

Hepatic miR-215 target Rictor and modulation of hepatic insulin signalling in rats

WEI-DONG LI¹, JIN-RONG XIA¹ and YAN-SHU LIAN²

¹Department of Gastroenterology, Zhongda Hospital, School of Medicine, Southeast University, Nanjing, Jiangsu 210009;

²Department of Preventive Medicine, Jiangsu Health Vacation College, Nanjing, Jiangsu 210036, P.R. China

Received June 14, 2018; Accepted March 6, 2019

DOI: 10.3892/mmr.2019.10031

Abstract. Increasing evidence has suggested that hepatic lipid accumulation is associated with hepatic insulin resistance; however, the underlying mechanism is yet to be determined. It was demonstrated that the levels of microRNA-215 (miR-215) expression in the liver of rats fed a high-fat diet were significantly increased compared with rats on a control diet. Additionally, it was revealed via luciferase assays and western blotting that miR-215 targets rapamycin-insensitive companion of mammalian target of rapamycin (Rictor), an important protein in the hepatic insulin signalling pathway. Following overexpression of miR-215 in the H4IIE rat hepatocarcinoma cell line, it was reported that the intracellular insulin signalling pathway was inhibited; conversely, inhibition of miR-215 expression induced this pathway. Furthermore, it was demonstrated via reverse transcription-quantitative polymerase chain reaction analysis that free fatty acids promoted the expression of miR-215. The present study provided a novel mechanistic insight into the association between nonalcoholic fatty liver and hepatic insulin resistance.

Introduction

Type 2 diabetes (T2D) poses a major threat to global health, with a prevalence of 8.5% in 2016 (1). The liver serves an important role in maintaining glucose homeostasis, as it controls 90% of glucose production of the body; dysregulated liver gluconeogenesis is a common factor contributing to the pathology of T2D (2). In T2D, hepatic glucose production (HGP) is increased and the inhibition of HGP by insulin is impaired (3); it was hypothesised that this phenomenon is due to hepatic insulin resistance. Nonalcoholic fatty liver disease (NAFLD) has been associated with hepatic insulin resistance; according

to the current literature, ~70% of patients with T2D (4,5) and almost all obese diabetic patients have NAFLD (6). Therefore, NAFL is a strong predictor of insulin resistance (7-10). Liver fat content has been closely associated with insulin resistance than visceral adipose tissue volume (11); however, the mechanisms underlying the association between NAFLD and hepatic insulin resistance requires further investigation.

MicroRNAs (miRNAs/miRs) are non-coding RNAs (~19-25 nucleotides) that regulate gene expression by binding to the 3'-untranslated regions (3'-UTR) of target mRNAs. A number of studies have demonstrated that miRNAs serve important roles in regulating insulin signalling pathways (12,13). miR-215 has been reported to serve roles in the pathogenesis of various human malignancies; the expression of miR-215 was significantly increased in cervical cancer (14), hepatocellular carcinoma (15) and gastric cancer (16). On the contrary, significantly decreased miR-215 expression was reported in oesophageal adenocarcinoma (17) and colon cancer (18). To the best of our knowledge, the contributions of miR-215 in the pathogenesis of T2D, hepatic glucose metabolism and NAFL remain unknown.

Rapamycin-insensitive companion of mammalian target of rapamycin (Rictor) is a subunit of the mammalian target of rapamycin complex 2 (mTORC2), a key activator of protein kinases that act downstream of insulin and growth factor signaling (19). Previous studies have reported roles for mTORC2 in the phosphorylation of AGC kinase family members, including protein kinase B (AKT), serum- and glucocorticoid-regulated kinase, and protein kinase C (PKC) (20-23). Rictor is required for the phosphorylation and activation of AKT; loss of Rictor in worm, fly, mouse and human cells results in depleted AKT phosphorylation at Ser473 (23-26). Furthermore, systemic knockout of mTORC2 components results in embryonic lethality (24,25,27). Yuan *et al* (20) reported that knockout of Rictor in the livers of mice induced systemic insulin resistance, indicating that Rictor in the liver serves an important role in maintaining the homeostasis of glucose metabolism in the body.

In the present study, it was reported that rats fed a high-fat diet (HFD) for 30 days exhibited hepatic insulin resistance and NAFLD. Furthermore, it was revealed that fatty acids upregulated the levels of miR-215 expression. Additionally, miR-215 inhibited insulin signalling via targeting Rictor, leading to

Correspondence to: Professor Yan-Shu Lian, Department of Preventive Medicine, Jiangsu Health Vacation College, 129 Hanzhong Road, Nanjing, Jiangsu 210036, P.R. China
E-mail: lianyanshu99@163.com

Key words: microRNA-215, hepatic steatosis, rapamycin-insensitive companion of mammalian target of rapamycin, insulin resistance

hepatic insulin resistance. These findings may provide novel insight for the treatment of T2D.

Materials and methods

Ethics statement. All animal protocols were approved by the Animal Care Committee of Southeast University (approval no. 2017-AN-1). All *in vivo* experiments described in the present study were in accordance with institutional guidelines for the care and use of animals (28).

In vivo study. Male Sprague Dawley rats (aged 8-12 weeks, 230-260 g) purchased from Kay Biological Technology Co., Ltd. (Shanghai, China) were maintained at a temperature of $23\pm3^{\circ}\text{C}$ and a humidity of $35\pm5\%$ under a 12 h dark/light cycle in a specific pathogen-free animal facility. A total of 14 rats were randomly separated into two groups, an HFD group and a control diet (CD) group. The HFD group was fed with D12451 formula (Research Diets, Inc., New Brunswick, NJ, USA), and the CD group received a standard diet. All rats were provided free access to water. Body weight and random glucose blood levels were measured every 5 days. On day 30, all rats were sacrificed. The livers, gastrocnemius (GAS) and epididymal white adipose tissue (eWAT) were immediately excised and placed in liquid nitrogen and stored at -80°C . Blood was also collected from the heart to investigate the components present in the serum.

Tolerance tests. The glucose tolerance test (GTT) and pyruvate tolerance test (PTT) were performed by administering an intraperitoneal injection of glucose (2 g/kg) or sodium pyruvate (2 g/kg), respectively, into rats following a 12-16-h period of starvation. The blood glucose levels of the rats were measured at 0, 15, 30, 60, 90 and 120 min following treatment. In the insulin tolerance test (ITT), rats were starved for 6 h prior to intraperitoneal injection of insulin (0.8 U/kg), and the blood glucose levels of the rats were measured 0, 15, 30, 60, 90 and 120 min later (Abbott Diabetes Care; Abbott Pharmaceutical Co., Ltd., Lake Bluff, IL, USA) (29).

Measurement of metabolic profile. Triglycerides and free fatty acids (FFAs) in the liver were analysed according to the manufacturer's protocols (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Blood samples were centrifuged at $3,000 \times g$ at 4°C for 15 min to isolate the serum, and insulin levels were detected using an ELISA kit (EZRMI-13K, EMD Millipore, Billerica, MA, USA).

