Gene 570 (2015) 50–56

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Asymptomatic individuals with high HDL-C levels overexpress *ABCA1* and *ABCG1* and present miR-33a dysregulation in peripheral blood mononuclear cells

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ARTICLE INFO

Article history:
Received 11 December 2014
Received in revised form 19 May 2015
Accepted 31 May 2015
Available online 4 June 2015

Keywords:
ABCA1
ABCG1
MicroRNA
HDL

ABSTRACT

Considering the growing knowledge and perspectives on microRNAs (miRNAs) that control high-density lipoprotein cholesterol (HDL-C) levels and metabolism, this study aimed at evaluating whether hsa-miR-33a and hsa-miR-128a are differentially expressed in peripheral blood mononuclear cells from asymptomatic individuals with low and high HDL-C, as well as at investigating the potential relationships with ATP binding cassete transporter A1 (*ABCA1*) expression, cholesterol efflux capacity and other parameters related with reverse cholesterol transport. In addition, the associations with cardiovascular risk were investigated by carotid-intima media thickness (cIMT). Asymptomatic volunteers of both genders (n = 51) were classified according to HDL-C (mg/dL) in hypoalphalipoproteinemics (hypo, HDL-C ≤ 39), hyperalphalipoproteinemics (hyper, HDL-C ≥ 68) and controls (CTL, HDL-C ≥ 40 < 68). cIMT, lipids, lipoproteins, HDL size and volume, C reactive protein and insulin were determined, as well as the activities of several proteins and enzymes related to HDL metabolism. In a subgroup of 19 volunteers the cellular cholesterol efflux and HDL composition were determined. Total RNA was extracted from peripheral blood mononuclear cells for relative quantification experiments. Hypo volunteers presented significantly higher levels of triglycerides, VLDL-C and insulin; in addition, HDL size and volume decreased when compared with CTL and hyper. Regarding gene expression analysis, the hyper group presented a decrease of 72% in hsa-miR-33a and higher mRNA expression of *ABCA1* and *ABCG1* when compared with CTL. No significant differences in hsa-miR-128a expression, cholesterol efflux, cIMT or plaques were found. Further studies are necessary to elucidate the mechanisms underlying the complex miRNA network, regulating cellular cholesterol homeostasis in humans and its clinical repercussions.

Abbreviations: *ABCA1*, ATP binding cassete transporter A1; *ABCG1*, ATP binding cassete transporter G1; ANCOVA, analysis of covariance; ANOVA, analysis of variance; Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; BAI, body adiposity index; BMI, body mass index; cDNA, complementary DNA; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; cIMT, carotid-intima media thickness; CRP, C reactive protein; CT, comparative threshold; CTL, controls; CVD, cardiovascular disease; DBP, diastolic blood pressure; DLS, dynamic light scattering; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FAFA, fatty acid-free albumin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; HL, hepatic lipase; HOMA, homeostatic model assessment; *HPRT1*, hypoxanthine guanine phosphoribosyltransferase 1; Hyper, hyperalphalipoproteinemics; Hypo, hypoalphalipoproteinemics; LAP, lipid accumulation product; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein (a); LPL, lipoprotein lipase; miRNA, microRNAs; mRNA, messenger RNA; N, number of individuals; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG, polyethylene glycol; *PGK1*, phosphoglycerate kinase 1; PLTP, phospholipid transfer protein; PON-1, paraoxonase-1; RCT, reverse cholesterol transport; RNA, ribonucleic acid; RPMI, Roswell Park Memorial Institute; SBP, systolic blood pressure; *SCARB1*, scavenger receptor class B type I; snRNA, small nuclear RNA; *SREBF1*, sterol regulatory element-binding transcription factor 1; *SREBF2*, sterol regulatory element-binding transcription factor 2; SREBP1, sterol regulatory element-binding protein 1; SREBP2, sterol regulatory element-binding protein 2; UTR, untranslated region; UV, ultraviolet; VLDL, very low-density lipoprotein; VLDL-C, very low-density lipoprotein cholesterol.

