Received Date : 25-May-2016

Revised Date : 17-Jul-2016

Accepted Date : 18-Jul-2016

Article type : Brief Communication

Donor graft microRNAs: a newly identified player in the development of new-onset diabetes after liver transplantation

Qi Ling [1,2], Haiyang Xie [2], Jie Li [1], Jimin Liu [1,3], Jili Cao [2], Fan Yang [1], Chao Wang [1], Qichao Hu [2], Xiao Xu [1,2], Shusen Zheng [1,2]

[1] Department of Surgery, Collaborative innovation center for diagnosis and treatment of infectious diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China

[2] Key Lab of Combined Multi-Organ Transplantation, Ministry of Public Health, China

[3] Department of Pathology and Molecular Medicine, Faculty of Health Sciences, McMaster University, Hamilton, Ontario, Canada

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ajt.13984

Corresponding author emails

Prof. Shusen Zheng: zyzss@zju.edu.cn

Prof. Xiao Xu: zjxu@zju.edu.cn

Running title: Donor graft microRNAs and NODALT

Abbreviations: NODALT, New-onset diabetes after liver transplantation; miRNA, microRNA; mRNA, message RNA; TAC, tacrolimus; BMI, body mass index; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CREB, cAMP responsive element binding protein; NFAT, nuclear factor of activated T cell;

ABSTRACT

New-onset diabetes after liver transplantation (NODALT) is a frequent complication with unfavorable outcome. We previously demonstrated a crucial link between the donor graft genetics and the risk of NODALT. Here, we selected 15 matched pairs of NODALT and non-NODALT liver recipients using propensity score matching analysis. The donor liver tissues were tested for the expression of ten miRNAs regulating human hepatic glucose homeostasis. The biological functions of potential target genes were predicted using Gene Ontology and KEGG enrichment analysis. Both miR-103 and miR-181a were significantly highly expressed in NODALT group as compared to non-NODALT

group. The predicted target genes (e.g., *Irs2*, *Pik3r1*, *Akt2*, and *Gsk3b*) were involved in glucose import and insulin signaling pathway. We also observed dysregulation of miRNAs (e.g., let-7, miR-26b, miR-145, and miR-183) in cultured human hepatocytes treated with tacrolimus or high glucose, the two independent risk factors of NODALT identified in this cohort. The altered hepatic miRNA profiles by tacrolimus or hyperglycemia were associated with insulin resistance and glucose homeostatic imbalance as revealed by enrichment analysis. The disease-susceptibility miRNA expressive pattern could be imported directly from the donor, and consolidated by the transplant factors.

New-onset diabetes after liver transplantation (NODALT) is a frequent complication in liver recipients and associated with an unfavorable clinical outcome (1, 2). Some well-known risk factors in recipients have been identified such as overweight, family history of diabetes mellitus, hepatitis C viral infection and immunosuppressive regimens (1, 2). However, the underlying mechanism for the development of NODALT remains poorly understood.

In our recent review, we proposed that the liver graft, itself, could be the origin of NODALT (3). The liver is a well-known metabolic centre and plays a key role in the glucose metabolism and homeostasis in liver transplant recipient. Increased evidences have emerged that both phenotype and genotype of the graft are involved in the development of NODALT (4, 5).

MicroRNA (miRNA) is a small non-coding single-stranded RNA and functions in RNA silencing and post-transcriptional regulation. It plays important roles in maintaining normal physiology as well as in disease processes. Compared to message RNA (mRNA), miRNA is well

conserved among vast species and highly stable in tissues, which makes it a promising modality in disease diagnosis, prognosis and therapy. It has been revealed that graft miRNA profiles could identify the risk of hepatitis C virus recurrence after liver transplantation (6). It is also known that miRNAs involve in hepatic glucose metabolism. For instance, let-7 (7), miR-29a (8), miR-103/107 (9, 10), miR-143/145 (11, 12), miR-181a (13), miR-183 (14) and miR-802 (15) impair hepatic insulin sensitivity, whereas miR-130a (16) and miR-26a (17) increase insulin signaling. In addition, miR-22-3p (18) and miR-26a (17) suppress gluconeogenesis. The dysregulation of hepatic miRNAs is closely associated with metabolic diseases. Therefore, in this study, we aimed to evaluate the impact of donor graft miRNAs in the development of NODALT.

PATIENTS AND METHODS

Patients

A total of 213 adult patients, non-preexisting diabetes, who underwent liver transplantation between September 2011 and December 2014 at the First Affiliated Hospital, College of Medicine, Zhejiang University, China, were included. There were 193 males and 19 females with a mean age of 46.8 ± 11.3 years. The majority of patients had hepatitis B-induced cirrhosis (89.6%). All patients were given lamivudine and low-dose intramuscular hepatitis B immunoglobulin as a prophylactic measure. Immunosuppressive regimen was composed of tacrolimus (TAC), mycophenolate, and steroid (4). All surgical procedures, including the organ procurement and transplantation, were performed by the same surgical team. This study was approved by the Institutional Review Board, the First Affiliated

Hospital, Zhejiang University, under the guidelines of the Declaration of Helsinki. Informed consent was obtained. No donor livers were harvested from the executed prisoners.