Cell culture. The rat hepatocarcinoma cell line, H4IIE (ml-cs-0524; American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Lonsera Science, Canelones, Uruguay), 1 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C .

Mimics for miR-215 (5'-AUGACCUAUGAUUUGACA GACA-3') and inhibitors for miR-215 (Ant-215; 5'-UGUCUG UCAAAUAGGUCAU-3') were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Nonsense sequences

were used as mimics negative control (NC; 5'-UCACAACCU CCUAGAAAGAGUAGA-3') and Ant-NC (5'-UCUACUCUU UCUAGGAGGUUGUGA-3'). Cells were transfected with mimics or inhibitors (100 nM) using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc.) when the cells reached 70-80% confluence and treated for 48 h prior to RNA and protein isolation. Cells were starved for 4 or 12 h in serum-free DMEM containing 0.5% bovine serum albumin (BSA, Sangon Biotech Co., Ltd., Shanghai, China) prior to FFA treatment. FFAs, including palmitic acid (PA), linoleic acid (LA) and oleic acid (OA) were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and diluted to 0.5 mM; BSA (0.5 mM) was used as a control. Cells were treated with FFA for 48 h at 37°C according to the manufacturer's protocols.

For the overexpression of Rictor, Rictor was cloned without its 3'-UTR into a pcDNA3.1 plasmid (VT1001, YouBio, Hunan, China) using the forward primer 5'-TTGCGGCCG CATGAGAAAGCTGGGCCATCTG-3' and reverse primer 5'-CCGCTCGAGTCAGGATTCGGCAGATTCGT-3', and the restriction enzymes *NotI* (R3189S, New England BioLabs, Inc., Ipswich, MA, USA) and *XhoI* (R0146S, New England BioLabs, Inc.). Cells were transfected with pcDNA3.1-Rictor using Lipofectamine 2000 as aforementioned and lysed 48 h later for subsequent experiments.

Insulin signalling analysis. H4IIE cells were transfected with mimics or plasmids for 36 h, starved for 12 h, and treated with 100 nM insulin for 5-20 min at 37°C prior to protein collection for western blot analysis, as described below.

Bioinformatics analysis. Putative target genes of miR-215 were screened using TargetScan 7.2 (http://www.targetscan.org/vert_72/).

Plasmid and luciferase assay. To construct reporter plasmids, the 3'-UTR sequence of Rictor containing the miR-215 regulatory elements (MRE) was cloned into the pRL-TK plasmid (YouBio). Mutations in the MRE were made using the KOD-Plus mutagenesis kit (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocols. H4IIE cells were cultured in a 48-well plate and co-transfected with miR-215 mimics (100 or 200 nM) or mimics-NC (100 nM), and pRL-TK-Rictor-3'-UTR-wild-type (wt) or pRL-TK-Rictor-3'-UTR-mutant (mut) by Lipofectamine 2000; pGL3basic (YouBio) served as an internal reference. Following culture for 48 h, cells were lysed and relative luciferase activity was analysed with the Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) on a luminometer (Promega Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.) from cells and rat livers, and 500 ng of RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent Kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocols. qPCR was performed on an Applied Biosystems[™] 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using 4.6 μl cDNA, 5 μl SYBR[®] Green (Roche Diagnostics, Basel, Switzerland) and 0.4 μl (10 μM) primers. qPCR was performed under the

following conditions: 95°C for 10 min, then 40 cycles of 95°C for 25 sec, 60°C for 30 sec and 72°C for 30 sec. The relative expression of genes was calculated using the $2^{-\Delta\Delta C_q}$ method and normalised to GAPDH (30). The primer sequences used were as follows: miR-215, forward 5'-ACACTCCAGCTGGGATGACCTATGATTTGA-3', reverse, 5'-CTCAACTGGTGTCGTGGAGTCGG-3'; glucose 6-phosphatase (G6Pase), forward 5'-AAGCCAACGTATGGATTCCG-3', reverse, 5'-ACAGCAATGCCTGACAAGACT-3'; phosphoenolpyruvate carboxykinase (PEPCK), forward 5'-GTGCTGGAGTGGATGTTCGG-3', reverse, 5'-CTGGCTGATTCTCTGTTTCAGG-3'; peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α), forward 5'-CAATGAATGCAGCGGTCTTA-3', reverse, 5'-GTGTGAGGAGGGTCATCGTT-3'; and GAPDH, forward 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Western blotting. Cells or tissues were lysed in radioimmunoprecipitation lysis buffer [10 mM Tris-HCl (pH 7.5), 1% SDS, 1 mM Na_3VO_4 , 10 mM NaF] and protease inhibitor cocktail (Roche Diagnostics), and total protein was quantified using the Bradford assay. Protein samples (20 μg) were separated via 80 V under constant pressure 10% SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes with 300 mA for 3 h and blocked with 5% skimmed milk for 1 h at room temperature. Membranes were probed with the following primary antibodies (diluted with 5% BSA, Sangon Biotech Co., Ltd.) at 4°C overnight: Anti-AKT (1:1,000; cat. no. 4685, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphorylated (p)-AKT (Ser473; 1:1,000; cat. no. 4060, Cell Signaling Technology, Inc.), anti-Rictor (1:1,000; cat. no. 5379, Cell Signaling Technology, Inc.), anti-GSK3 β (1:1,000; BM2974, Boster Biological Technology, Pleasanton, CA, USA), anti-p-GSK3 β (1:1,000; cat. no. 9323, Cell Signaling Technology, Inc.), anti-actin (1:1,000; ab8227, Abcam, Cambridge, UK) and anti-GAPDH (1:5,000; HPA040067, Sigma-Aldrich; Merck KGaA). Membranes were then washed with TBS-0.1% Tween 20 buffer and probed with horseradish peroxidase-conjugated secondary antibodies (HAF008, Novus Biologicals, LLC, Littleton, CO, USA) diluted with 5% skim milk (1:2,000) for 1 h at 37°C. Protein bands were visualised using SuperSignal Ultra (36209ES01, Shanghai Yeasen Biotechnology Corporation Co., Ltd., Shanghai, China). ImageJ V1.49 (National Institutes of Health, Bethesda, MD, USA) was employed for densitometry analysis.

Liver histology. The livers were extracted from rats and fixed in 4% paraformaldehyde at 37°C for 24–36 h. Paraffin-embedded sections (10 μm) were subjected to H&E and Oil-Red staining for 10 min at room temperature with aid from Wuhan Google (Wuhan, China). Images of Oil-Red and H&E staining were acquired using an inverted microscope (magnification, x20; Olympus Corporation, Tokyo, Japan); three fields per view were analysed.