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http://dx.doi.org/10.1016/j.gene.2015.05.074
0378-1119/

1. Introduction

Plasma levels of high-density lipoprotein cholesterol (HDL-C) are considered a significant and independent predictor of cardiovascular disease (CVD) risk, according to evidences derived from clinical and epidemiological studies (Gordon et al., 1989; Amarenco et al., 2008). Furthermore, HDL-C levels are under strong genetic control, and despite monogenic disorders, most cases result from the interaction between multiple genes and environmental factors (Weissglas-Volkov and Pajukanta, 2010).

The protective role of HDL particles is explained by their multiple properties, such as cholesterol efflux capacity, and antiinflammatory, antioxidant, antiapoptotic, vasodilatory, anti-thrombotic, as well as anti-infectious activities (Kontush and Chapman, 2006). However, reverse cholesterol transport (RCT), in which HDL particles remove cholesterol from peripheral tissues and macrophages from the liver for excretion, has a particular key role in the development of atherosclerosis.

Recent discoveries of microRNAs (miRNAs) that control HDL levels and function expanded the knowledge of the mechanisms regulating its biogenesis, uptake and RCT (Rayner and Moore, 2014). In this regard, several miRNAs targets the ATP binding cassette transporter A1 (ABCA1), a transporter that controls the rate of cholesterol efflux to apolipoprotein A-I (apo A-I) and consequently HDL biogenesis (Yokoyama, 2006). In fact, *ABCA1* has a long 3′ untranslated region (3′ UTR) as compared to other common genes involved in cholesterol and HDL metabolism, raising the probability of posttranscriptional regulation by miRNAs (Davalos and Fernandez-Hernando, 2013). The first to be identified as ABCA1 regulators were miR-33a and miR-33b (Rayner et al., 2010; Najafi-Shoushtari et al., 2010), which are embedded in intronic regions of sterol regulatory element-binding transcription factor 2 (*SREBF2*) and *SREBF1*, that code for sterol regulatory element-binding protein 2 (SREBP2) and SREBP1. In addition, miRNAs as miR-758, miR-106b, miR-26, miR-144, miR-10b, miR-145 and miR-128a were also experimentally validated for their importance to cholesterol efflux and RCT (Rayner and Moore, 2014).

Since the cholesterol efflux from peripheral cells (first and key step in RCT pathway) represent the most clinically relevant atheroprotective function of HDL, probably forming the basis between HDL-C levels and CVD (von Eckardstein et al., 2001) and that ABCA1 has a fundamental role in this process, this study aimed at evaluating whether miRNAs hsa-miR-33a and hsa-miR-128a are differentially expressed in peripheral blood mononuclear cells from asymptomatic individuals with low and high HDL-C levels, as well as investigating their potential relationships with *ABCA1* expression and other parameters related with reverse cholesterol transport, as cholesterol efflux. We also aimed at determining their relationships with carotid atherosclerosis.

2. Materials and methods

2.1. Study subjects

Volunteers of both genders were recruited from primary health care centers in Campinas (SP-Brazil). Nonsmokers, asymptomatic individuals with body mass index (BMI) lower than 30 kg/m^2, without regular use of any medications that interfere with lipid metabolism and daily intake of alcohol lower than 14 g, as previously described, were included (Parra et al., 2013).

Thus, 51 volunteers were selected and classified, according to plasma HDL-C levels (mg/dL), into three groups: hypoalphalipoproteinemics (hypo, HDL-C ≤ 39, n = 17), hyperalphalipoproteinemics (hyper, HDL-C ≥ 68, n = 17) and controls (CTL, HDL-C ≥ 40 < 68, n = 17). They were invited to a blood collection for biochemical measurements and real-time PCR analysis.

The study was approved by the Research Ethics Committee of the Faculty of Medical Sciences, University of Campinas and each participant provided written informed consent.