Data collection and definition

Patient demographics and clinical characters such as body mass index (BMI), primary liver disease, comorbidities, and biochemistry parameters (collected 24 hours before transplantation) were retrieved from the hospital electronic medical records. The post-transplant blood glucose levels and TAC concentrations were monitored as closely as possible within the first 3 months after liver transplantation (4). NODALT was defined as a fasting blood glucose of ≥ 7 mmol/L, or a non-fasting blood glucose of ≥ 11.1 mmol/L confirmed on at least 2 occasions or a need for antidiabetic drugs persisting beyond the first month after transplantation (4). Early hyperglycemia was defined as fasting blood glucose of ≥ 7.0 mmol/L confirmed on at least 2 occasions within the first post-transplant month (< 30 days) (19). Extended criteria donors include those with age > 50 years, steatosis > 30%, and cold ischemia time > 12 hours (20).

Cell culture

Human hepatocellular carcinoma cell lines HepG2 and HUH7, purchased from China Center for Type Culture Collection, were cultured in DMEM (Life technologies) containing 1.0 g/L of glucose and 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. Cell line authentication has been carried out by STR profiling in China Center for Type Culture Collection. TAC injection was supplied by Astellas Ireland Co., Ltd. (Killorglin, Co. Kerry, Ireland). Cells were seeded at 2.5×10^5 cells per well in 6-well plates and treated with different concentration of TAC (0, 5, 20 ng/ml) or glucose (5.5, 10, 30 mM). Protein was extracted after 72 h of culture.

Quantitative real-time PCR analysis

Total RNA was extracted from the donor graft tissues and cultured human cell lines with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). MicroRNA detection was performed using SYBR PrimeScript miRNA RT-PCR Kit (TaKaRa) according to the manufacturer's instructions. Ten miRNAs (miR-22 (18), miR-26a (17), miR-29a (8), miR-103 (9), miR-107 (10), miR-130a (16), miR-145 (12), miR-181a (13), miR-183 (14) and miR-802 (15) that reported to be associated with hepatic glucose metabolism in human tissue or cell lines were included (Table S1). Specific miRNA reverse-transcription primers were purchased from Shanghai GenePharma Co., Ltd. Real-time PCR was performed using a SYBR PCR kit in an Applied Biosystems 7900 Sequence Detection System. All tests were run in triplicate. The expression of miRNA/mRNA was plotted as the average cycle threshold value for each triplicate sample minus the average triplicate value for U6/GAPDH using the $2^{-\Delta\Delta CT}$ method.

miRNA microarray and bioinformatics analysis

Microarray profiles were obtained using a human Affymetrix GeneChip miRNA 4.0 Array (Affymetrix Technologies, USA) in different concentrations (0, 5, 20 ng/ml) of TAC treated HepG2 cells. After the significance (ANOVA) and false discovery rate (FDR) analyses, differentially expressed genes were selected according to a criterion (*P* value < 0.05, Q value < 0.2, and fold change > 1.2). Potential target genes of miRNAs were predicted by databases (miRanda and Targetscan) and underwent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses by KOBAS 2.0 (21). The glucose metabolism and insulin signaling associated pathways were identified. Gene-Cloud of Biotechnology Information

(https://www.gcbi.com.cn/gclib/html/index) was used to performed microarray data analysis. The microarray data have been uploaded to Gene Expression Omnibus (GSE81767, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE81767).

Western blot analysis

Western blot analysis was performed as described previously. The primary antibodies used were: anti-human IRS2 antibody (Catalog # bs-0173R, 1:500, bioss, Beijing, China), anti-human AKT antibody (Catalog # 4685, 1:2000, Cell Signaling, Danvers, MA, US), anti-human p-AKT (Ser473) antibody (Catalog # 4060, 1:2000, Cell Signaling, Danvers, MA, US), anti-human FoxO1 antibody (Catalog # 2880, 1:1000, Cell Signaling, Danvers, MA, US), anti-human TCF7L2 (TCF4) antibody (Catalog # 2565, 1:1000, Cell Signaling, Danvers, MA, US), and anti-β actin antibody (Catalog # 3700, 1:3000, Cell Signaling, Danvers, MA, US). Actin was used as a loading control.

Statistical analysis

Quantitative variables were described as the mean ± SD while categorical variables were presented as values (percentages). Quantitative variables were compared by independent-samples *t* test or Mann-Whitney test, and categorical variables were compared by Pearson's χ^2 test (Fisher's exact test). Cumulative survival was compared by Kaplan-Meier analysis with the log-rank method. The risk factors were evaluated by logistic regression analysis. Patient selection was performed by propensity score matching (22). Pearson analysis was performed to evaluate the correlation. SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used to complete analyses. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Donor graft miRNAs express differently in NODALT and non-NODALT patients

Out of 213 liver recipients, 61 (28.2%) developed NODALT with a median duration of 38 days (range: 30-423 days) post-operatively. From logistic analysis, early hyperglycemia and high blood TAC concentration were two independent risk factors of NODALT, with increased disease risk by 1.7- and 2.1-fold, respectively (Table 1).