Statistics. Data were presented as the mean \pm standard deviation of at least three independent experiments. Animal sample size for each study was selected on the basis of literature documentation of similar experiments, and no statistical method was used to pre-determine the sample size. Excel (Microsoft

Corporation, Redmond, WA, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. Differences between multiple groups were determined using one-way analysis of variance followed by a Dunnett's post-hoc test. Differences between two groups were analysed using Student's t-tests. Samples were not excluded during the animal experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HFD leads to hepatic steatosis in rats. A rat model of short-term fatty liver was generated to investigate the association between fatty liver and hepatic insulin resistance. Rats were randomly separated into two groups; one group was fed a CD and the other was fed a HFD. The blood glucose levels and liver condition of rats were evaluated after 30 days. As presented in Fig. 1A, HFD rats exhibited significantly increased body weight following 25 days compared with the CD group. Oil-Red and H&E staining of livers following 30 days on the respective diets revealed that the lipid content of the livers of HFD rats was markedly increased compared with CD rats (Fig. 1B). Additionally, the liver weight: Body weight ratio of HFD rats was significantly increased compared with CD rats (Fig. 1C). The lipid content of the livers was determined via an ELISA. As presented in Fig. 1D, the lipid content of livers from the HFD group was significantly increased compared with that of the CD group. The results suggest that the HFD treatment successfully induced fatty liver symptoms in rats.

HFD rats develop hepatic insulin resistance. A number of studies reported an association between fatty liver and T2D (31,32). To investigate whether the hepatic insulin signalling pathway was impaired in rats with fatty liver, blood glucose levels were regularly monitored (Fig. 2A). Random blood glucose levels in HFD rats were significantly elevated on days 25 and 30 compared with in the CD group. Furthermore, HFD treatment resulted in significantly impaired glucose, pyruvate and insulin tolerance compared with CD treatment (Fig. 2B–D). As presented in Fig. 2B and D, there were no significant differences in glucose and insulin tolerance reported between the two groups 60 and 90 min following injection. This may be due to two reasons; the HFD group was not fed for a sufficient amount of time to develop severe insulin resistance, or the serum glucose level of rats may be highly variable. The number of rats in each group may be insufficient to counteract this variability; however, the maximum possible number of rats were included in each group. On the contrary, the area under the curves across the 120-min period indicate significant differences in the glucose and insulin tolerances between the two groups.

Furthermore, the expression of the major molecular nodes of the insulin signalling pathways in the livers of HFD and CD rats were determined by western blot analysis. It was revealed that the expression levels of p-AKT and p-GSK3 β protein in the liver of HFD rats were significantly decreased compared with CD rats (Fig. 2E and F). The liver modulates glucose homeostasis of the body via the regulation of gluconeogenesis (33). Therefore, the expression of gluconeogenesis-associated genes, including G6PASE,

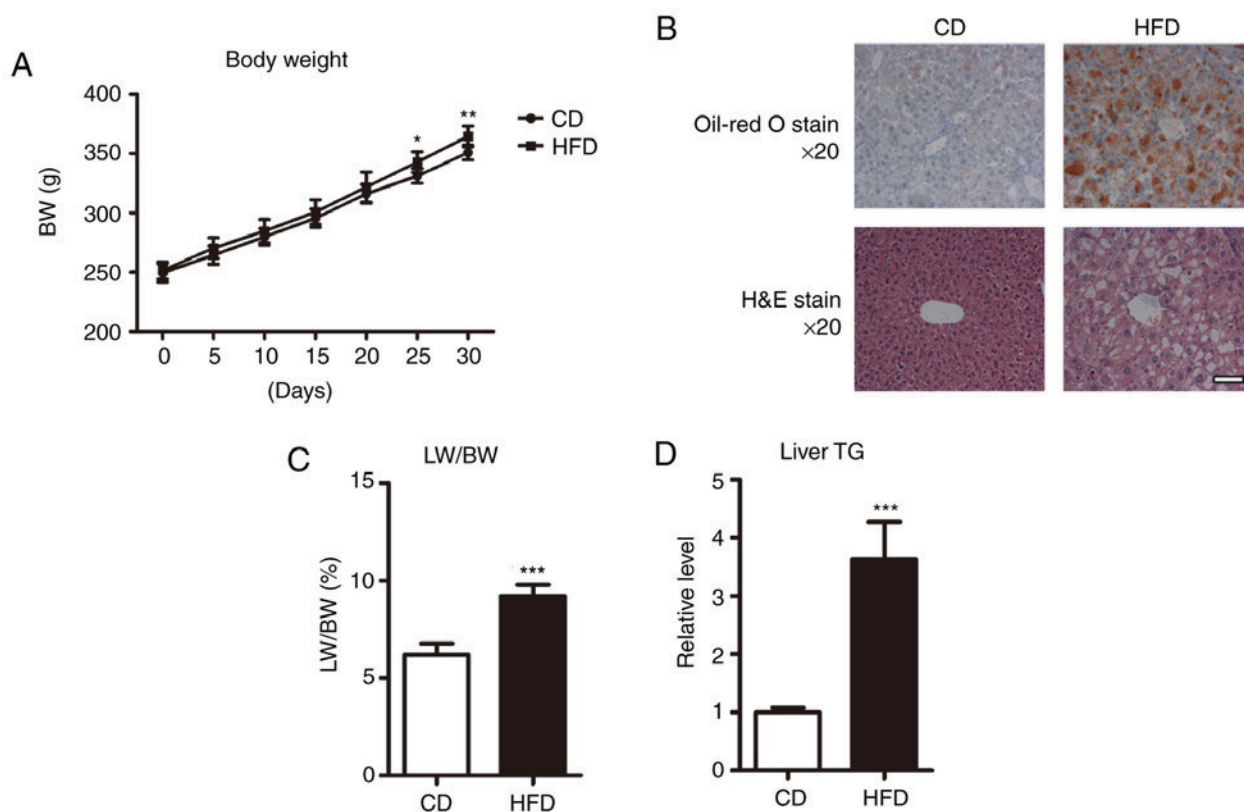


Figure 1. Effects of CD and HFD on the bodies and livers of rats. (A) Time course of the BW of rats fed with a CD or HFD. (B) Oil-Red O and HE staining of the livers of CD and HFD rats. Scale bar, 50 mm. (C) LW/BW ratio of the rats fed with a CD or HFD. (D) Levels of TG in the liver, normalised to LW. Data are presented as the mean \pm standard deviation of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. CD (Student's t-test). All experiments were repeated at least twice. BW, body weight; CD, control diet; HFD, high-fat diet; LW, liver weight; TG, triglyceride.

