

Original

G1359A polymorphism of the cannabinoid receptor gene (CNR1) and insulin resistance in patients with diabetes mellitus type 2

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Abstract

Background: A silent intragenic biallelic polymorphism (1359 G/A) (rs1049353) of the CB1 gene resulting in the substitution of the G to A at nucleotide position 1359 in codon 435 (Thr), was reported as a common polymorphism in Caucasian populations.

Objective: The aim of our study was to investigate the influence of the missense polymorphism (G1359A) of CB1 receptor gene on obesity anthropometric parameters, cardiovascular risk factors and adipocytokines in patients with obesity and diabetes mellitus type 2.

Design: A population of 60 naïve diabetic patients was analyzed. An indirect calorimetry, tetrapolar electrical bioimpedance, blood pressure, a serial assessment of nutritional intake with 3 days written food records and biochemical analysis (lipid profile, adipocytokines, insulin, CRP and lipoprotein-a) were performed. The statistical analysis was performed for the combined G1359A and A1359A as a group and wild type G1359G as second group, with a dominant model.

Results: The mean age was 57.44 ± 11.7 years and the mean BMI 37.84 ± 6.4 , with 14 males and 46 females. Thirty-five patients (58.3%) had genotype G1359G (wild type group) and 25 (42.7%) patients G1359A (mutant type group). Age was similar in both groups (wild type: 56.3 ± 11.8 years vs mutant group: 58.7 ± 10 years:ns). Sex distribution was similar in both groups (wild vs mutant type groups), males (22.9% vs 24%) and females (77.1% vs 76%). No differences were detected between groups in anthropometric parameters, cardiovascular risk factors, dietary intake and adipocytokines levels.

Conclusion: The finding of this study is the lack of association of G1359A polymorphism of CB receptor 1 gene with obesity, cardiovascular risk factors and adipocytokines.

(Nutr Hosp. 2010;25:34-38)

DOI:10.3305/nh.2010.25.1.4356

Key words: *Cannabinoid receptor gene. Diabetes mellitus. Insulin resistance. Polymorphism.*

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Recibido: 25-V-2009.
Aceptado: 25-V-2009.

RELACIÓN DEL POLIMORFISMO G1359A DEL RECEPTOR ENDOCANABINOIDE CB1 Y LA RESISTENCIA A LA INSULINA EN PACIENTES CON DIABETES MELLITUS TIPO 2

Resumen

Introducción: Se ha descrito un polimorfismo bialélico silente (1359 G/A) del receptor CB1 endocanabinoide, produciendo una sustitución del nucleótido G por el A en la posición 1359 en el codón 435 (Thr), siendo frecuente en la población Caucásica.

Objetivo: El objetivo de nuestro estudio fue investigar la influencia de este polimorfismo sobre parámetros relacionados con la obesidad, factores de riesgo cardiovascular y adipocitoquinas en pacientes con obesidad y diabetes mellitus tipo 2.

Material y métodos: Se evaluó una muestra de 60 pacientes diabéticos tipo 2 de reciente diagnóstico con obesidad. Se practicó una calorimetría indirecta, una impedanciometría bioeléctrica, determinación de presión arterial, encuesta nutricional de 3 días y valoración bioquímica nutricional. El análisis estadístico se realizó, combinando el genotipo G1359A y A1359A como grupo mutante y como grupo salvaje G1359G (modelo dominante).

Resultados: La edad media fue de $57,44 \pm 11,7$ años y el IMC medio de $37,84 \pm 6,4$, con una distribución por sexos de 14 varones y 46 mujeres. Un total de 35 pacientes (58,3%) presentaron el genotipo G1359G (genotipo salvaje) y 25 pacientes (42,7%) G1359A (genotipo mutante). La edad fue similar en ambos grupos (grupo salvaje: $56,3 \pm 11,8$ años vs grupo mutante: $58,7 \pm 10$ años: ns). La distribución por sexos fue similar (genotipo salvaje vs mutante), varones (22,9% vs 24%) y mujeres (77,1% vs 76%). No existieron diferencias entre ambos grupos en variables antropométricas, factores de riesgo cardiovascular, ingesta dietética y niveles de adipocitoquinas.

Conclusión: Nuestros datos muestran una ausencia de relación entre el polimorfismo G1359A del receptor endocanabinoide CB 1 y las variables antropométricas, factores de riesgo cardiovascular y niveles séricos de adipocitoquinas.

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Palabras clave: *Gen del receptor cannabinoide. Diabetes mellitus. Insulin resistencia. Polimorfismo.*

Introduction

Adipose tissue is an active secretory organ, sending out and responding to signals that modulate appetite, insulin sensitivity, energy expenditure, inflammation and immunity.¹ Obesity and insulin resistance are associated with cardiovascular risk factors, including altered levels of inflammatory markers and adipocytokines.

In this topic area, the important role played by endocannabinoid system is emerging: it controls food intake, energy balance and glucose metabolism through both central and peripheral effects, and stimulated lipogenesis and fat accumulation.^{2,3} This system consists of endogenous ligands 2-arachidonoylglycerol (2-AG) and anandamide (ADA) and two types of G-protein-coupled cannabinoid receptors: cannabinoid type-1 receptor (CB1), located in several brain areas and in a variety of peripheral tissues including adipose tissue, and CB2, present in the immune system.⁴ A greater insight into the endocannabinoid system has been derived from studies in animals with a genetic deletion of the CB1 receptor, that have a lean phenotype and are resistant to diet-induced obesity and the associated insulin resistance induced by a high palatable high-fat diet.⁵ A silent intragenic polymorphism (1359 G/A) of the CB1 gene resulting in the substitution of the G to A at nucleotide position 1359 in codon 435 (Thr), was reported as a common polymorphism in the German population,⁶ reaching frequencies of 24-32% for the rarer allele (A).