Matched by the above two risk factors as well as age and gender, 43 pairs of NODALT and non-NODALT patients were initially selected. After eliminating recipients with extended criteria donors, 15 matched NODALT and non-NODALT cases were chosen (Table 2). Compared to the non-NODALT group, the NODALT patients had significantly higher miR-103 ($P = 0.013$, fold change = 2.6) and miR-181a ($P = 0.002$, fold change = 3.5) expression (Figure 1A). The expression of other 8 miRNAs did not differ significantly between the two groups (Figure S1). Furthermore, donor graft miR-103 and miR-181a levels were positively correlated with fasting blood glucose levels following liver transplantation ($P < 0.05$) (Figure 1B). According to GO and KEGG pathway analyses, the predicted target genes of miR-103 and miR-181a were involved in glucose import and insulin signaling pathway, e.g., *Irs1*, *Irs2*, *Pik3r1*, *Akt2*, *Gsk3b*, *Gys2*, *G6pc2*, *Hnf1a*, *Tcf7l2*, and *Foxo1* (Table S2).

TAC and hyperglycemia induce dysregulation of hepatic miRNAs

TAC has been considered as a predominant contributor of new-onset diabetes after solid organ transplantation. To evaluate the impact of TAC on hepatic miRNA profiles, we treated HepG2 cells with 0, 5 and 20 ng/ml of TAC for 24 h and compared the expression of miRNAs using Microarrays.

We observed 124 and 60 different expressed miRNAs between low-dose TAC group and control group, as well as high-dose TAC group and control group, respectively (Table S3). Twenty-seven miRNAs were significantly up-regulated by both low- and high-dose TAC, mostly, in a dose-related manner (Table 3). Their predicted target genes were undergone GO and KEGG pathway analyses (Figure S2). We could observe that TAC inhibited the calcium signaling pathway, e.g., *Calm1*, *Camk2b*, and *Camk2g*. Furthermore, two well described molecular targets of TAC in regulating insulin secretion, cAMP responsive element binding protein (CREB) transcriptional coactivator and nuclear factor of activated T cell (NFAT), were predicted as the targets of miR-4492 and miR-320e, respectively. The main glucose metabolism- and insulin signaling-associated pathways are presented in Table 4. Out of the 27 miRNAs, Let-7 (7) and miR-183 (14) were reported to impair hepatic insulin sensitivity, and miR-26b (23) was revealed to promote lipid synthesis and accumulation. Also, we noted that *Tcf7l2* and *Foxo1*, both of which are key molecules in activation of the gluconeogenesis, are targeted by miR-183. In addition, *Irs2*/*Akt2* and *Gsk3b*, which are associated with insulin signal transduction, are targeted by let-7 and miR-26b. We then verified the results in both HepG2 and HUH7 cells using real-time PCR that TAC increased the expressions of Let-7a, miR-26b and miR-183 in a dose-related manner (Figure 2A). We also found that prolonged incubation time resulted in elevated expressions of Let-7a, miR-26b and miR-183 only after high concentration of TAC treatment (20 ng/ml) but not in low concentration (5 ng/ml) (Figure 2B and 2C). Furthermore, we tested the mRNA levels and protein contents of the potential targets and found they were decreased by TAC (Figure 2D).

Early hyperglycemia was the other independent risk factor of NODALT besides TAC. To assess the effect of high glucose on hepatic miRNA levels, we treated HepG2 and HUH7 cells with 5.5, 10 and 30 mM of glucose for 24 h and tested for the expression of miRNAs. The expressions of miR-145 and miR-183 were increased after high glucose exposure in both cell lines (Figure 3), whereas the other eight miRNAs did not (Figure S3). Furthermore, we found the elevation of miR-145 and miR-183 was time-related in 10mM glucose medium (Figure 3). The predicted target genes are key regulators involved in gluconeogenesis (e.g., *Tcf7l2, Foxo1*), glycogen synthesis (e.g., *Gyg2, Gys1*), and insulin signaling (e.g., *Irs1, Irs2, Akt1, Akt2, Akt3*).

DISCUSSION

We previously have demonstrated that donor graft brings disease-susceptibility genes to the recipients and contributes to the development of metabolic disorders (4, 24). This study, from the view of miRNA, further confirmed our previous findings and demonstrated that donor grafts with certain miRNA profiles had greater risk of developing NODALT. We found markedly higher hepatic miR-103 and miR-181a expression in liver recipients with NODALT as compared to those without NODALT in a propensity-matched cohort. Elevated miR-103 and miR-181a levels in the donor grafts correlated with increased blood glucose level following liver transplantation. The possible underlying mechanism is that the two miRNAs target several key genes involved in insulin signal transduction and glucose homeostasis as revealed by the enrichment analysis. Among these predicted targets, some have been verified by previous studies. Trajkovski et al. have demonstrated that miR-103 directly targets *caveolin-1*, thereby diminishes the number of insulin receptors and reduces downstream

insulin signaling in both human and rodent models (10). Zhou et al. have shown that miR-181a directly targets *sirtuin-1* and induces hepatic insulin resistance in human hepatocytes and transgenic mouse models (13). Therefore, we suppose that the gaining of miR-103 and miR-181a in the donor graft impairs hepatic insulin sensitivity, which could be the "first hit" in the development of NODALT.