PEPCK and PGC-1 α , in the liver of the two groups was investigated by RT-qPCR. It was demonstrated that the expression levels of gluconeogenesis-associated genes in HFD rat livers were significantly increased compared with control rats (Fig. 2G). In addition to the liver, muscle and adipose tissues are involved in glucose homeostasis (34). The levels of p-AKT and p-GSK3 β expression in rat GAS and eWAT were determined. It was demonstrated that the levels of p-AKT and p-GSK3 β expression in HFD GAS (Fig. 2H) and eWAT (Fig. 2I) were not markedly altered. Additionally, it was revealed that the serum expression levels of insulin in HFD rats were significantly increased compared with in CD rats (Fig. 2J). The results indicated that HFD rats may have developed hepatic insulin resistance and hyperglycaemia.

miR-215 is upregulated in rat fatty liver and targets Rictor. Numerous studies reported that miRNAs were involved in the regulation of glucose homeostasis (29,35), and that the expression of miR-215 in fatty liver was aberrant, as observed via array analysis (36,37). The contribution of miR-215 to the development of T2D and fatty liver requires further study. The involvement of miR-215 in hepatic insulin resistance was investigated and the expression levels of miR-215 in the livers of the CD and HFD groups were determined. It was revealed that miR-215 expression in the livers of the HFD group was significantly increased compared with in the CD group (Fig. 3A). Via bioinformatics analysis, an miR-215 binding site was reported in the 3'-UTR of Rictor that is conserved across

several species (Fig. 3B). Rictor is an important regulatory protein in the insulin signalling pathway (25). By transfecting H4IIE cells with mimics-215, miR-215 was successfully overexpressed and significantly reduced the levels of Rictor protein expression compared with the control (Fig. 3C and D). Conversely, following transfection of H4IIE cells with Ant-NC or Ant-215, it was demonstrated that Ant-215 significantly suppressed the expression of miR-215 and increased the levels of Rictor protein expression compared with the control (Fig. 3E and F). To investigate whether Rictor is a direct target for miR-215, the 3'-UTR of Rictor, including the MRE, was cloned; luciferase reporter assays demonstrated that miR-215 mimics significantly reduced the activity of the reporter gene containing Rictor 3'-UTR-wt but not Rictor 3'-UTR-mut (Fig. 3G), suggesting that Rictor was a target of miR-215. Additionally, Rictor protein expression was determined in the liver of HFD and CD rats; it was revealed that the levels of Rictor protein expression were significantly decreased in the livers of the HFD group compared with the CD group (Fig. 3H). The results indicated that Rictor is a direct target gene of miR-215.

Overexpression of miR-215 impairs insulin signalling. Insulin signalling was investigated in H4IIE cells following overexpression of miR-215 (Fig. 4A). It was revealed that overexpression of miR-215 markedly reduced the levels of Rictor expression and decreased the insulin-stimulated phosphorylation of AKT compared with the NC. The expression of

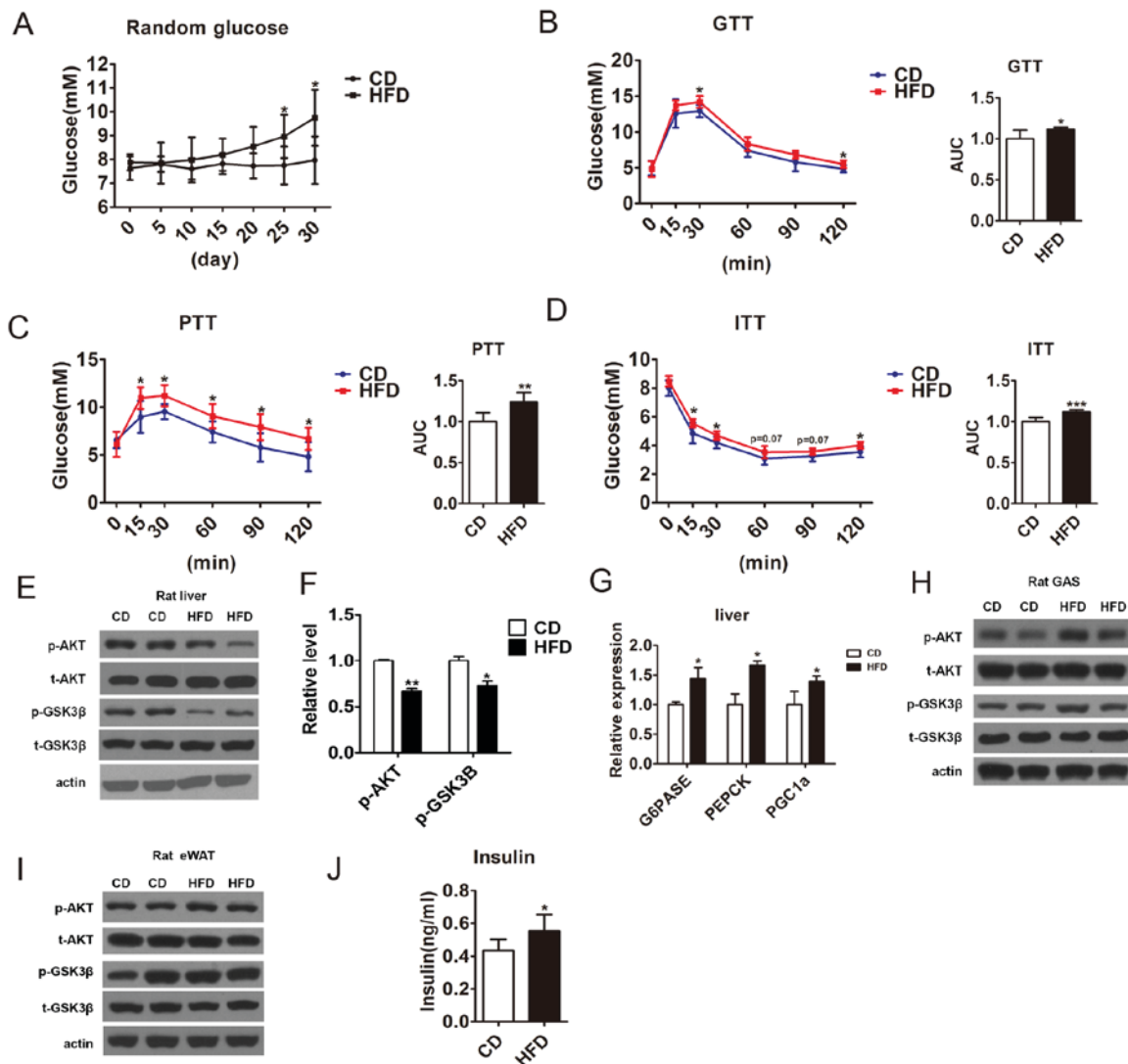


Figure 2. Alterations in blood glucose levels and insulin signalling pathway activity in CD and HFD rats. (A) Time course of random glucose levels of rats fed with a CD or HFD. (B) GTT, (C) PTT and (D) ITT experiments were performed in CD and HFD rats. AUC data were calculated for each test. Western blot analysis of protein phosphorylation involved in the insulin signalling pathway in the (E and F) liver of CD and HFD rats. (G) Expression levels of G6Pase, PEPCK and PGC-1 α in the liver of CD and HFD rats were determined via reverse transcription-quantitative polymerase chain reaction. Western blot analysis of protein phosphorylation involved in the insulin signalling pathway in the (H) GAS and (I) eWAT of CD and HFD rats. (J) Serum insulin levels of CD and HFD rats were determined via ELISA. Data are presented as the mean \pm standard deviation of at least three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 vs. CD (Student's t -test). AKT, protein kinase B; AUC, area under the curve; CD, control diet; eWAT, epididymal white adipose tissue; G6Pase, glucose 6-phosphatase; GAS, gastrocnemius; GSK3 β , glycogen synthase kinase 3 β ; GTT, glucose tolerance test; HFD, high-fat diet; ITT, insulin tolerance test; p, phosphorylated; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1 α , peroxisome proliferator-activated receptor γ co-activator 1 α ; PTT, pyruvate tolerance test.

