Clin. Lab. 2019;65:XXX-XXX

ORIGINAL ARTICLE

Peripheral Blood miR-937 May Serve as a Biomarker for Metabolic Disorders by Targeting AMPKα

Chunxiao Yang [1], Jun Guo [2], Yajun Lin [2], Gang Hu [2], Jie Wie [2], Xin Zhang [2], Jingyu Sun [2], Jian Li [1, 2]

[1] *Peking University Fifth School of Clinical Medicine, Beijing, China*
[2] *The MOH Key Laboratory of Geriatrics, Beijing Hospital, National Center of Gerontology, Beijing, China*

SUMMARY

Background: **The current study mainly evaluated whether peripheral blood miR-937 could be a biomarker to differentiate patients with metabolic disorders and healthy controls.**
Methods: **The peripheral blood was collected with patients with hyperglycemia, hyperlipidemia and healthy control. The relative peripheral blood miR-937 level in patients with metabolic disorders and healthy individuals were evaluated by real-time PCR. Receiver operating characteristic curve (ROC) analysis and Spearman's correlation coefficient were applied to evaluate whether miR-937 could be a potential biomarker for metabolic disorders. Dual luciferase reporter assay was performed to identify the possible target genes of miR-937.**
Results: **First, miR-937 was significantly increased (8.02 ± 8.27) in the peripheral blood of hyperglycemia patients. The level of miR-937 of patients with hyperlipidemia (13.7 ± 14.72) was also enhanced obviously compared with healthy controls (1 ± 1.35). ROC analysis showed that the peripheral blood levels of miR-937 could screen patients with hyperglycemia or hyperlipidemia from healthy controls. Furthermore, peripheral blood miR-937 level positively correlated with serum glucose level ($r = 0.556$, $p < 0.01$) as well as total serum TG/TC levels ($r = 0.455$, $p < 0.01$). Dual luciferase reporter assay indicated that miR-937 suppressed the relative luciferase activity of pmirGLO-AMPKα-3'UTR.**
Conclusions: **The upregulation of circulating miR-937 level may cause a metabolism disorder by suppressing the expression of AMPKα. miR-937 could be a potential biomarker to differentiate patients with metabolism syndrome from healthy controls.**
(Clin. Lab. 2019;65:xx-xx. DOI: 10.7754/Clin.Lab.2018.181113)

Correspondence:
Dr. Jian Li
The MOH Key Laboratory of Geriatrics
Beijing Hospital
National Center of Gerontology
Beijing 100730
China
Phone: +86 10 58115048
Fax: +86 10 65237929
Email: lijian@bjhmoh.cn

Manuscript accepted December 4, 2018

KEY WORDS

metabolic disorder, MiR-937, AMPKα, biomarker

INTRODUCTION

Metabolic syndrome is a group of interrelated disorders including hyperglycemia, hyperlipidemia, and obesity and hypertension together, which is becoming a major public and clinical problem worldwide [1]. Metabolic syndrome is tightly related to cardiovascular disease, and is considered a major risk factor for type 2 diabetes mellitus (T2DM), which affected nearly 30% of Europeans [2], almost 40% of Americans [3], and threatens public health worldwide. Hyperinsulinemia is a main characteristic of metabolic syndrome. It is caused by an

over secretion of insulin and is considered one important pathogenesis of T2DM and cardiovascular dysfunction [4-7].

miRNAs are noncoding endogenous, single-stranded RNAs. They consist of 18 - 25 nucleotides and regulate gene expressions by repression of target genes [8]. miRNAs participate in many cell processes, which are correlated with many diseases such as T2DM, obesity-induced hyperlipidemia, and nonalcoholic fatty liver disease (NAFLD) [9]. Recently, increasing evidence suggests that miRNA plays an important role in the development of metabolic syndrome [10,11]. For example, miR-223 is a reporter of diet-induced obesity [10], miR-103 accelerates fat cell development [11]. Therefore, miRNAs are potential biomarkers of metabolic syndrome and endocrine signaling molecules.