Furthermore, we propose that hyperglycemia and TAC, the two identified independent risk factors of NODALT in this study, act as a "second hit" in the development of NODALT. Immunosuppressive drugs have been long regarded as the major contributor in the development of diabetes in almost all types of solid organ transplantation. Since the implementation of the steroid-free protocol among the organ transplantation recipients, the calcineurin inhibitors, mostly TAC, have been regarded as one of the main diabetogenic agents (19, 25). Clinical studies as well as our current work have showed the significance of TAC in the development of NODALT (4, 19, 25, 26). However, the underlying mechanism has not been fully elucidated yet. Previous experimental studies have demonstrated that TAC reduces insulin secretion in pancreatic β cells (27) and may also induce insulin resistance (28). We here provide evidence that the liver is another target organ of TAC. Hepatic glucose homeostasis could be altered by TAC via regulating miRNA expression. As revealed by the enrichment analyses, TAC could potentially up-regulate 27 miRNA expression and affect glucose metabolic process and insulin signaling transduction. We further proved that TAC could induce hepatic insulin resistance via up-regulation of let-7 and miR-26b, which target IRS2/PI3K/AKT pathway. TAC also attenuated gluconeogenesis by up-regulation of miR-183, which targets TCF7L2 and FoxO1. Of note, only high concentration of TAC could induce time-dependent

elevation of certain miRNA profile that are involved in hepatic glucose metabolism. It may explain the higher risk of developing NODALT in patients with high concentration of TAC.

Hyperglycemia in the immediate post-transplant period has been considered as a major predictor of NODALT in our recent National Report (19). In kidney transplant recipients, immediate post-transplant insulin therapy, leading to better blood glucose control, was found to reduce the risk of developing new-onset diabetes (29). One possible explanation is the glucose's direct toxicity in pancreatic β cells (30). Another is hyperglycemia-induced peripheral insulin resistance (31). *In vitro* experiments usually require high glucose exposure to establish insulin resistant models. In human and rodent hepatocytes, high glucose treatment significantly inhibits Akt phosphorylation and IRS-1 expression, and subsequently reduces glucose uptake (31, 32). A recent gene network analysis in human liver cancer cells displays that high glucose concentration regulates the transcription of genes involved in several signaling pathways, including glycolysis, regulators of reactive oxygen species production, e.g., glucose oxidase, cyclooxygenase 2, AMPK and second messenger signaling pathways, e.g., PI3K/Akt (33). In this study, we proved that high glucose treatment modulated the expression of hepatic miRNAs, which presented as an insulin resistant pattern. In high glucose stressed human hepatocytes, the expressions of miR-29a and miR-145 were significantly increased in a dose- and time-dependent manner. The predicted target genes are involved in hepatic glucose homeostasis and insulin signal transduction. Therefore, hyperglycemia may induce the alteration of hepatic miRNAs and subsequently lead to hepatic insulin resistance and glucose homeostatic imbalance.

We have to admit that this study has some limitations. First, this was a single center study and the sample size was small. In order to control confounding factors and better elucidate the effect of genetic profile, only a limited number of cases were selected for the study. Therefore, the results need to be validated in large cohorts, preferably including other ethnic populations. Second, although the diagnostic criteria of brain death was defined by the Chinese Ministry of Health in 2003, brain death in organ donation has not been widely accepted by the general public in China because of the culture barrier. Donation after circulatory death provides the predominant source of organs for transplantation during the study period. We excluded extended criteria donors such as aged liver (34), fatty liver (35), and grafts with prolonged ischemia time (36), which would potentially bring diabetes susceptibility genes to the recipients. But donation after circulatory death graft itself might increase the risk of developing NODALT due to warm ischemia (5). Therefore, the results also need to be verified among the patients receiving liver grafts from brain death donors.

In summary, donor graft miRNAs, targeting multiple genes involved in hepatic glucose metabolism and insulin signaling, are associated with the development of NODALT. The disease-susceptibility miRNA expressive pattern could be imported directly from the donor, and greatly consolidated and augmented by the transplant factors such as early hyperglycemia and immunosuppressive drugs. The two-hit mechanism indicates that miRNA targeted therapy in donor grafts maybe a novel and promising strategy for the prophylaxis and treatment of NODALT and even other post-transplant complications.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (81470892, 81570589 and 91542205).

