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Research paper

Circulating microRNAs in obese and lean heart failure patients: A case–control study with computational target prediction analysis

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ABSTRACT

Aims: MicroRNAs (miRs) regulate processes involved in both cardiac remodeling and obesity. We investigated if the expression of selected miRs in patients with heart failure (HF) is influenced by the presence of obesity.
Methods: In this case–control study, we compared plasma levels of miR-21, -130b, -221, -423-5p, and the -221/-130b ratio in 57 age- and gender-matched subjects: 40 HF patients (20 obese HF and 20 lean HF) and 17 lean healthy controls. Body composition was estimated by bioelectrical impedance analysis. MiRs were measured by quantitative reverse transcription-PCR. Bioinformatics analysis was performed based on miRs findings to predict their putative targets and investigate their biological function.
Results: HF was associated with increased miR-423-5p levels in both lean and obese patients ($P < 0.05$ vs. controls) without differences between HF groups. MiR-130b levels were reduced in obese HF patients compared with HF lean ($P = 0.036$) and controls ($P = 0.025$). MiR-221 levels were non-significantly increased in obese HF patients. MiR-21 levels were not different among the groups. MiR-221/-130b ratio was increased in obese HF patients, and was positively associated with body fat percentage ($r = 0.43$; $P = 0.002$), body mass index ($r = 0.44$; $P = 0.002$), and waist circumference ($r = 0.40$; $P = 0.020$). Computational prediction of target genes followed by functional enrichment analysis indicated a relevant role of miR-130b and miR-221 in modulating the expression of genes associated to cardiovascular and endocrine diseases, and suggested their influence in important signaling mechanisms and in numerous processes related to the circulatory and endocrine systems.
Conclusions: In HF patients, the presence of obesity is associated with a differential expression of selected miRs and the miR-221/-130b ratio had significant correlations with adiposity parameters. Computational target prediction analysis identified several interrelated pathways targeted by miR-130b and miR-221 with a known relationship with endocrine and cardiovascular diseases, representing potential mechanisms to be further validated.

1. Introduction

Both heart failure (HF) and obesity are major public health problems worldwide that are associated with significant mortality, morbidity and healthcare expenditures (Roger, 2013; Cornier et al., 2011). Obesity is a well-known risk factor for cardiovascular diseases and significantly increases the chance of developing HF (Oreopoulos et al., 2008). However, overweight and obesity were also associated with lower all-cause and cardiovascular mortality rates in HF patients (Oreopoulos et al., 2008; Von Haehling et al., 2011), which has been termed the *obesity paradox*. The mechanisms underlying this finding have been difficult to ascertain.

MicroRNAs (miRs) are small non-coding endogenous RNAs that regulate gene expression at the post-transcriptional level by inhibiting translation or promoting the degradation of target mRNAs (Kumarswamy and Thum, 2013). It has been confirmed that extracellular mature miRs

Abbreviations: miR, microRNA; HF, heart failure; LV, left ventricle; LVEF, left ventricular ejection fraction; BMI, body mass index; NYHA, New York Heart Association; ACE, angiotensin-converter enzyme; ARB, angiotensin receptor blocker; GAD, Genetic Association Database; KEGG, Kyoto Encyclopedia of Genes and Genomes; TF, transcription factor; HTRIdb, Human Transcriptional Regulation Interactions Database; FDR, false discovery rate; HDL, high density lipoprotein; LDL, low density lipoprotein; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; MRA, mineralocorticoid-receptor antagonist; JC, Jaccard Coefficient; PPARG, proliferator-activated receptor-gamma; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K-Akt, phosphatidylinositol 3-kinase/protein kinase B; TGF-beta, transforming growth factor-beta.

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circulate in the bloodstream with remarkable stability, thus making them an attractive class of disease biomarkers (Creemers et al., 2012). The occurrence of miRs in the bloodstream may be due either to a cell membrane disruption after an insult or to an active releasing process. Despite the process involved, miRs are signaling molecules that regulate processes in distant or neighboring cells (Chen et al., 2012). Previous works have demonstrated that miR-130b-enriched microvesicles can be transported into adipocytes and downregulate protein peroxisome proliferator-activated receptor-gamma (PPARG), influencing in the receptor cell phenotype (Pan et al., 2014). Similarly, fibroblast and endothelial-derived microvesicle containing miRs were shown to target mRNA in cardiomyocytes both *in vitro* and *in vivo*, influencing in molecular pathways related to inflammation and cell survival (Das and Halushka, 2015).

MiRs were shown to be implicated in modulating various aspects related to cardiac remodeling (Topkara and Mann, 2011). Our group has previously demonstrated that levels of miR-423-5p are elevated in the coronary sinus of HF patients, suggesting that it has a cardiac origin (Goldraich et al., 2014). Other miRs, such as miR-21 and miR-221, have also been implicated in ventricular hypertrophy and HF pathogenesis (Tatsuguchi et al., 2007; Wang et al., 2012).

Circulating miRs have also been studied in obesity (Kilic et al., 2015; Ortega et al., 2013), and are involved in obesity-related processes such as disruption of fatty acid metabolism, cellular hypertrophy, inflammation and systemic insulin resistance (Alexander et al., 2011). Deregulation of miRs expression was observed in the adipose tissue from obese patients, such as lower levels of miR-130 and increased levels of miR-221, which are associated with opposite changes in activation of major regulators of adipogenesis (Lee et al., 2011; Meerson et al., 2013).