In the present study, we mainly focused on miR-937, which is shown to inhibit the proliferation of gastric cancer cells [12] and contribute to the proliferation of lung cancer cells [13]. However, whether circulating miR-937 regulates the progression of metabolic disorders has been poorly understood. For the first time, we showed that peripheral blood miR-937 level is significantly increased in patients with metabolic syndrome. Receiver operating characteristic curve (ROC) analysis confirmed that miR-937 could differentiate patients with hyperglycemia or hyperlipidemia from healthy controls. Further study showed that AMPK was the target gene of miR-937. These results indicate that circulating miR-937 could potentially be a non-invasive biomarker of metabolic disorders.

MATERIALS AND METHODS

Patient samples

Patient samples were collected from the Research Ethics Committee of Beijing Hospital, and written consent was obtained from all patients.

Peripheral blood from patients was divided into three groups, including hyperlipidemia (fasting blood TG higher than 1.7 mM and fasting blood TC higher than 6.0 mM, n = 27), hyperglycemia (fasting blood glucose higher than 6.1 mM, n = 24), and healthy controls (n = 33). Physical examinations, biochemical measurements, and body mass index (BMI) calculations were listed in Table 1.

One milliliter of blood was collected from each patient into a anticoagulation tube with ethylenediaminetetraacetic acid.

RNA isolation and real time PCR

Total RNA was harvested with RNAVzol LS (Vigorous, Beijing, China) according to the specific instructions to isolate small RNAs. The concentration and the purity of the RNA samples were monitored by a NanoDrop spectrophotometer (ND-1000, Nanodrop Technologies). RNA was reverse transcribed into cDNA using the Prime-Script one-step qRT-PCR kit (C28025-032, Invitrogen). Detailed qRT-PCR procedure was described as follows: 50 cycles of 95°C (10 seconds), 55°C (10 seconds), 72°C (5 seconds); 99°C (1 second); 59°C (15 seconds); 95°C (1 second), then 95°C for 10 minutes; then cooling to 40°C. The cDNA preparations were routinely tested by real-time PCR based on the SYBR Green I method, according to the manufacturer's instructions (TaKaRa). The amplification and detection of specific products were performed according to the manufacturer's protocol with the iQ5 system (BioRad). The U6 small nucleolar RNA was used as an internal control. The relative gene expression was normalized to U6 small nucleolar RNA. Each reaction was performed in triplicate, and analysis was performed by the $2^{-\Delta\Delta CT}$ method. The primers used in the current study were listed as follows:

miR-937-RT:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACCCAGCC;
U6-RT:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGACAAATATG;
miR-937-F: GCGCGCGTGAGTCAGGGTGG;
U6-F: GCGCGTCGTGAAGCGTTC;
Universal reverse primer: GTGCAGGGTCCGAGGT.

Cell culture

Two hundred ninety-three T cells and HepG2 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal calf serum (FCS; Gibco), streptomycin (100 mg/mL; Gibco), and penicillin (100 units/mL; Gibco) to a density of 2 x 10^3 cells/cm^2 in flasks at 37°C with 5% CO_2.

miRNA target prediction and dual-luciferase reporter assay

TargetScan (https://www.targetscan.org) was used to determine the potential target gene of miR-937. The 3' untranslated region (3'UTR) of AMPK containing the binding site of miR-937, was cloned into the pmirGLO plasmid. After 293 cells were seeded for 24 hours, miR-937 or scramble were cotransfected with blank pmirGLO or pmirGLO-AMPKα-3'UTR by vigofect (Vigorous, Beijing, China) according to the instructions. The luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (E1910; Promega).

Western Blot

Cell lysates (15 μg of protein) were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore), blocked with 8% nonfat dry milk, and probed with the antibodies at 4°C overnight. The blots were incubated with HRP-conjugated anti-IgG, followed by detection with ECL (Millipore). The antibodies against AMPKα and GAPDH were purchased from Cell Signaling.