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

FIGURE LEGENDS

Figure 1 Donor graft miR-103 and miR-181a were associated with NODALT. (A) Donor graft miR-103 and miR-181a were significantly differentially expressed between 15 propensity-matched patients with and without NODALT (both $P < 0.05$); (B) Donor graft miR-103 and miR-181a expression were significantly correlated with fasting glucose levels at 1 week, 1 month and 3 month following liver transplantation. NODALT, new-onset diabetes after liver transplantation.

Figure 2 TAC induced dysregulation of miRNAs and their potential targets. (A) The expressions of let-7a, miR-26b and miR-183 were significantly increased after different concentration of TAC treatment (5, 20 ng/ml vs. 0 ng/ml); (B) The expressions of let-7a, miR-26b and miR-183 did not significantly change after different culture time (48, 72 h vs. 24 h) in physiological concentration of

TAC treatment (5 ng/ml); (C) The expressions of let-7a, miR-26b and miR-183 were increased in a time-related manner (48, 72 h vs. 24 h) in extremely high concentration of TAC treatment (20 ng/ml); (D) The selected glucose metabolism-associated targets (*Akt*, *Irs2*, *Gsk3*, *Tcf7l2* and *Foxo1*) of let-7, miR-26b and miR-183 were significantly decreased after TAC treatment; The protein content of p-AKT, AKT, IRS2, TCF7L2 and FoxO1 decreased after TAC treatment. HepG2 and HUH7 cells were seeded at 2.5×10^5 cells per well in 6-well plates and treated with different concentration of TAC (0, 5, 20 ng/ml). Protein was extracted after 72 h of culture. TAC, tacrolimus. *: $P < 0.05$ vs. control group.

Figure 3 High glucose led to changed miRNA levels in a dose- and time-dependent manner. (A) The expressions of miR-145, miR-183 and miR-29a significantly increased after high glucose culture. HepG2 and HUH7 cells were seeded at 2.5×10^5 cells per well in 6-well plates and treated with different concentration of glucose (5.5, 10, 30 mM). (B) The expressions of miR-145 and miR-183 elevated in a time-dependent manner when cultured with 10 mM of glucose. RNA was extracted after 24, 48 and 72 h of culture. *: $P < 0.05$ vs. control group.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1 The PCR primers

Table S2 The glucose metabolism-associated gene ontology (GO) and KEGG pathway analyses of potential target genes of miR-103 and miR-181a

Table S3 The significant dysregulated miRNAs by tacrolimus

Table S4 The glucose metabolism-associated gene ontology (GO) and KEGG pathway analyses of potential target genes of miR-29a and miR-145

Figure S1. The expression of miR-22, miR-26a, miR-29a, miR-107, miR-130a, miR-145, miR-183 and miR-802 did not differ significantly between patients with and without new-onset diabetes after liver transplantation (NODALT).

Figure S2. Gene ontology (GO) and KEGG pathway analyses for the target genes of the 29 miRNAs significantly up-regulated by both low- and high-dose tacrolimus. Top 50 pathways with *P* value < 0.05 and false discovery rate (FDR) value < 0.05 are shown.

Figure S3. The expression of miR-22, miR-26a, miR-103, miR-107, miR-130a and miR-181a did not differ significantly after high glucose exposure for 24 h in both cell lines (HepG2 and HUH7). miR-802 was undetectable. *: $P < 0.05$.

REFERENCES

1. Sarno G, Mehta RJ, Guardado-Mendoza R, Jimenez-Ceja LM, De Rosa P, Muscogiuri G. New-onset diabetes mellitus: predictive factors and impact on the outcome of patients undergoing liver transplantation. Curr Diabetes Rev 2013;9(1):78-85.

2. Parekh J, Corley DA, Feng S. Diabetes, hypertension and hyperlipidemia: prevalence over time and impact on long-term survival after liver transplantation. Am J Transplant 2012;12(8):2181-2187.

3. Ling Q, Xu X, Wang B, Li L, Zheng S. The Origin of New-Onset Diabetes After Liver Transplantation: Liver, Islets, or Gut? Transplantation 2016;100(4):808-813.

4. Ling Q, Xie H, Lu D, Wei X, Gao F, Zhou L et al. Association between donor and recipient TCF7L2 gene polymorphisms and the risk of new-onset diabetes mellitus after liver transplantation in a Han Chinese population. J Hepatol 2013;58(2):271-277.

5. Hartog H, May CJ, Corbett C, Phillips A, Tomlinson JW, Mergental H et al. Early occurrence of new-onset diabetes after transplantation is related to type of liver graft and warm ischaemic injury. Liver Int 2015;35(6):1739-1747.

6. Gehrau RC, Mas VR, Villamil FG, Dumur CI, Mehta NK, Suh JL et al. MicroRNA signature at the time of clinical HCV recurrence associates with aggressive fibrosis progression post-liver transplantation. Am J Transplant 2013;13(3):729-737.

7. Frost RJ, Olson EN. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. Proc Natl Acad Sci U S A 2011;108(52):21075-21080.

8. Pandey AK, Verma G, Vig S, Srivastava S, Srivastava AK, Datta M. miR-29a levels are elevated in the db/db mice liver and its overexpression leads to attenuation of insulin action on PEPCK gene expression in HepG2 cells. Mol Cell Endocrinol 2011;332(1-2):125-133.

