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Circulating and visceral adipose miR-100 is down-regulated in patients with obesity and Type 2 diabetes

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ABSTRACT

Obesity is a major public health problem conferring substantial excess risk for Type 2 diabetes (T2D). The role of microRNAs (miRNAs) in obesity and adipose tissue is not clearly defined. We hypothesize that circulating miRNA expression profiles vary according to differences in body mass index (BMI) and T2D and circulating miRNAs may reflect adipose tissue expression. Compared to healthy, lean individuals, circulating miR-100 was significantly lower in obese normoglycemic subjects and subjects with T2D. In visceral adipose tissue, expression of miR-100 was lower from obese subjects with T2D compared to obese subjects without T2D. miR-100 expression was significantly lower after adipogenic induction in human visceral, subcutaneous adipocytes and 3T3-L1 adipocytes. miR-100 reduced expression of mammalian target of rapamycin (mTOR) and Insulin Growth Factor Receptor (IGFR) directly. Differentiation of 3T3-L1 was accelerated by inhibition of miR-100 and reduced by miR-100 mimic transfection. Our data provide the first evidence of an association of circulating miR-100 with obesity and diabetes. Additionally, our in-vitro findings, and the miR-100 expression patterns in site-specific adipose tissue suggest miR-100 to modulate IGFR, mTOR and mediate adipogenesis.

1. Introduction

Obesity is a major public health problem worldwide, conferring substantial excess risk for morbidity and mortality, especially for Type 2 diabetes (T2D), cardiovascular disease and cancers (Kopelman, 2007). Although environmental factors have driven the rise in the number of people who are obese, genetic and epigenetic factors are estimated to account for 40–90% of the variation in body mass index (BMI) (Hjelmborg et al., 2008; Wardle et al., 2008; Maes et al., 1997). Identifying these factors underlying the risk of obesity may contribute to our understanding of the biology, pathophysiology of disease and also highlight molecules and pathways that can be targeted for therapeutic intervention.

MicroRNAs (miRNAs) are small non-coding RNAs (~22 nucleotides) that bind to regulatory sites of target mRNA and modify their expression, either by translational repression or target mRNA degradation, resulting in decreased protein production (Bartel, 2004). miRNAs play important roles in regulation of insulin secretion, insulin response, glucose homeostasis, are required for

Abbreviations: aP2, adipocyte fatty acid binding protein 2; BMI, Body Mass Index; DBP, Diastolic Blood Pressure; DMEM, Dulbecco's Modified Eagle Medium; EDTA, Ethylenediaminetetraacetic acid; HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; IGFR, Insulin Growth Factor Receptor; IBMX, 3-isobutyl-ʟ-methylxanthine; KRB, Kreb's ringer Buffer; LDL, low-density lipoprotein; LPL, lipoprotein lipase; miRNAs, microRNAs; mTOR, mechanistic target of rapamycin; PPARγ, peroxisome proliferator-activated receptors-γ; Prodh, proline dehydrogenase; qPCR, real-time PCR validation (qPCR); SBP, Systolic Blood Pressure; Smoc2, SPARC related modular calcium binding 2; Smpdl3b, sphingomyelin phosphodiesterase, acid-like 3B; T2D, Type 2 diabetes; TG, triglyceride; TC, total cholesterol; VLDLR, Very light density lipoprotein receptor.

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pancreatic development, regulation of glucose-stimulated insulin secretion and amino acid catabolism (Tavintharan et al., 2009). They are also involved in functional aspects of insulin target tissues, such as adipocyte differentiation, myoblast differentiation and fatty acid synthesis in hepatocytes (Poy et al., 2004). Recent studies have documented the presence of circulating RNAs in serum, protected from RNase activity (El-Hefnawy et al., 2004; Tsui et al., 2002; Chen et al., 2008). Conventional clinical and blood biomarkers, such as body mass index (BMI), fasting blood glucose or glycated hemoglobin (HbA1c) levels, are well-established predictors but remain imperfect, and provide limited insights regarding underlying pathophysiology. The use of miRNAs as novel biomarkers in circulation represents an attractive approach for early screening to identify individuals at risk of developing T2D and potentially explain molecular mechanisms for its pathophysiology.

Several studies have examined changes in miRNA in blood or in specific tissues from subjects with T2D (Ortega et al., 2010). Few studies have attempted to explore a combination of both adipose tissue and circulating miRNA, comparing obese and non-obese patients (Heneghan et al., 2011). The aim of the study was to compare microRNA expression profiles in obese and non-obese subjects with and without T2D. A secondary aim was to establish if circulating miRNAs reflected adipose tissue expression and to determine possible function of identified miRNA.

2. Methods

2.1. Patients and sample collection

We selected lean and obese participants, without diabetes and those who were newly diagnosed with T2D for miRNA profiling (Cohort 1, n = 32). Observed miRNA deregulation was further evaluated in paired visceral or subcutaneous adipose depots from obese subjects with or without T2D (Cohort 2, n = 83). T2D was defined as fasting glucose ≥7.0 mmol/l or HbA1c ≥ 6.5% (ADA, 2015).