Table 1. Biochemical index for patients and healthy controls.

Variable	Normal	Hyperglycemia	Hyperlipidemia
Total subjects (n)	33	24	27
Gender (male/female)	17/16	9/15	10/17
Age (years)	57.3 ± 3.91	58.6 ± 4.29	55.08 ± 5.25
BMI (kg/m^2)	25.00 ± 3.12	26.73 ± 3.20	25.52 ± 3.80
GLU (mmol/L)	4.99 ± 0.98	8.01 ± 1.20	5.82 ± 0.82
HBA1C (%)	5.35 ± 0.07	6.26 ± 0.63	6.06 ± 0.47
AST (U/L)	18.90 ± 7.33	21.36 ± 8.16	20.11 ± 8.40
ALT (U/L)	14.27 ± 6.32	20.51 ± 11.60	15.67 ± 11.72
AST/ALT	1.44± 0.54	1.18 ± 0.40	1.47 ± 0.47
HDL-cholesterol (mmol/L)	1.56 ± 0.86	1.50 ± 0.29	1.76 ± 0.26
LDL-cholesterol (mmol/L)	2.27 ± 0.34	3.64 ± 1.08	3.88 ± 0.90
Total cholesterol (mmol/L)	4.26 ± 0.53	5.53 ± 0.89	7.02 ± 0.25
Triglycerides (mmol/L)	0.90 ± 0.31	1.66 ± 0.64	1.53 ± 1.57

*** - p < 0.05, ** - p < 0.01, *** - p < 0.001 vs. healthy controls.**

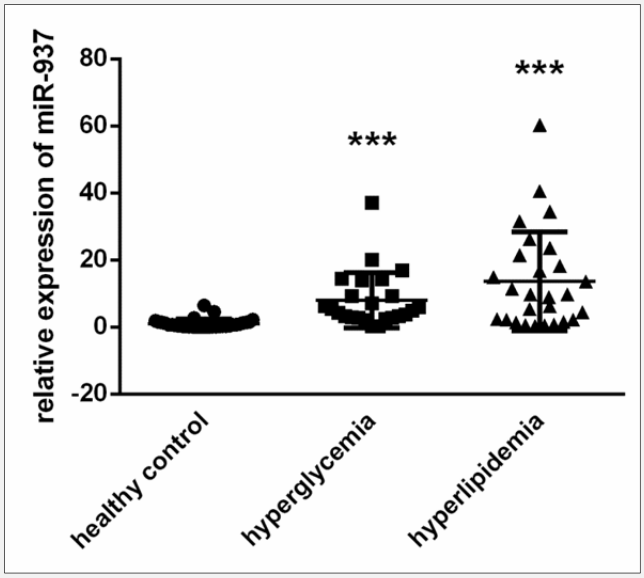

Figure 1. Upregulation of miR-937 in the peripheral blood of patients with hyperglycemia and hyperlipidemia.

***** - p < 0.001 vs. healthy controls.**

Statistics

The data are represented as the mean ± standard error (SD). The two-tailed unpaired Student's *t*-tests were used for comparisons of two groups. ROC curves were used to assess miR-937 as a biomarker, and the area under the curve (AUC) was reported (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA). Spearman's correlation coefficient was used to test the correlation between the expression of miRNA-937 and clinical index (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA). $p < 0.05$ was considered significant.