9. Xu Q, Li Y, Shang YF, Wang HL, Yao MX. miRNA-103: molecular link between insulin resistance and nonalcoholic fatty liver disease. World J Gastroenterol 2015;21(2):511-516.

10. Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M et al. MicroRNAs 103 and 107 regulate insulin sensitivity. Nature 2011;474(7353):649-653.

11. Jordan SD, Kruger M, Willmes DM, Redemann N, Wunderlich FT, Bronneke HS et al. Obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism. Nat Cell Biol 2011;13(4):434-446.

12. Wen F, Yang Y, Jin D, Sun J, Yu X, Yang Z. MiRNA-145 is involved in the development of resistin-induced insulin resistance in HepG2 cells. Biochem Biophys Res Commun 2014;445(2):517-523.

13. Zhou B, Li C, Qi W, Zhang Y, Zhang F, Wu JX et al. Downregulation of miR-181a upregulates sirtuin-1 (SIRT1) and improves hepatic insulin sensitivity. Diabetologia 2012;55(7):2032-2043.

14. Motino O, Frances DE, Mayoral R, Castro-Sanchez L, Fernandez-Velasco M, Bosca L et al. Regulation of MicroRNA 183 by Cyclooxygenase 2 in Liver Is DEAD-Box Helicase p68 (DDX5) Dependent: Role in Insulin Signaling. Mol Cell Biol 2015;35(14):2554-2567.

15. Kornfeld JW, Baitzel C, Konner AC, Nicholls HT, Vogt MC, Herrmanns K et al. Obesity-induced overexpression of miR-802 impairs glucose metabolism through silencing of Hnf1b. Nature 2013;494(7435):111-115.

16. Xiao F, Yu J, Liu B, Guo Y, Li K, Deng J et al. A novel function of microRNA 130a-3p in hepatic insulin sensitivity and liver steatosis. Diabetes 2014;63(8):2631-2642.

17. Fu X, Dong B, Tian Y, Lefebvre P, Meng Z, Wang X et al. MicroRNA-26a regulates insulin sensitivity and metabolism of glucose and lipids. J Clin Invest 2015;125(6):2497-2509.

18. Kaur K, Vig S, Srivastava R, Mishra A, Singh VP, Srivastava AK et al. Elevated Hepatic miR-22-3p Expression Impairs Gluconeogenesis by Silencing the Wnt-Responsive Transcription Factor Tcf7. Diabetes 2015;64(11):3659-3669.

19. Ling Q, Xu X, Xie H, Wang K, Xiang P, Zhuang R et al. New-onset diabetes after liver transplantation: a national report from China Liver Transplant Registry. Liver Int 2016;36(5):705-712.

20. Chinese Society of Organ Transplantation; Section of Organ Transplantation, Chinese Society of Surgery; Chinese College of Transplant Doctors. Expert consensus on evaluation and application of organ donated after cardiac death in China. Chin J Transplant 2014;8(3):117-122.

21. Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S et al. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 2011;39(Web Server issue):W316-322.

22. Jo JY, Choi SS, Yi JM, Joo EY, Kim JH, Park SU et al. Differential Postoperative Effects of Volatile Anesthesia and Intraoperative Remifentanil Infusion in 7511 Thyroidectomy Patients: A Propensity Score Matching Analysis. Medicine (Baltimore) 2016;95(7):e2764.

23. Wang H, Luo J, Zhang T, Tian H, Ma Y, Xu H et al. MicroRNA-26a/b and their host genes synergistically regulate triacylglycerol synthesis by targeting the INSIG1 gene. RNA Biol 2016:1-11.

24. Ling Q, Xu X, Wang K, Wang C, Xiang P, Zhang X et al. Donor PPARalpha Gene Polymorphisms Influence the Susceptibility to Glucose and Lipid Disorders in Liver Transplant Recipients: A Strobe-Compliant Observational Study. Medicine (Baltimore) 2015;94(35):e1421.

25. Kuo HT, Sampaio MS, Ye X, Reddy P, Martin P, Bunnapradist S. Risk factors for new-onset diabetes mellitus in adult liver transplant recipients, an analysis of the Organ Procurement and Transplant Network/United Network for Organ Sharing database. Transplantation 2010;89(9):1134-1140.

26. Lankarani KB, Eshraghian A, Nikeghbalian S, Janghorban P, Malek-Hosseini SA. New onset diabetes and impaired fasting glucose after liver transplant: risk analysis and the impact of tacrolimus dose. Exp Clin Transplant 2014;12(1):46-51.

27. Chakkera HA, Mandarino LJ. Calcineurin inhibition and new-onset diabetes mellitus after transplantation. Transplantation 2013;95(5):647-652.

28. Larsen JL, Bennett RG, Burkman T, Ramirez AL, Yamamoto S, Gulizia J et al. Tacrolimus and sirolimus cause insulin resistance in normal sprague dawley rats. Transplantation 2006;82(4):466-470.

