Clinical Biochemistry 61 (2018) 12–17

Contents lists available at ScienceDirect

Clinical Biochemistry

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

Autophagy-related 16-like 1gene polymorphism, risk factors for cardiovascular disease and associated carotid intima-media thickness in postmenopausal women

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ARTICLE INFO

Keywords:
ATG16L1
Postmenopausal
Gene polymorphism
Atherosclerosis
Inflammatory

ABSTRACT

Background: Early identification of asymptomatic postmenopausal women (PW), who are more predisposed to developing cardiovascular disease (CVD), is an important preventive strategy. Autophagy-related 16-like 1 (ATG16L1) is an autophagy gene known to control host immune responses and is associated with a variety of diseases, including CVD.

Objective: The aim of the study was to associate the ATG16L1 polymorphism variant with subclinical carotid atherosclerosis in asymptomatic PW.

Study design: This cross-sectional study included 210 Brazilian postmenopausal women (age ≥ 45 years with amenorrhea ≥12 months). Clinical, anthropometric and biochemical assessments were performed to evaluate the cardiovascular risk factors. DNA was extracted from buccal cells and the ATG16L1 (T300A) polymorphism was determined by the polymerase chain reaction (PCR). The carotid intima-media thickness and/or the presence of plaques were evaluated by carotid duplex ultrasound. For statistical analysis, the *t*-test, logistic regression and analysis of covariance (ANCOVA) were used.

Results: The presence of the polymorphic allele forATG16L1 (T300A) was found in 77.47% (A/G = 49.87%, G/G = 27.60%). The ATG16L1 (T300A) polymorphism is significantly associated with increased carotid intima-media thickness (IMT) after adjustments of the confounding variables ($P < .037$). No significant associations were observed between the polymorphism with other risk factors for CVD in PW.

Conclusion: In postmenopausal women, the ATG16L1 (T300A) polymorphism is significantly associated with increased carotid IMT (marker of atherosclerotic disease) after adjustments of the confounding variables ($P < .037$). Thus, identifying the ATG16L1 polymorphism is an important strategy for screening asymptomatic PW who are more predisposed to developing CVD.

1. Introduction

In women, aging and menopause are considered cardiovascular risk factors [1] because of estrogen deprivation due to ovarian insufficiency [2]. Coronary heart disease (CHD) is often fatal and over one-half of postmenopausal women (PW) with CHD do not present early symptoms [3]. Therefore, early identification of asymptomatic PW, who are more predisposed to developing CHD, is an important preventive strategy [4]. Detecting early markers enables recognition of subclinical atherosclerotic disease and early intervention in modifiable risk factors [5].

In CHD, conventional risk factors - dyslipidemia, hypertension, diabetes, obesity, smoking - are important [6]. However, individual differences in the immune-genetic profile may modulate the severity of the CHD process [7,8]. The main mechanisms in the installation and development of systemic inflammatory response comprise the complement cascade system, the activation of immune cells by releasing cytokines and the endothelial dysfunction [9]. Studies have shown an

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https://doi.org/10.1016/j.clinbiochem.2018.09.006
Received 25 October 2017; Received in revised form 13 September 2018; Accepted 16 September 2018
Available online 17 September 2018

immune influence associated with the development of CHD in postmenopausal women by cytokine production and expression of receptors [10,11], *heat-shock proteins* [12] and genetic polymorphism [13]. The expression of inflammatory molecules is stimulated by risk factors and are under the control of inflammatory cytokines and hemodynamic factors, making its characterization extremely important [14].

Autophagy is an important host defense mechanism, interacting with the innate and acquired immunity to both identify and defend against aggressors [15]. Autophagy is a self-protecting cellular catabolic pathway. At a basal level, autophagy is involved in maintaining normal cellular homeostasis [16,17]. Autophagy is associated with a variety of diseases, including cardiovascular diseases, tumors, neurodegenerative diseases and disorders of the immune system [18,19] in response to environmental stress, such as a sedentary lifestyle, hypoxia or oxidative stress [16,19]. Autophagy-related 16-like 1 (ATG16L1) is an autophagy gene known to control host immune responses [20]. ATG16L1 single nucleotide polymorphism (SNP), rs2241880, encodes a missense variant resulting in a substitution of threonine with alanine at amino acid position 300 (T300A) [20]. The T300A variant is the most prevalent of all ATG16L1 variants, representing approximately 55% of the alleles in the European population and 20–40% of alleles in other populations [20]. A SNP in this gene results in decreased autophagy activity [20]. Evidence shows that autophagy plays an important role in inflammation and inhibition of apoptosis and it has been demonstrated that autophagy plays a protective role in atherosclerosis [21], which acts as a cholesterol efflux promoter [22,23], suggesting that autophagy is a potential mechanism in atherosclerosis [24]. However, there is little evidence regarding the contribution of ATG16L1 polymorphism variants to the regulation of atherosclerosis.

To assess the relationship between autophagy and atherosclerosis, we investigated whether the ATG16L1 polymorphism variant is associated with subclinical carotid atherosclerosis and risk factors in asymptomatic postmenopausal women. This paper presents an association between the ATG16L1 polymorphism and carotid intima-media thickness in the development of atherosclerosis in PW and discusses the theory behind autophagy in the atherosclerosis process.