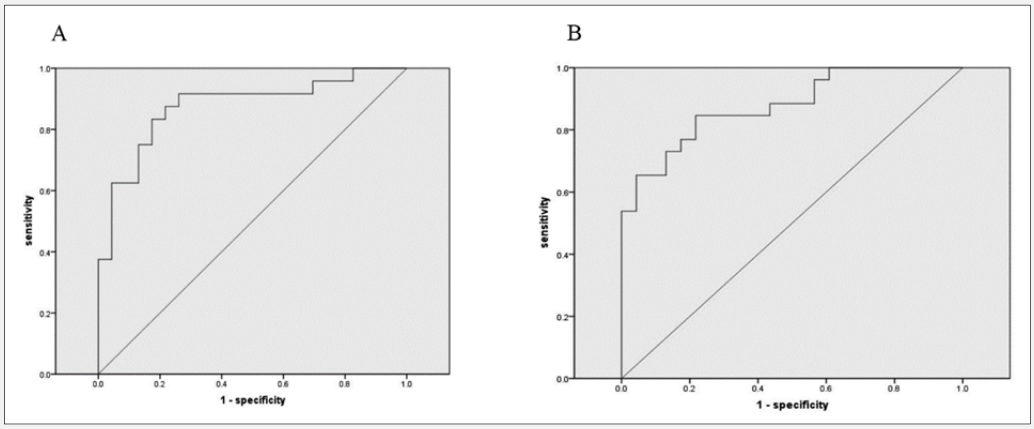

Figure 2. miR-937 could screen patients with metabolic disorders from healthy controls.

(A) ROC analysis showed that the peripheral blood levels of miR-937 may screen patients with hyperglycemia with healthy controls. (B) The peripheral blood levels of miR-937 could differentiate patients from hyperlipidemia from healthy controls.

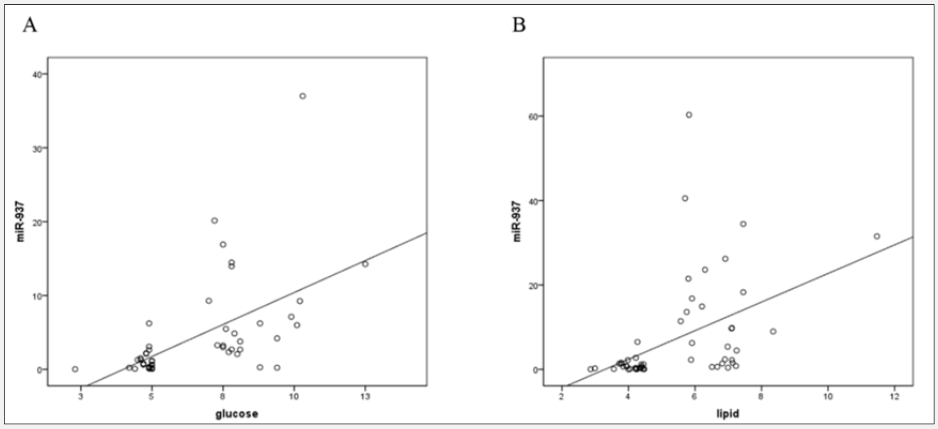

Figure 3. Peripheral blood miR-937 positively correlated with serum GLU and TG/TC levels.

(A) The level of miR-937 in peripheral blood was positively related to serum glucose level ($r = 0.592$, $p < 0.01$). (B) The level of peripheral blood miR-937 significantly correlated with total serum TG/TC levels ($r = 0.423$, $p < 0.01$).

RESULTS

Upregulation of miR-937 in the peripheral blood of patients with hyperglycemia and hyperlipidemia

We evaluated the peripheral blood level of miR-937 in patients with hyperglycemia or hyperlipidemia as well as controls by using real-time PCR. The level of miR-937 in the peripheral blood of hyperglycemia patients was increased to 8.02 ± 8.27 compared with healthy controls (1 ± 1.35). Meanwhile, the level of miR-937 was much higher in the peripheral blood of hyperlipidemia patients (13.7 ± 14.72) (Figure 1) compared with healthy controls. These results indicated that miR-937 was upregulated in patients with metabolic disorders.