Therefore, the present case–control study aimed at comparing levels of miR-21, miR-130b, miR-221, miR-423-5p, and the miR-221/-130b ratio in blood samples from obese and lean patients with systolic HF and in healthy lean subjects to investigate if the expression of miRs in patients with HF is influenced by the presence of obesity. Through bioinformatics analysis, we explored the potential role of differentially expressed miRs in major cardiovascular, endocrine and cell signaling pathways in order to shed light on possible mechanisms that link the activity of these miRs with the obesity paradox.

2. Methods

2.1. Patients and controls

Patients were recruited at the Heart Failure and Transplant Clinic of our institution. Subjects with stable HF, 18 to 80 years old and a left ventricular ejection fraction (LVEF) lower than 45% were included. Control subjects were selected from donors at the Hemotherapy Center of our institution. All controls underwent a focused clinical interview and had no history of structural cardiac disease or HF symptoms.

Exclusion criteria for both controls and HF patients were cell dyscrasias, active inflammation, malignant disease and severe hepatic or renal disease (creatinine >3 mg/dl). Exclusion criteria for HF patients were as follows: (1) episode of decompensation within the previous 30 days, (2) acute coronary syndrome within the previous three months and (3) presence of implantable devices such as pacemakers, implantable cardioverter defibrillators and cardiac resynchronization therapy-defibrillators, because they were not eligible for bioelectrical impedance analysis.

Obesity was defined as body mass index (BMI) ≥30 kg/m^2 and body fat percentage >25% in men and >35% in women (Cornier et al., 2011), measured by a bioimpedance analysis. Lean subjects were included if BMI was <25 kg/m^2 and body fat percentage was ≤22% in men and ≤32% in women (Pi-Sunyer, 2000).

Subjects were classified into three groups: (1) lean healthy controls, (2) lean HF patients, and (3) obese HF patients. All groups were matched for age and sex, and groups 2 and 3 were also matched for LVEF, heart failure etiology (ischemic and non-ischemic), New York Heart Association (NYHA) class, use of beta-blockers and of angiotensin-converter enzyme (ACE) inhibitor or angiotensin receptor blocker (ARB).

2.2. Data collection

Demographic data, clinical history, co-morbidities, echocardiographic, electrocardiographic and laboratory data were collected. Body mass index was calculated as weight in kilograms divided by squared height in meters (kg/m^2). Bioimpedance analysis was performed with a tetrapolar bioimpedance equipment (Model 450, Tetrapolar, Biodynamics, USA). Waist circumference for HF patients was measured by a trained examiner and determined on the lesser curvature located between the ribs and the iliac crest after expiration.

2.3. Measurement of circulating miRs

Peripheral venous blood samples from all subjects were collected in EDTA-coated tubes. Blood samples were centrifuged at 1500 rpm for 15 min at 4 °C within 1 h from collection and stored at −80 °C until analysis.

Plasma samples were freeze–thawed and miR isolation was performed using the commercially available *mir*Vana PARIS Kit (Ambion, Austin, TX, USA) using 495 μL of plasma. After protein denaturation, 50 pM (fixed volume of 5 μL) of synthetic, non-human miR-39 from *Caenorhabditis elegans* (cel-miR-39) (Qiagen, Valencia, CA, USA) was spiked into plasma samples to control for potential technical variations throughout the extraction and measurement procedures. Cel-miR-39 was simultaneously analyzed in the final miR eluate, providing a standard control for the concentration of studied miRs obtained from subject samples.

The concentration of miRs was determined by spectrophotometric analysis (NanoDrop 1000, Thermo Scientific, Wilmington, DE, USA). Reverse transcription (RT) reactions were performed according to manufacturer's instructions, using miRs Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). MicroRNAs of interest (miR-21, -130b, -221 and -423-5p) were measured by quantitative RT-polymerase chain reaction (qRT-PCR) using TaqMan® miRs Expression Assays probes (Life Technologies) on the 7500 Real Time PCR System (Life Technologies). All miR qRT-PCR reactions were run in triplicate, and the relative expression values were determined using the $2^{-\Delta\Delta CT}$ method, previously described in detail (Livak and Schmittgen, 2001).

2.4. Computational prediction of miR targets

A list of putative target genes was obtained for miRs differentially expressed among the groups based on computational predictions from the TargetScan algorithm (Lewis et al., 2005) (release 6.2; accessed July 2014). TargetScan works by searching mRNAs sites with high complementarity to the seed region (nucleotides 2–7) of the miRs, assessing sequence features derived from empirically defined rules and site conservation across species. This bioinformatics analysis helps in the interpretation of the biological and molecular role of miRs (Bazil et al., 2014). TargetScan was specifically used because it is suggested to be one of the most reliable miR target prediction algorithms available, presenting a distinguished tradeoff between sensitivity and specificity (Sethupathy et al., 2006; Baek et al., 2008; Alexiou et al., 2009). More importantly, target prediction scores provided by TargetScan were shown to correlate with protein downregulation in a comparison between *in vivo* and *in silico* findings (Baek et al., 2008).

As an attempt to evaluate the quality of TargetScan predictions, we carried out a consensus analysis among several resources for gathering miRNA-target interactions to investigate the extent to which predictions by TargetScan are supported by other computational prediction tools, as well as by experimentally validated data. It is likely that miR targets predicted by different tools represent more solid hypotheses