gluconeogenesis-associated genes was also evaluated; it was demonstrated that overexpression of miR-215 significantly increased the expression levels of G6Pase, PEPCK and PGC-1 α compared with the NC (Fig. 4B). Conversely, knock-down of miR-215 by Ant-215 markedly increased the levels of Rictor and p-AKT expression following treatment with insulin compared with the NC (Fig. 4C), and significantly decreased the expression levels of gluconeogenesis-associated genes (Fig. 4D). Rictor was subsequently cloned without its 3'UTR into the pcDNA3.1 plasmid; as presented in Fig. 4E, transfection of H4IIE cells with pcDNA3.1-Rictor significantly increased the expression levels of Rictor mRNA and protein compared with empty vector. Following restoration of Rictor expression, the suppressive effects of mimics-215 on the phosphorylation of AKT were eliminated (Fig. 4F), indicating that regulating the

expression of miR-215 affects the insulin signalling pathway. Then, the mechanisms promoting enhanced miR-215 expression in fatty liver were investigated. As triglyceride levels were significantly increased in livers from HFD rats, it was hypothesised that fatty acids may regulate the expression of miR-215. H4IIE cells were stimulated with various fatty acids; it was revealed that PA, LA and OA markedly upregulated the levels of miR-215 expression compared with control BSA treatment (Fig. 4G). Furthermore, the protein expression levels of Rictor were markedly decreased in H4IIE cells following treatment with LA, PA and OA compared with BSA treatment (Fig. 4H). The levels of FFAs in the livers of HFD and CD rats were determined via an ELISA. It was demonstrated that the levels of FFAs in the liver were significantly increased in HFD rats compared with CD rats (Fig. 4I).

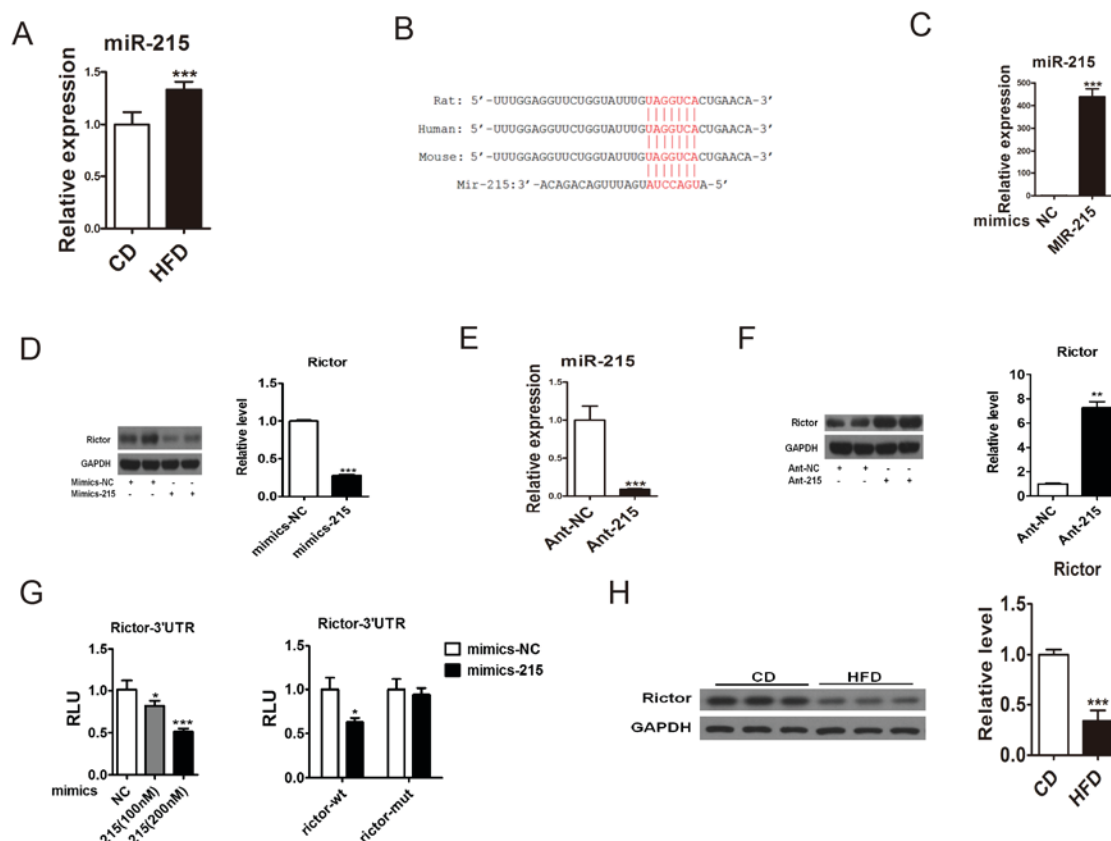


Figure 3. miR-215 is upregulated in the livers of HFD rats and targets Rictor. (A) Expression levels of miR-215 in the liver of rats fed a CD or HFD. (B) Sequence alignment of the 3'-UTR of the Rictor in different organisms and miR-215; seed sequences are highlighted in red. (C) Expression of miR-215 in H4IIE cells transfected with mimics-NC (100 nM) or mimics-215 (100 nM). (D) Western blot analysis of Rictor expression following transfection with mimics-NC or mimics-215 (n=3). (E) Expression of miR-215 in H4IIE cells transfected with Ant-NC (100 nM) or Ant-215 (100 nM). (F) Western blot analysis of Rictor expression following transfection with Ant-NC or Ant-215 (n=3). (G) Activity of the reporter plasmid containing the 3'-UTR-wt of Rictor was determined in H4IIE cells in the presence of increasing amounts of miR-215 mimics (left panel). Effects of mimics-NC and mimics-215 on the activity of reporters containing Rictor 3'-UTR-wt or 3'-UTR-mt (right panel; n=3). (H) Western blot analysis of Rictor expression in the liver of CD and HFD rats. Data are presented as the mean \pm standard deviation of at least three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 vs. CD or transfection with NC (Student's *t*-test or one-way analysis of variance). Ant, inhibitor of miR-215; CD, control diet; HFD, high-fat diet; miR-215, microRNA-215; mut, mutant; NC, negative control; Rictor, rapamycin-insensitive companion of mammalian target of rapamycin; 3'-UTR, 3'-untranslated region; wt, wild-type.

Discussion

Numerous studies have demonstrated an association between fatty liver and hepatic insulin resistance (7,38); however, the mechanistic basis of this association remains unclear. Using a rat model of a HFD, it was revealed that miR-215 exhibited increased expression in fatty liver and affected the hepatic insulin signalling pathway by targeting Rictor. In addition, it was demonstrated that increased levels of FFAs upregulated the expression of miR-215. Therefore, the findings of the present study may provide novel insight into the controversial association between NAFLD and hepatic insulin resistance.