29. Hecking M, Haidinger M, Doller D, Werzowa J, Tura A, Zhang J et al. Early basal insulin therapy decreases new-onset diabetes after renal transplantation. J Am Soc Nephrol 2012;23(4):739-749.

30. Kawahito S, Kitahata H, Oshita S. Problems associated with glucose toxicity: role of hyperglycemia-induced oxidative stress. World J Gastroenterol 2009;15(33):4137-4142.

31. Hu Y, Hou Z, Liu D, Yang X. Tartary buckwheat flavonoids protect hepatic cells against high glucose-induced oxidative stress and insulin resistance via MAPK signaling pathways. Food Funct 2016;7(3):1523-1536.

32. Lee CC, Hsu WH, Shen SR, Cheng YH, Wu SC. Fagopyrum tataricum (buckwheat) improved high-glucose-induced insulin resistance in mouse hepatocytes and diabetes in fructose-rich diet-induced mice. Exp Diabetes Res 2012;2012:375673.

33. Berger E, Vega N, Weiss-Gayet M, Geloen A. Gene Network Analysis of Glucose Linked Signaling Pathways and Their Role in Human Hepatocellular Carcinoma Cell Growth and Survival in HuH7 and HepG2 Cell Lines. Biomed Res Int 2015;2015:821761.

34. Kwekel JC, Desai VG, Moland CL, Branham WS, Fuscoe JC. Age and sex dependent changes in liver gene expression during the life cycle of the rat. BMC Genomics 2010;11:675.

35. Sobolewski C, Calo N, Portius D, Foti M. MicroRNAs in fatty liver disease. Semin Liver Dis 2015;35(1):12-25.

36. Farid WR, Pan Q, van der Meer AJ, de Ruiter PE, Ramakrishnaiah V, de Jonge J, et al. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after livertransplantation. Liver Transpl 2012;18(3):290-297

TABLES

Table 1 Logistic regression analysis of risk factors associated with NODALT

	Univariate analysis		Multivariate analysis	
	P	OR (95% CI)	*P*	OR (95% CI)
Recipient age > 55 years (0 = no, 1 = yes)	0.039	2.052 (1.038-4.056)		
Hyperglycemia < 30 days (0 = no, 1 = transient, 2 = persistent)	0.003	1.802 (1.217-2.669)	0.006	1.743 (1.170-2.596)
Acute rejection (steroid pulse) < 30 days (0 = no, 1 = yes)	0.033	4.111 (1.117-15.13)		
TAC level at 1-month > 10 ng/ml (0 = no, 1 = yes)	0.021	2.190 (1.127-4.254)	0.036	2.067 (1.049-4.073)

NODALT, new-onset diabetes after liver transplantation; OR, odds ratio; CI, confidence interval; TAC, tacrolimus

Table 2 Demographics and clinical data for NODALT and non-NODALT groups

	NODALT group (n = 15)	non-NODALT group (n = 15)	*P*
Donor characteristics			
Age (y)	30.3 ± 7.6	33.8 ± 8.7	0.195
Male/females (n)	15/0	15/0	/
Hepatic steatosis (n)	0	0	/
WIT [a] (min)	19.3 ± 4.0	20.6 ± 3.2	0.488
CIT [b] (h)	8.9 ± 1.1	8.6 ± 1.7	0.387
DCD/DBD/LDLT	15/0/0	15/0/0	/
Causes of injury (n)			0.425
Trauma	12	11	
Stroke	2	4	
Anoxia	1	0	
Recipient characteristics			
Age (y)	47.9 ± 10.2	47.5 ± 9.2	0.899
Male/females (n)	15/0	15/0	/
BMI (kg/m^2)	24.2 ± 3.7	23.3 ± 3.4	0.435
HBV-cirrhosis	15	15	/
HCC	4	3	1.000
MELD score	16.0 ± 10.4	19.0 ± 8.9	0.465
Kidney dysfunction	1	2	1.000
Dialysis	0	0	/

NODALT, new-onset diabetes after liver transplantation; WIT, warm ischemia time; CIT, cold ischemia time; DBD, Donation after brain death; DCD, circulatory death donation; LDLT, living donor liver transplantation; BMI, body mass index; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; MELD, model for end-stage liver diseases.

Kidney dysfunction was defined as serum creatinine > 1.5 mg/dL.

[a] Time from hypotension (SBP < 50 mm Hg) until organ flushing.

[b] Time from organ flushing until implantation.