Cohort 1 consisted of consenting adult subjects (18–70 years of age) with no past medical history, seen at our institution for health screening and newly diagnosed patients with T2D, from outpatient clinic, during the period of July 2008 to March 2012. Apart from past history of hypertension or hypercholesterolemia, those with any other illnesses, and those with medications likely to modify blood glucose levels, e.g. glucocorticoids, thiazides, beta-blockers, were excluded from the study.

For Cohort 2, morbidly obese patients referred to bariatric surgeons for weight management at our institution were recruited. Paired *visceral and subcutaneous adipose* samples were harvested intra-operatively from each patient during bariatric surgery.

Both studies were approved by our institutional ethics review board and all study participants signed written informed consent.

2.2. Clinical and biochemical measurements

All participants underwent physical examination for anthropometric measures and blood pressure and fasting blood was collected. Fasting plasma glucose (FPG), triglyceride (TG), total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) levels, insulin and high-sensitivity C-reactive protein (hsCRP) were measured as previously reported (Pek et al., 2013). Homeostasis model assessment of insulin resistance (HOMA-IR) index for insulin resistance was estimated by formula: [FPG (mmol/L) × insulin (μU/ml)]/22.5. Whole blood from EDTA-tubes was added with RNAlater® solution (Ambion, USA) and stored at −80 °C for RNA isolation.

2.3. Pre-adipocytes isolation and culturing

Pre-adipocytes were isolated from human visceral and subcutaneous adipose tissues are described previously (McTernan et al., 2002). Fresh paired adipose tissues from 3 random patients (Cohort 2) were minced and incubated in collagenase I (Sigma, USA). Cell suspension was filtered through a 250 μm nylon mesh and washed with Kreb's ringer Buffer (KRB), Penicillin, Streptomycin and Amphoterin B (Sigma, USA). The filtrate was centrifuged and final pellet was obtained after 4 washes with KRB. To eliminate erythrocytes the pellet was treated with red blood cell lysis buffer (154 mM NH_4Cl, 10 mM KHCO3, 0.1 mM EDTA). The final pellet was resuspended and cultured in pre-adipocyte media (Promocell, GmbH, Germany) supplemented with antibiotics. Primary cell cultures were used within 5 passages.

2.4. Primary culture and 3T3-L1 differentiation

Human primary adipocyte differentiation from both visceral and subcutaneous depots were induced 2 days post−confluence (designated day 0), with 3-isobutyl-L-methylxanthine (IBMX)(0.5 mM), dexamethasone (1 μM), 10 μM insulin and 200 μM Indomethacin (Sigma, USA) in DMEM+10% heat-inactivated fetal bovine serum (FBS), similar to (Back and Arnqvist, 2009). Differentiation media was replaced every 2–3 days for 14 days and 21 days for visceral adipocytes.

3T3-L1 adipocytes (ATCC, USA) were maintained in basal media (DMEM+10%FBS). 3T3-L1 adipogenesis was induced 2 days post−confluence, with standardized differentiation cocktail [IBMX (0.5 mM), dexamethasone (1 μM) and bovine insulin (1 μg/ml)] in basal media. After 3 days, the media was changed to maintenance media [basic media supplemented with insulin (1 μg/ml)] for an additional 3 days as reported (Bak et al., 2010).

Cells were lysed in Qiazol for RNA extraction or RIPA buffer (Pierce, USA), supplemented with protease inhibitors (Roche, USA) for immunoblot analyses. *3T3*-L1 adipogenesis was visualized by *Oil Red O staining* (Sigma, USA) (Bak et al., 2010).

2.5. Total RNA isolation

Total RNA, including miRNA from whole blood was extracted using RiboPureTM-Blood kit (Ambion, USA), as reported previously (Karolina et al., 2011). For adipose tissue and cell culture experiments, total RNA and miRNA were extracted using the miRNeasy Mini Kit (Qiagen, Germany). Purity and quantity of total RNA were assessed by NanoDrop ND-1000 Spectrophotometry (Thermo Scientific, USA) and Agilent's 2100 Bioanalyzer.

2.6. miRNA microarray and quantitative real-time PCR validation(qPCR)

miRNA was profiled from whole blood of the 32 Chinese male patients. Briefly, 100 ng of total RNA was labeled with Cyanine3-pCp and hybridized to Agilent Sureprint G3 Human miRNA microarray (version16.0), incubated, washed and scanned in Agilent high resolution microarray scanner. Data was analyzed using Genespring software (Agilent Technologies, USA) (Zhuang et al., 2015). Differences in blood miRNAs were analyzed using DIANA miRPATH (Vlachos et al., 2012) for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses.

Validation of microarray results was done by qPCR on whole blood of all 32 participants, visceral and subcutaneous adipose tissues obtained from diabetic or non-diabetic obese patients (Cohort 2). miRNA was reverse transcribed (Exiqon, Denmark) and expression levels of miRNA were performed using Locked nucleic