Triglycerides are not signalling molecules; however, the lipid molecule diacylglycerol (DAG) has been hypothesised to be involved in hepatic steatosis and hepatic insulin resistance (38). It was reported that liver lipid content was a more reliable predictor of insulin resistance than other variables, including body mass index, long chain fatty acyl-coenzyme A content, ceramide content, endoplasmic reticulum stress markers and plasma concentrations of various inflammatory cytokines (31). Accumulation of DAG in the liver may promote the development of insulin resistance by activating

the downstream PKC signalling pathway (38). It has also been reported that inhibition or knockdown of PKC improves liver insulin sensitivity (32,39). There are numerous hepatokines that have been suggested to be involved in the association between fatty liver and hepatic insulin resistance, including adropin (40), angiopoietin-like 4 (41), fibroblast growth factor 21 (42), heparinase (43) and leukocyte cell-derived chemotaxin-2 (44).

Conversely, the accumulation of hepatic triglycerides may not directly lead to hepatic insulin resistance (45). For example, knockout of comparative gene identification-58 induces an increase in the levels of DAG in the livers of mice and promotes the development of NAFL; however, the livers remained sensitive to insulin (46). Similar phenotypes were observed in mice lacking microsomal triglyceride transfer protein (47) and mice with a liver-specific deletion of histone deacetylase 3 (48). These studies are inconsistent with the hypothesis that liver lipid accumulation is associated with hepatic insulin resistance.

Various studies have demonstrated that miRNAs regulate liver insulin sensitivity. A study reported that in the livers of ob/ob mice or mice with HFD-induced obesity, the

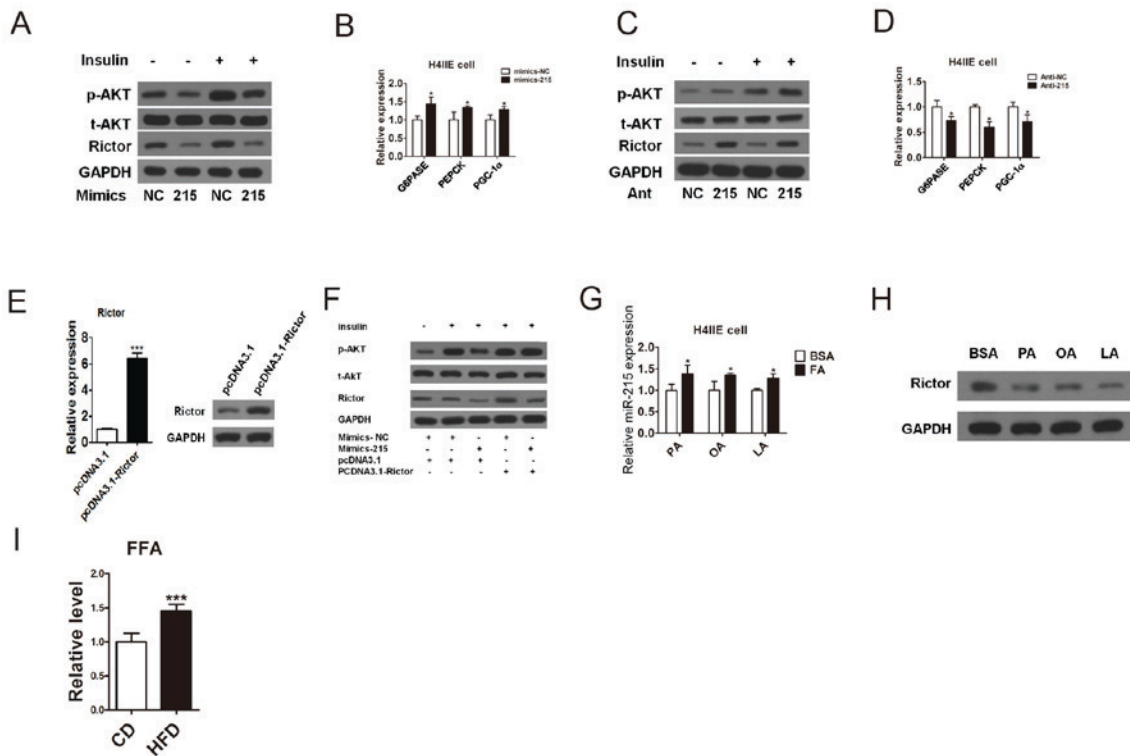


Figure 4. miR-215 expression affects insulin signalling and is regulated by FFAs. (A) Western blot analysis of p-AKT, AKT and Rictor expression in insulin-stimulated H4IIE cells transfected with mimics-NC (100 nM) or mimics-215 (100 nM). (B) Expression levels of G6Pase, PEPCK and PGC-1α in H4IIE cells transfected with mimics-NC or mimics-215. (C) Western blot analysis of p-AKT, AKT and Rictor expression in insulin-stimulated H4IIE cells transfected with Ant-NC (100 nM) or Ant-215 (100 nM). (D) Expression levels of G6Pase, PEPCK and PGC-1α in H4IIE cells transfected with Ant-NC or Ant-215. (E) Relative expression levels of Rictor mRNA and protein in H4IIE cells transfected with pcDNA3.1 or pcDNA3.1-Rictor. (F) Western blot analysis of p-AKT, AKT and Rictor expression in H4IIE cells transfected with mimics-215 with or without pcDNA3.1-Rictor following insulin stimulation. (G) Relative expression of miR-215 in H4IIE cells treated with BSA (5%), PA (0.5 mM), LA (0.5 mM) or OA (0.5 mM). (H) Western blot analysis of Rictor expression in H4IIE cells treated with BSA, PA, LA or OA. (I) Relative levels of FFA in the livers of rat fed with a CD or HFD. Data are presented as the mean \pm standard deviation of at least three independent experiments. * P <0.05 and ** P <0.001 vs. control (Student's *t*-test). AKT, protein kinase B; Ant, inhibitor of miR-215; BSA, bovine serum albumin; CD, control diet; FFA, free fatty acid; G6Pase, glucose 6-phosphatase; HFD, high-fat diet; LA, linoleic acid; miR-215, microRNA-215; NC, negative control; OA, oleic acid; p, phosphorylated; PA, palmitic acid; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor γ co-activator 1α; Rictor, rapamycin-sensitive companion of mammalian target of rapamycin.

expression levels of miR-103 and miR-107 were significantly increased (35). Furthermore, overexpression of miR-103 and miR-107 in the livers of normal mice induced impaired glucose homeostasis, hyperglycaemia and hyperinsulinaemia, whereas inhibiting the expression of miR-103 and miR-107 improved glucose homeostasis and insulin sensitivity. It has also been reported that caveolin-1, an important regulator of the insulin receptor, stabilised the insulin receptor in the cell membrane, and that overexpression of miR-103 and miR-107 inhibited caveolin-1 expression at the cell membrane, thereby inducing hepatic insulin resistance (35). Jordan *et al* (49) demonstrated that the levels of miR-143 expression were elevated in the livers of genetically altered or diet-induced models of insulin resistance. Overexpression of miR-143 was observed to result in glucose intolerance and insulin resistance in mice, whereas inhibition of miR-143 expression improved glucose tolerance and increased insulin sensitivity (43). MiR-215 is involved in the pathogenesis of various types of human malignant tumour (50,51); however, certain studies have reported the aberrant expression of miR-215 in fatty liver (36,37). The potential involvement of miR-215 in the development of T2D and fatty liver requires further investigation.