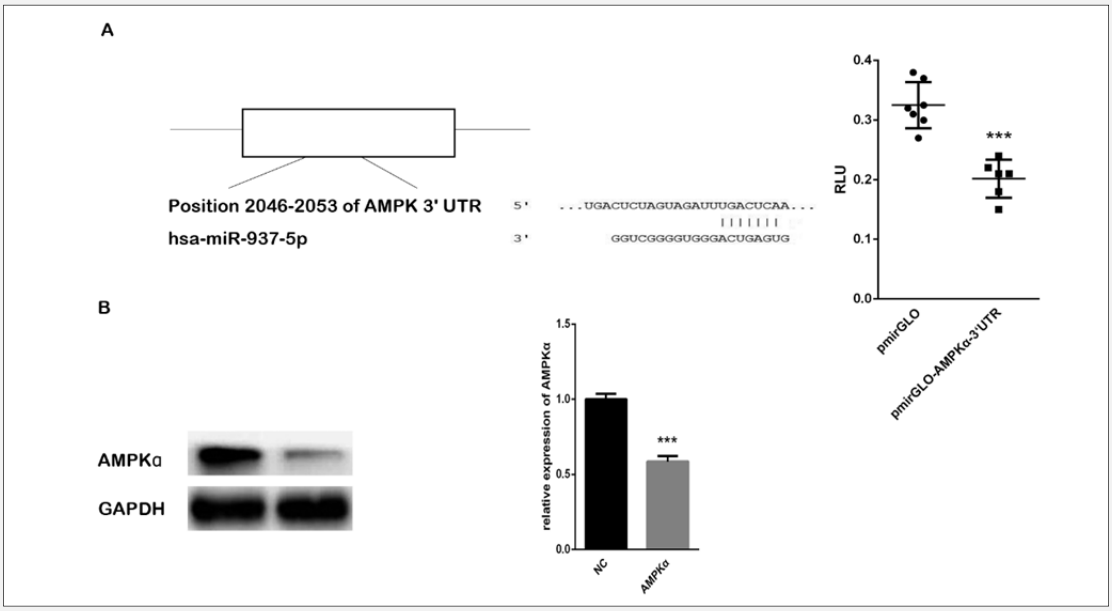

Figure 4. miR-937 targets AMPKα and regulates its expression.

(A) Dual luciferase reporter assay indicated that miR-937 significantly suppressed the relative luciferase activity of pmirGLO-AMPKα-3'UTR. (B) The expression of AMPKα was decreased after HepG2 cells were transfected with miR-937 mimics for 48 hours. * - $p < 0.001$ vs. control.**

miR-937 could screen patients with metabolic disorders from healthy controls

We then evaluated whether miR-937 could be a potential biomarker for patients with metabolic disorders. ROC analysis showed that the peripheral blood levels of miR-937 could differentiate patients with hyperglycemia from healthy controls, with a ROC curve area of 0.875 (95% confidence interval: 0.771 - 0.979; $p < 0.001$) (Figure 2A). Furthermore, the peripheral blood level of miR-937 could distinguish patients with hyperlipidemia from healthy controls, with a ROC curve area of 0.878 (95% confidence interval: 0.784 - 0.971; $p < 0.001$) (Figure 2B).

Peripheral blood miR-937 positively correlated with serum glucose and TG/TC levels

We evaluated whether the level of peripheral blood miR-937 was related to serum TG/TC or glucose levels by correlation analysis. The level of miR-937 in peripheral blood was related to serum glucose level ($r = 0.556$, $p < 0.01$) (Figure 3A). Meanwhile, the level of peripheral blood miR-937 was significantly correlated with total serum TG/TC levels ($r = 0.455$, $p < 0.01$) (Figure 3B).

miR-937 targets AMPKα and regulates its expression

Based on TargetScan, there is a conserved binding site identified in the 3'UTR of AMPKα, which is a key regulator in glucose and lipid metabolism. Then, we cloned the 3'UTR containing the binding sites of miR-937 into the pmirGLO plasmid (Figure 4A). Dual luciferase reporter assay showed that miR-937 obviously suppressed the relative luciferase activity of pmirGLO-AMPKα-3'UTR (Figure 4A). We then transfected miR-937 mimics into HepG2 cells for 48 hours. The expression level of AMPKα decreased compared with negative controls (Figure 4B). These results confirmed that mir-937 targeted AMPKα and regulated its expression.

