 1794-1808, 2010.
- Hildebrandt M, Rose M, Ruter J, *et al*: Dipeptidyl peptidase IV (DP IV, CD26) in patients with inflammatory bowel disease. *Scand J Gastroenterol* 36: 1067-1072, 2001.
- Begriche K, Igoudjil A, Pessayre D, *et al*: Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* 6: 1-28, 2006.
- Tilg H and Moschen AR: Inflammatory mechanisms in the regulation of insulin resistance. *Mol Med* 14: 222-231, 2008.
- Okamoto Y, Tanaka S and Haga Y: Enhanced GLUT2 gene expression in an oleic acid-induced in vitro fatty liver model. *Hepatol Res* 23: 138-144, 2002.
- Enjoji M, Machida K, Kohjima M, *et al*: NPC1L1 inhibitor ezetimibe is a reliable therapeutic agent for non-obese patients with nonalcoholic fatty liver disease. *Lipids Health Dis* 12: 9-29, 2010.
- Enjoji M and Nakamuta M: Is the control of dietary cholesterol intake sufficiently effective to ameliorate nonalcoholic fatty liver disease? *World J Gastroenterol* 16: 800-803, 2010.
- Higuchi N, Kato M, Shundo Y, *et al*: Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. *Hepatol Res* 38: 1122-1129, 2008.
- Firneisz G, Lakatos PL, Szalay F, *et al*: Serum dipeptidyl peptidase IV (DPP IV, CD26) activity in chronic hepatitis C. *Scand J Gastroenterol* 36: 877-880, 2001.
- Andrieu T, Thibault V, Malet I, *et al*: Similar increased serum dipeptidyl peptidase IV activity in chronic hepatitis C and other viral infections. *J Clin Virol* 27: 59-68, 2003.
- Itou M, Kawaguchi T, Taniguchi E, *et al*: Altered expression of glucagon-like peptide-1 and dipeptidyl peptidase IV in patients with HCV-related glucose intolerance. *J Gastroenterol Hepatol* 23: 244-251, 2008.
- Lakatos PL, Firneisz G, Borcsiczky D, *et al*: Elevated serum dipeptidyl peptidase IV (CD26, EC 3.4.14.5) activity in experimental liver cirrhosis. *Eur J Clin Invest* 30: 793-797, 2000.
- Firneisz G, Varga T, Lengyel G, *et al*: Serum dipeptidyl peptidase-4 activity in insulin resistant patients with non-alcoholic fatty liver disease: a novel liver disease biomarker. *PLoS One* 18: e12226, 2010.
- Balaban YH, Korkusuz P, Simsek H, *et al*: Dipeptidyl peptidase IV (DDP IV) in NASH patients. *Ann Hepatol* 6: 242-250, 2007.
- Ryskjaer J, Deacon CF, Carr RD, *et al*: Plasma dipeptidyl peptidase-IV activity in patients with type-2 diabetes mellitus correlates positively with HbA1c levels, but is not acutely affected by food intake. *Eur J Endocrinol* 155: 485-493, 2006.
- McKillop AM, Duffy NA, Lindsay JR, *et al*: Decreased dipeptidyl peptidase-IV activity and glucagon-like peptide-1(7-36) amide degradation in type 2 diabetic subjects. *Diabetes Res Clin Pract* 79: 79-85, 2008.
- Yang J, Campitelli J, Hu G, *et al*: Increase in DPP-IV in the intestine, liver and kidney of the rat treated with high fat diet and streptozotocin. *Life Sci* 81: 272-279, 2007.