In the present study, a rat model with NAFLD and liver insulin resistance was generated by feeding rats with HFD for 1 month. It was revealed that the levels of miR-215 expression were significantly increased in HFD-fed rat livers. Furthermore, it was demonstrated using a luciferase reporter assay that Rictor was a direct target gene of miR-215. Overexpression of miR-215 in H4IIE cells suppressed the insulin signalling pathway in insulin-stimulated cells; conversely, inhibiting the expression of miR-215 increased the activity of the intracellular insulin signalling pathway. Whether the activity of the insulin signalling pathway can be promoted by regulating the levels of miR-215 expression *in vivo* requires further investigation. Additionally, it was demonstrated that FFAs upregulated the expression of miR-215 *in vitro*; however, the molecular mechanism has not yet been determined.

A number of studies have demonstrated that miRNAs are involved in glucose and lipid metabolism in the liver and the body. Therefore, it was hypothesised that miRNAs serve roles in NAFL and T2D. Various miRNAs have been screened by microarray analyses, and were reported as significantly differentially expressed in normal liver and NAFL. In the present study, the expression levels of not only miR-215, but other miRNAs were determined in NAFLD (data not shown).

Numerous studies reported that miRNAs were involved in the regulation of glucose homeostasis (29,35), and that the expression of miR-215 in fatty liver was aberrant, as observed via array analysis (36,37). Therefore, miR-215 was selected for further experimentation. Additional miRNAs likely serve important roles in NAFL and T2D. The findings of the present study suggest that miRNAs may be a novel target for investigating NAFLD and T2D.

In conclusion, the present study demonstrated that the expression levels of miR-215 were increased in fatty liver, potentially due to the enhanced production of FFAs, leading to a decrease in hepatic insulin sensitivity. Furthermore, a specific molecular mechanism was identified that may link fatty liver to hepatic insulin resistance. These findings demonstrate the importance of improving diagnostic screening of T2D to aid the early identification of patients with NAFLD.

Acknowledgements

The authors would like to thank the Institutional Technology Service Center of Southeast University (Nanjing, China) for technical support.

Funding

The present study was supported by the programme of Nanjing Medical Science Development (grant no. YKK16277).

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

WDL and YSL designed the experiments and analyzed the data. WDL conducted the majority of the experiments. JRJ made substantial contributions to the acquisition of data and drafting the manuscript. WDL and YSL drafted the manuscript.

Ethics approval and consent to participate

All animal protocols were approved by the Animal Care Committee of Southeast University (approval no. 2017-AN-1). All *in vivo* experiments described in the present study were in accordance with institutional guidelines for the care and use of animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- World Health Organization (WHO): Global report on diabetes 2016. WHO, Geneva, 2016. <http://www.who.int>.
- Magnusson I, Rothman DL, Katz LD, Shulman RG and Shulman GI: Increased rate of gluconeogenesis in type-II diabetes-mellitus-a C-13 nuclear-magnetic-resonance study. *J Clin Invest* 90: 1323-1327, 1992.
- Basu R, Chandramouli V, Dicke B, Landau B and Rizza R: Obesity and type 2 diabetes impair insulin-induced suppression of glycogenolysis as well as gluconeogenesis. *Diabetes* 54: 1942-1948, 2005.
- Leite NC, Salles GF, Araujo AL, Villela-Nogueira CA and Cardoso CR: Prevalence and associated factors of non-alcoholic fatty liver disease in patients with type-2 diabetes mellitus. *Liver Int* 29: 113-119, 2009.
- Targher G, Bertolini L, Padovani R, Rodella S, Tessari R, Zenari L, Day C and Arcaro G: Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care* 30: 1212-1218, 2007.
- Silverman JF, O'Brien KF, Long S, Leggett N, Khazanie PG, Pories WJ, Norris HT and Caro JF: Liver pathology in morbidly obese patients with and without diabetes. *Am J Gastroenterol* 85: 1349-1355, 1990.
- Marchesini G, Brizi M, Morselli-Labate AM, Bianchi G, Bugianesi E, McCullough AJ, Forlani G and Melchionda N: Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 107: 450-455, 1999.
- Bugianesi E, McCullough AJ and Marchesini G: Insulin resistance: A metabolic pathway to chronic liver disease. *Hepatology* 42: 987-1000, 2005.
- Korenblat KM, Fabbri E, Mohammed BS and Klein S: Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. *Gastroenterology* 134: 1369-1375, 2008.
- Tiikkainen M, Tamminen M, Hakkinen AM, Bergholm R, Vehkavaara S, Halavaara J, Teramo K, Rissanen A and Yki-Järvinen H: Liver-fat accumulation and insulin resistance in obese women with previous gestational diabetes. *Obes Res* 10: 859-867, 2002.
- Chu MP, Klopstein BJ, Krisky CM, Urbanski HF, Rooney WD, Kohama SG and Purnell JQ: Intrahepatic lipid, not visceral or muscle fat, is correlated with insulin resistance in older, female rhesus macaques. *Obesity (Silver Spring)* 21: 2021-2028, 2013.
- Bartel DP: MicroRNAs: Target recognition and regulatory functions. *Cell* 136: 215-233, 2009.
- Rottiers V and Näär AM: MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol* 13: 239-250, 2012.
- Liang H, Li Y, Luo RY and Shen FJ: MicroRNA-215 is a potential prognostic marker for cervical cancer. *J Huazhong Univ Sci Technolog Med Sci* 34: 207-212, 2014.
- Liu F, You X, Chi X, Wang T, Ye L, Niu J and Zhang X: Hepatitis B virus X protein mutant HBx Delta 127 promotes proliferation of hepatoma cells through up-regulating miR-215 targeting PTPRT. *Biochem Biophys Res Commun* 444: 128-134, 2014.
- Deng Y, Huang Z, Xu Y, Jin J, Zhuo W, Zhang C, Zhang X, Shen M, Yan X, Wang L, *et al*: MiR-215 modulates gastric cancer cell proliferation by targeting RB1. *Cancer Lett* 342: 27-35, 2014.
- Wijnhoven BP, Hussey DJ, Watson DI, Tsykin A, Smith CM, Michael MZ and South Australian Oesophageal Research Group: MicroRNA profiling of Barrett's oesophagus and oesophageal adenocarcinoma. *Br J Surg* 97: 853-861, 2010.
- Karaayvaz M, Pal T, Song B, Zhang C, Georgakopoulos P, Mehmood S, Burke S, Shroyer K and Ju J: Prognostic significance of miR-215 in colon cancer. *Clin Colorectal Cancer* 10: 340-347, 2011.
- Facchinetti V, Ouyang W, Wei H, Soto N, Lazorchak A, Gould C, Lowry C, Newton AC, Mao Y, Miao RQ, *et al*: The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. *EMBO J* 27: 1932-1943, 2008.
- Yuan M, Pino E, Wu L, Kacergis M and Soukas AA: Identification of Akt-independent regulation of Hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2. *J Biol Chem* 287: 29579-29588, 2012.
- Garcia-Martinez JM and Alessi DR: mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochem J* 416: 375-385, 2008.
- Ikenoue T, Inoki K, Yang Q, Zhou X and Guan KL: Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J* 27: 1919-1931, 2008.
- Sarbassov DD, Guertin DA, Ali SM and Sabatini DM: Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098-1101, 2005.