DISCUSSION

At present, the number of obese people has increased dramatically, rendering metabolic syndrome as an increasingly important problem [14]. Many studies have shown that miRNAs are involved in the regulation of metabolism by affecting insulin signaling, lipogenesis, and lipid metabolism [15,16]. Therefore, miRNAs may be potential therapeutic targets. miRNAs are widely present in human peripheral blood, urine, and other liquid samples [17-19]. miRNAs in peripheral blood can

be stably present at room temperature [20]. In the current study, we assessed whether miR-937 could distinguish between normal and metabolic syndrome populations. MiR-937 showed a statistically significant increase in hyperglycemia and hyperlipidemia patients compared to healthy controls. ROC analysis also demonstrated that miR-937 could distinguish between healthy controls and patients with metabolic disorders. At the same time, miR-937 was positively correlated with blood glucose and TG/TC levels. These results indicate that peripheral blood miR-937 appears to be a candidate biomarker for metabolic disorders. It is well known that miRNAs regulate metabolism by regulating target genes [21,22]. Therefore, we predicted possible target genes of miR-937. Interestingly, a conserved binding site was identified in the 3'UTR of an important metabolic regulator, AMPKα. AMPKα regulates the activities of a number of key metabolic enzymes, inhibits the activation of autophagy, inflammation, ER and oxidative stress, which are involved in the pathogenesis of insulin resistance [23-25]. We confirmed that AMPKα is the target of miR-937 gene by luciferase assay and western blot. We then suggest that enhanced circulating miR-937 levels may result in a disorder of glycolipid metabolism in multiple organs by inhibiting the expression of AMPKα.

CONCLUSION

In summary, enhanced peripheral blood miR-937 could be used as a biomarker for metabolic disorder patients via targeting AMPKα. Since miR-937 is significantly elevated in peripheral blood, it is meaningful to further investigate the changes in various metabolic pathways after silencing miR-937. This may be full of difficulties due to the presence of the off-target effect. However, exploring the relationship between miR-937 and metabolic syndrome still has great significance for patients.

Acknowledgment:
This work was supported by grants (81570789, 81700 765 and 81671391) from the National Natural Science Foundation of China.

Declaration of Interest:
We declare no conflicts of interest.

References:

1. Grundy SM. Metabolic syndrome pandemic. Arterioscler Thromb Vasc Biol 2008;28:629-36 (PMID: 18174459).

2. Cameron AJ, Shaw JE, Zimmet PZ. The metabolic syndrome: prevalence in worldwide populations. Endocrinol Metab Clin North Am 2004;33:351-75, table of contents (PMID: 15158523).

3. Ford ES, Giles WH, Mokdad AH. Increasing prevalence of the metabolic syndrome among u.s. Adults. Diabetes Care 2004;27: 2444-9 (PMID: 15451914).

4. Battiprolu PK, Gillette TG, Wang ZV, Lavandero S, Hill JA. Diabetic Cardiomyopathy: Mechanisms and Therapeutic Targets. Drug Discov Today Dis Mech 2010;7:e135-e143 (PMID: 212744 25).

5. Cao W, Liu HY, Hong T, Liu Z. Excess exposure to insulin may be the primary cause of insulin resistance. Am J Physiol Endocrinol Metab 2010;298:E372 (PMID: 20075432).

6. Qi Y, Xu Z, Zhu Q, et al. Myocardial loss of IRS1 and IRS2 causes heart failure and is controlled by p38alpha MAPK during insulin resistance. Diabetes 2013;62:3887-900 (PMID: 241590 00).

7. Reaven GM. Why Syndrome X? From Harold Himsworth to the insulin resistance syndrome. Cell Metab 2005;1:9-14 (PMID: 16054040).

8. Chan LS, Yue PY, Wong YY, Wong RN. MicroRNA-15b contributes to ginsenoside-Rg1-induced angiogenesis through increased expression of VEGFR-2. Biochem Pharmacol 2013;86: 392-400 (PMID: 23688497).