Table 3 MicroRNAs significantly up-regulated by both low- and high-dose TAC

	Low-dose TAC				High-dose TAC			
	Fold change	*P* value	Q value	Trend	Fold change	*P* value	Q value	Trend
hsa-let-7a-5p	1.278	0.019	0.081	up	1.544	0.021	0.177	up
hsa-let-7c-5p	1.388	0.011	0.081	up	1.492	0.031	0.177	up
hsa-let-7f-5p	1.732	0.020	0.081	up	2.036	0.007	0.177	up
hsa-miR-26b-3p	1.518	0.002	0.000	up	1.686	0.010	0.177	up
hsa-miR-26b-5p	1.969	0.037	0.084	up	2.108	0.025	0.177	up
hsa-miR-146b-5p	1.440	0.007	0.062	up	1.249	0.036	0.177	up
hsa-miR-183-5p	1.239	0.019	0.081	up	1.296	0.023	0.177	up
hsa-miR-320e	1.265	0.022	0.081	up	1.285	0.035	0.177	up
hsa-miR-374b-5p	3.199	0.003	0.000	up	2.805	0.008	0.177	up
hsa-miR-502-5p	1.642	0.002	0.000	up	1.558	0.002	0.177	up
hsa-miR-584-5p	2.697	0.050	0.084	up	3.087	0.039	0.177	up
hsa-miR-885-3p	1.888	0.009	0.081	up	2.259	0.019	0.177	up

hsa-miR-1184	2.237	0.005	0.053	up	2.166	0.017	0.177	up
hsa-miR-1343-5p	1.479	0.033	0.084	up	1.435	0.048	0.177	up
hsa-miR-3147	1.589	0.014	0.081	up	1.662	0.027	0.177	up
hsa-miR-3197	2.435	0.006	0.062	up	2.547	0.011	0.177	up
hsa-miR-3652	1.987	0.003	0.000	up	2.046	0.006	0.177	up
hsa-miR-4284	1.422	0.046	0.084	up	1.759	0.019	0.177	up
hsa-miR-4492	1.712	0.039	0.084	up	2.181	0.031	0.177	up
hsa-miR-4667-5p	1.582	0.032	0.084	up	1.671	0.017	0.177	up
hsa-miR-6735-5p	1.974	0.020	0.081	up	2.537	0.017	0.177	up
hsa-miR-6795-5p	1.650	0.004	0.053	up	1.829	0.019	0.177	up
hsa-miR-6861-5p	1.472	0.023	0.081	up	1.771	0.030	0.177	up
hsa-miR-6875-5p	2.079	0.006	0.062	up	2.163	0.017	0.177	up
hsa-miR-6887-5p	1.731	0.011	0.081	up	1.806	0.012	0.177	up
hsa-miR-7106-5p	2.213	0.036	0.084	up	2.795	0.026	0.177	up
hsa-miR-7111-5p	3.352	0.028	0.084	up	3.176	0.030	0.177	up

TAC, tacrolimus.

Table 4 The glucose metabolism-associated pathways identified by GO and KEGG pathway analysis using potential targets of miRNAs up-regulated by both low- and high-dose TAC

ID	Name	Enrichment score	P value	FDR	Gene symbols
GO analysis					
0006006	Glucose metabolic process	4.327	5.91E-08	4.09E-06	PGAM1, GPI, PGM1, BRS3, PRKACA, PKLR, SERP1, KCNJ11, TNF, PFKFB2, PFKM, AKT2, PHKG2, PFKFB4, ADIPOQ, GYG2, IGFBP5, CALM1, IRS2, WDTC1
0042593	Glucose homeostasis	3.938	4.42E-05	1.08E-03	TCF7L2, STAT3, MLXIPL, RPH3AL, IGFBP5, PFKM, ADIPOQ, PRKAA2, HNF4A, NGFR, SLC2A4, NCOR2, CACNA1E
0008286	Insulin receptor signaling pathway	3.684	6.87E-08	4.51E-06	IRS2, FOXC2, TSC1, FOXO4, PRKAA2, NAMPT, AKT2, EIF4G1, NRAS, PIK3R1, MAPK1, IDE, SOCS7, EEF2K, PRKAB2, PRKAG1, EIF4B, PDPK1, IGF1R, APPL1, STXBP4, FOXO1, FGFR1, EIF4EBP2
0046326	Positive regulation of glucose import	7.573	2.78E-06	1.10E-04	PRKCD, GPC3, AKT2, PIK3R1, ARPP19, CREBL2, PRKCI, IRS2, ADIPOQ
0009749	Response to glucose stimulus	4.309	7.87E-05	1.70E-03	HNF4A, PRKCD, THBS1, TCF7L2, ADIPOQ, NNAT, ACVR2B, IRS2, PFKFB2, VAMP2, PKLR
0032869	Cellular response to insulin stimulus	5.274	1.44E-06	6.35E-05	SLC2A4, ACSL6, ADIPOQ, PKLR, AKT2, PRKCI, PDPK1, PRKCD, HDAC9, IRS2, VAMP2, PAK1, WDTC1

KEGG Pathway analysis					
04910	Insulin signaling pathway	4.219	9.39E-10	1.42E-08	SOCS4, PHKG2, CALM3, IRS2, ACACA, RAPGEF1, PKLR, AKT3, PRKACA, PDPK1, PRKAB2, PIK3R1, CBL, CALM1, AKT2, SLC2A4, FOXO1, GSK3B, PPP1R3D, PRKAA2, NRAS, PRKAG1, MAPK1, ELK1, PRKCI, TSC1

GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; TAC, tacrolimus; FDR, false discovery rate.