24. Yang Q, Inoki K, Ikenoue T and Guan KL: Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev* 20: 2820-2832, 2006.
25. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ and Sabatini DM: Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC alpha but not S6K1. *Dev Cell* 11: 859-871, 2006.
26. Soukas AA, Kane EA, Carr CE, Melo JA and Ruvkun G: Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans*. *Genes Dev* 23: 496-511, 2009.
27. Frias MA, Thoreen CC, Jaffe JD, Schroder W, Sculley T, Carr SA and Sabatini DM: mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr Biol* 16: 1865-1870, 2006.
28. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals, 8th edition. National Academies Press (US), Washington, DC, 2011.
29. Liu W, Cao H, Ye C, Chang C, Lu M, Jing Y, Zhang D, Yao X, Duan Z, Xia H, *et al*: Hepatic miR-378 targets p110alpha and controls glucose and lipid homeostasis by modulating hepatic insulin signalling. *Nat Commun* 5: 5684, 2014.
30. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
31. Kumashiro N, Erion DM, Zhang D, Kahn M, Beddow SA, Chu X, Still CD, Gerhard GS, Han X, Dziura J, *et al*: Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. *Proc Natl Acad Sci USA* 108: 16381-16385, 2011.
32. Samuel VT, Liu ZX, Wang A, Beddow SA, Geisler JG, Kahn M, Zhang XM, Monia BP, Bhanot S and Shulman GI: Inhibition of protein kinase C epsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. *J Clin Invest* 117: 739-745, 2007.
33. Rines AK, Sharabi K, Tavares CD and Puigserver P: Targeting hepatic glucose metabolism in the treatment of type 2 diabetes. *Nat Rev Drug Discov* 15: 786-804, 2016.
34. Czech MP: Insulin action and resistance in obesity and type 2 diabetes. *Nat Med* 23: 804-814, 2017.
35. Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, Heim MH and Stoffel M: MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* 474: 649-653, 2011.
36. Li S, Chen X, Zhang H, Liang X, Xiang Y, Yu C, Zen K, Li Y and Zhang CY: Differential expression of microRNAs in mouse liver under aberrant energy metabolic status. *J Lipid Res* 50: 1756-1765, 2009.
37. Park JH, Ahn J, Kim S, Kwon DY and Ha TY: Murine hepatic miRNAs expression and regulation of gene expression in diet-induced obese mice. *Mol Cells* 31: 33-38, 2011.
38. Petersen MC and Shulman GI: Roles of diacylglycerols and ceramides in hepatic insulin resistance. *Trends Pharmacol Sci* 38: 649-665, 2017.
39. Raddatz K, Turner N, Frangioudakis G, Liao BM, Pedersen DJ, Cantley J, Wilks D, Preston E, Hegarty BD, Leitges M, *et al*: Time-dependent effects of Prkce deletion on glucose homeostasis and hepatic lipid metabolism on dietary lipid oversupply in mice. *Diabetologia* 54: 1447-1456, 2011.
40. Zhao LP, Xu WT, Wang L, You T, Chan SP, Zhao X and Yang XJ: Serum adipon level in patients with stable coronary artery disease. *Heart Lung Circ* 24: 975-979, 2015.
41. Dijk W, Dijk W, Beigneux AP, Larsson M, Bensadoun A, Young SG and Kersten S: Angiopoietin-like 4 (ANGPTL4) promotes intracellular degradation of lipoprotein lipase in adipocytes. *J Lipid Res* 57: 1949-1949, 2016.
42. Zhang X, Yeung DC, Karpisek M, Stejskal D, Zhou ZG, Liu F, Wong RL, Chow WS, Tso AW, Lam KS and Xu A: Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans. *Diabetes* 57: 1246-1253, 2008.
43. Wu HT, Lu FH, Ou HY, Su YC, Hung HC, Wu JS, Yang YC, Wu CL and Chang CJ: The role of hepassocin in the development of non-alcoholic fatty liver disease. *J Hepatol* 59: 1065-1072, 2013.
44. Lan F, Misu H, Chikamoto K, Takayama H, Kikuchi A, Mohri K, Takata N, Hayashi H, Matsuzawa-Nagata N, Takeshita Y, *et al*: LECT2 functions as a hepatokine that links obesity to skeletal muscle insulin resistance. *Diabetes* 63: 1649-1664, 2014.
45. Farese RV Jr, Zechner R, Newgard CB and Walther TC: The problem of establishing relationships between hepatic steatosis and hepatic insulin resistance. *Cell Metab* 15: 570-573, 2012.
46. Brown JM, Betters JL, Lord C, Ma Y, Han X, Yang K, Alger HM, Melchior J, Sawyer J, Shah R, *et al*: CGI-58 knockdown in mice causes hepatic steatosis but prevents diet-induced obesity and glucose intolerance. *J Lipid Res* 51: 3306-3315, 2010.
47. Minehira K, Young SG, Villanueva CJ, Yetukuri L, Oresic M, Hellerstein MK, Farese RV Jr, Horton JD, Preitner F, Thorens B and Tappy L: Blocking VLDL secretion causes hepatic steatosis but does not affect peripheral lipid stores or insulin sensitivity in mice. *J Lipid Res* 49: 2038-2044, 2008.
48. Sun Z, Miller RA, Patel RT, Chen J, Dhir R, Wang H, Zhang D, Graham MJ, Unterman TG, Shulman GI, *et al*: Hepatic Hdac3 promotes gluconeogenesis by repressing lipid synthesis and sequestration. *Nat Med* 18: 934-942, 2012.
49. Jordan SD, Krüger M, Willmes DM, Redemann N, Wunderlich FT, Brönneke HS, Merkwirth C, Kashkar H, Olkkonen VM, Böttger T, *et al*: Obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism. *Nat Cell Biol* 13: 434-446, 2011.
50. Zhou SW, Su BB, Zhou Y, Feng YQ, Guo Y, Wang YX, Qi P and Xu S: Aberrant miR-215 expression is associated with clinical outcome in breast cancer patients. *Med Oncol* 31: 259, 2014.
51. Zang Y, Wang T, Pan J and Gao F: miR-215 promotes cell migration and invasion of gastric cancer cell lines by targeting FOXO1. *Neoplasma* 64: 579-587, 2017.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.