9. Vienberg S, Geiger J, Madsen S, Dalgaard LT. MicroRNAs in metabolism. Acta Physiol (Oxf) 2017;219:346-61 (PMID: 27009 502).

10. Milagro FI, Miranda J, Portillo MP, et al. High-throughput sequencing of microRNAs in peripheral blood mononuclear cells: identification of potential weight loss biomarkers. PLoS One 2013;8:e54319 (PMID: 23335998).

11. Xie H, Lim B, Lodish HF. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. Diabetes 2009;58:1050-7 (PMID: 19188425).

12. Yu L, Chen J, Liu Y, Zhang Z, Duan S. MicroRNA-937 inhibits cell proliferation and metastasis in gastric cancer cells by downregulating FOXL2. Cancer Biomark 2017;21:105-16 (PMID: 290 60929).

13. Zhang L, Zeng D, Chen Y, et al. miR-937 contributes to the lung cancer cell proliferation by targeting INPP4B. Life Sci 2016;155: 110-5 (PMID: 27179609).

14. van Greevenbroek MM, Schalkwijk CG, Stehouwer CD. Dysfunctional adipose tissue and low-grade inflammation in the management of the metabolic syndrome: current practices and future advances. F1000Res 2016;5 (PMID: 27803798).

15. Yang Z, Cappello T, Wang L. Emerging role of microRNAs in lipid metabolism. Acta Pharm Sin B 2015;5:145-50 (PMID: 2657 9440).

16. Fernandez-Hernando C. Emerging role of microRNAs in the regulation of lipid metabolism. Hepatology 2013;57:432-4 (PMID: 22806606).

17. Ohyashiki K, Umezu T, Katagiri S, et al. Downregulation of Plasma miR-215 in Chronic Myeloid Leukemia Patients with Successful Discontinuation of Imatinib. Int J Mol Sci 2016;17:570 (PMID: 27092489).

18. Rainer TH, Leung LY, Chan CPY, et al. Plasma miR-124-3p and miR-16 concentrations as prognostic markers in acute stroke. Clin Biochem 2016;49:663-8 (PMID: 26968104).

19. Chen YP, Wang J, Zhao K, et al. The plasma miR-125a, miR-361 and miR-133a are promising novel biomarkers for Late-Onset Hypogonadism. Sci Rep 2016;6:23531 (PMID: 27000524).

20. Florczyk M, Brzuzan P, Krom J, Wozny M, Lakomiak A. miR-122-5p as a plasma biomarker of liver injury in fish exposed to microcystin-LR. J Fish Dis 2016;39:741-51 (PMID: 26345281).

21. Gotanda K, Hirota T, Saito J, et al. Circulating intestine-derived exosomal miR-328 in plasma, a possible biomarker for estimating BCRP function in the human intestines. Sci Rep 2016;6:32299 (PMID: 27571936).

22. Ghosh A, Ghosh A, Datta S, et al. Hepatic miR-126 is a potential plasma biomarker for detection of hepatitis B virus infected hepatocellular carcinoma. Int J Cancer 2016;138:2732-44 (PMID: 267 56996).

23. Kjobsted R, Pedersen AJ, Hingst JR, et al. Intact Regulation of the AMPK Signaling Network in Response to Exercise and Insulin in Skeletal Muscle of Male Patients With Type 2 Diabetes: Illumination of AMPK Activation in Recovery From Exercise. Diabetes 2016;65:1219-30 (PMID: 26822091).

24. Mandal SK, Shrestha PK, Alenazi FSH, Shakya M, Alhamami H, Briski KP. Role of hindbrain adenosine 5'-monophosphate-activated protein kinase (AMPK) in hypothalamic AMPK and metabolic neuropeptide adaptation to recurring insulin-induced hypoglycemia in the male rat. Neuropeptides 2017;66:25-35 (PMID: 28823463).

25. Ruderman NB, Carling D, Prentki M, Cacicedo JM. AMPK, insulin resistance, and the metabolic syndrome. J Clin Invest 2013; 123:2764-72 (PMID: 23863634).