2.2. Clinical and anthropometrical data

Clinical and anthropometrical data as body weight, height, BMI, waist and hip circumference, as well as systolic and diastolic blood pressures (SBP and DBP, respectively) were obtained at admission. Moreover, waist-to-hip and waist-to-height ratios, body adiposity index (BAI = ((hip circumference) / ((height)$^{1.5}$) − 18) and lipid accumulation product ((LAP, for males = (waist circumference − 65) × triglycertriglycerides), for females = (waist circumference − 58) × triglycerides)) (Bergman et al., 2011; Kahn, 2005) were calculated.

2.3. Biochemical analysis

Venous blood samples were drawn after a 12-hour fasting period. Serum and EDTA plasma were separated by centrifugation (4 °C, 1000 ×*g*, 10 min) and stored at −80 °C until analysis. Total cholesterol, triglycerides and HDL-C were measured in an automated system Modular Analytics® EVO (Roche Diagnostics, Burgess Hill, West Sussex, UK), using Roche Diagnostics® reagents (Mannheim, Germany). Low-density lipoprotein cholesterol (LDL-C) was calculated by Friedwald equation and very low-density lipoprotein cholesterol (VLDL-C) by triglycerides / 5 (Friedewald et al., 1972).

Apolipoproteins A-I, B (apo B) and lipoprotein (a) (Lp(a)) were determined by nephelometry in automated system BN II (Siemens Healthcare Diagnostics, Marburg, Germany), using commercially available assays (Dade-Boehringer®, Deerfield, Illinois, USA). C-reactive protein (CRP) was measured using the Tina-quant® CRP (latex) high sensitivity assay (Roche Diagnostics®, Mannheim, Germany) by immunoturbidimetry. Insulin was determined using an ELISA assay (Human Insulin ELISA kit, Millipore Corporation, MA, USA). Homeostasis Model Assessment (HOMA) Calculator 2.2.2 (University of Oxford, UK) was used to estimate the insulin sensitivity (HOMA-S) and β cell function (HOMA-β) (Caumo et al., 2006).

2.3.1. HDL particle size, volume and chemical composition

For particle size and volume determinations, HDL was isolated by the precipitation of apo B containing lipoproteins with polyethylene glycol (PEG) 8000 (Sigma-Aldrich, St. Louis, USA), as previously reported (Dias et al., 1988). After PEG precipitation, HDL samples were maintained at 25 °C in a heat block and immediately analyzed by the dynamic light scattering (DLS) technique on Nanotrac Particle Size Analyzer (Microtrac, North Largo, Florida, USA) (Lima and Maranhao, 2004). The measurements were performed in triplicate and each sample was analyzed three times with 30 s of running time. A 100 nm polymeric nanoparticle was used as the standard and a control sample obtained from the same individual plasma was used in all determinations. HDL volume (nm^3) was calculated using the Microtrac FLEX Software (Microtrac, USA).

The measurements of HDL composition were performed in a subgroup of 19 volunteers (6 hypo, 6 controls and 7 hyper) and determined after the lipoprotein isolation from plasma through density gradient ultracentrifugation, using a SW41Ti rotor (Chapman et al., 1981). All assays were performed in freshly isolated lipoproteins, kept refrigerated for a maximum period of 7 days. In 96-well microplates the particle content of total protein (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford, USA), total cholesterol (CHOD-PAP, Roche Diagnostics® reagents, Mannheim, Germany), free cholesterol (Free Cholesterol E, Wako Chemicals, Richmond, USA), phospholipids (Phospholipids C, Wako Chemicals, Richmond, USA), triglycerides (GPO-PAP, Roche Diagnostics® reagents, Mannheim, Germany) and apo A-I (TINA QUANT APO A1 V2, Roche Diagnostics® reagents, Mannheim, Germany) were measured using commercially available kits, in the microplate reader Power Wave XS (BioTek®, Winooski, USA). Cholesteryl ester was calculated as the difference between total cholesterol and free cholesterol times 1.67 (Chapman et al., 1981).