2. Methods

2.1. Study design and sample selection

This is a clinical, analytical and cross-sectional study. The study population was postmenopausal women, aged 45–70 years, seen at the Climacteric and Menopause Outpatient Clinic at the Botucatu Medical School-UNESP, who took part in a study that evaluated clinical and inflammatory markers of subclinical carotid atherosclerosis, which were previously described in detail [25]. Women whose last menstruation was at least 12 months prior to the beginning of the study and aged ≥ 45 years old were included (n = 311). Thirty five patients had insufficient buccal cell samples. Therefore, 276 patients were included in the study and the polymorphic test was performed. However, 93 patients were not analyzed as they did not have all the variables for the analysis. Thus, 210 patients were included and analyzed (Fig. 1). The exclusion criteria were: [1] known high cardiovascular risk due to existing or preexisting CHD, cerebrovascular arterial disease, abdominal aortic stenosis or aneurysm, peripheral artery disease, chronic kidney disease; [2] history of: hepatitis B and C, acute infection, genital tract infection, chronic inflammatory or autoimmune diseases (ulcerative colitis, Crohn's disease, rheumatoid arthritis, lupus), cancer and addiction to either alcohol or illicit drugs; [3] current medications (statin use) or menopausal hormone treatments. A written informed consent was obtained from all the participants and the study was approved by the Research Ethics Committee at Botucatu Medical School, Sao Paulo State University/UNESP (process number: 3362–2009). (See Table 1.)

2.2. Methodology

During the consultations, all participants were interviewed individually and the following data were collected: age, time since menopause, if they are currently smoking, use of hormone therapy (HT), personal history of hypertension, diabetes and physical activity, as well as family history of CHD (acute myocardial infarctionin1st degree relative male aged < 55 years and female aged < 65 years). Their blood pressure was measured using a standard aneroid sphygmomanometer on the right arm with patients in the sitting position, forearm resting at the level of the precordium and the palm of the hand facing upwards, after a five-minute rest. Smokers were defined as those who reported smoking regardless of the number of cigarettes smoked. Women who practiced aerobic physical exercise of moderate intensity for at least 30 min, five times a week (150/min/week) or resistance exercise three times a week were considered to be active [26]. Women showing three or more of the following diagnostic criteria proposed by the US National Cholesterol Education Program/Adult Treatment Panel III (NCEP-ATP III) [27] were diagnosed as positive for MetS: waist circumference > 88 cm; triglycerides ≥150 mg/dL; HDL cholesterol < 50 mg/dL; blood pressure ≥ 130/85 mmHg or under therapy; fasting glucose ≥100 mg/dL or under therapy.

2.3. Anthropometry

The anthropometric data included weight, height, body mass index (BMI = weight/height^2) and waist circumference (WC). The weight and height were determined using a standard balance beam scale (max. 150 kg, 0.1 kg accuracy) and a portable wall anthropometer (0.1 cm accuracy), respectively. Patients wore lightweight clothes and no shoes. BMI was classified according to the system used by the World Health Organization (2002): lower than 25 kg/m^2 was defined as normal, from 25 to 29.9 kg/m^2 as overweight and above 30 kg/m^2 as obese. The waist circumference was measured at the midpoint between the lowest rib and the top of the iliac crest. The patients were advised to remain in the orthostatic position and readings were taken when they exhaled. These measurements were performed by a single evaluator. Any WC exceeding 88 cm was considered high [27].

2.4. Laboratory tests

Blood samples were collected from each participant, after 12 h of fasting. After centrifugation, samples underwent biochemical analysis immediately and a serum aliquot was frozen and kept at −80 °C for cytokine determinations. Triglycerides (TG), total cholesterol (TC), HDL, glucose and C-reactive protein (CRP) measurements were processed by an automated analyzer, Model Vitros 950®, using the colorimetric dry-chemistry method (Johnson & Johnson®, Rochester, NY, USA). Low density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula in which the total cholesterol is subtracted from the sum of HDL cholesterol and triglycerides. The result is divided by five, which shows a usage limitation when TG values exceed 400 mg/dL. The values considered to be optimal were: TC < 200 mg/dL, HDL > 50 mg/dL, LDL < 100 mg/dL, TG < 150 mg/dL, glucose < 100 mg/dL and CRP < 1.0 mg/dL. Insulin, TSH and T_4-L were quantified using the Immulite System® (DPC®, USA), which uses a solid phase chemiluminescence immune-assay and were assessed in the designated automatic analyzer for quantitative reading. The normality rate ranged from 6.0 to 27.0μIU/mL (Insulin). The analytical sensitivity for TSH was 0.002 mIU/mL and T_4-L, 0.15 ng/dL. The intra-assay and inter assay variations for TSH were 7.0% and 11% and T_4, 7.2%, respectively. The normal rates for the exams are: TSH from 0.49 to 4.67 mIU/mL and T_4-Lfrom 0.71 to 1.85 ng/dL. Insulin resistance was determined by measurement of two plasma components (insulin and fasting glucose). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: insulin