Considering that endogenous cannabinoid system plays a role in metabolic aspects of body weight and there is not specific studies in patients with diabetes mellitus type 2,⁷ we decide to investigate the association of this CB1 receptor polymorphism with insulin resistance and adipocytokines in this population.

The aim of our study was to investigate the influence of the missense polymorphism (G1359A) of CB1 receptor gene on adipocytokines and insulin resistance in the fasted state in naïve patients with diabetes mellitus type 2.

Subjects and methods

Subjects

A population of 60 naïve patients with diabetes mellitus type 2 (fasting glucose > 126 mg/dl) and obesity (body mass index > 30) was analyzed in a prospective way (research protocol accepted by ethical committee). These patients were recruited in a Nutrition Clinic Unit and signed an informed consent. Exclusion criteria included history of cardiovascular disease or stroke during the previous 36 months, total cholesterol > 300 mg/dl, triglycerides > 400 mg/dl, blood pressure > 140/90 mmHg, as well as the use of sulphonilureas, thiazolidinedionas, metformin, insulin, glucocorti-

coids, antineoplastic agents, angiotensin receptor blocker, angiotensin converting enzyme inhibitors, psychoactive medications, drinking and/or smoking habit.

Procedure

All patients with a 2 weeks weight-stabilization period before recruitment were enrolled. Weight, blood pressure, basal glucose, c-reactive protein (CRP), insulin, insulin resistance (HOMA), total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides blood and adipocytokines (leptin, adiponectin, resistin, TNF alpha, and interleukin 6) levels were measured at basal time. A tetrapolar bioimpedance, an indirect calorimetry and a prospective serial assessment of nutritional intake with 3 days written food records were realized. Genotype of CB1 receptor gene polymorphism was studied.

Genotyping of CB1 gene polymorphism

Oligonucleotide primers and probes were designed with the Beacon Designer 4.0 (Premier Biosoft International®, LA, CA). The polymerase chain reaction (PCR) was carried out with 50 ng of genomic DNA, 0.5 uL of each oligonucleotide primer (primer forward: 5'-TTC ACA GGG CCG CAG AAA G-3' and reverse 5'-GAG GCA TCA GGC TCA CAG AG-3'), and 0.25 uL of each probes (wild probe: 5'-Fam-ATC AAG AGC ACG GTC AAG ATT GCC-BHQ-1-3') and (mutant probe: 5'-Texas red- ATC AAG AGC ACA GTC AAG ATT GCC -BHQ-1-3') in a 25 uL final volume (Termociclador iCycler IQ (Bio-Rad®), Hercules, CA). DNA was denaturated at 95°C for 3 min; this was followed by 50 cycles of denaturation at 95°C for 15 s, and annealing at 59.3°C for 45 s). The PCR were run in a 25 uL final volume containing 12.5 uL of IQTM Supermix (Bio-Rad®, Hercules, CA) with hot start Taq DNA polymerase. Hardy Weimberger equilibrium was assessed.

Assays

Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, California). Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5 mUI/L (normal range 0.5-30 mUI/L)⁸ and the homeostasis model assessment for insulin sensitivity (HOMA) was calculated using these values.⁹ CRP was measured by immunoturbimetry (Roche Diagnostcis GmbH, Mannheim, Germany), with a normal range of (0-7 mg/dl) and analytical sensitivity 0.5 mg/dl.

Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assay

(Technicon Instruments, Ltd., New York, N.Y., USA), while HDL cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulfate-magnesium. LDL cholesterol was calculated using Friedewald formula.

Resistin was measured by ELISA (Biovendor Laboratory, Inc., Brno, Czech Republic) with a sensitivity of 0.2 ng/ml with a normal range of 4-12 ng/ml.¹⁰ Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., Texas, USA) with a sensitivity of 0.05 ng/ml and a normal range of 10-100 ng/ml.¹¹ Adiponectin was measured by ELISA (R&D systems, Inc., Minneapolis, USA) with a sensitivity of 0.246 ng/ml and a normal range of 8.65-21.43 ng/ml.¹² Interleukin 6 and TNF alpha were measured by ELISA (R&D systems, Inc., Minneapolis, USA) with a sensitivity of 0.7 pg/ml and 0.5 pg/ml, respectively. Normal values of IL6 was (1.12-12.5 pg/ml) and TNFalpha (0.5-15.6 pg/ml).¹³⁻¹⁴

Indirect calorimetry and anthropometric measurements

For the measurement of resting energy expenditure, subjects were admitted to a metabolic ward. After a 12 h overnight fast, resting metabolic rate was measured in the sitting awake subject in a temperature-controlled room over one 20 min period with an open-circuit indirect calorimetry system (standardized for temperature, pressure and moisture) fitted with a face mask (MedGem; Health Tech, Golden, USA), coefficient of variation 5%. Resting metabolic rate (kcal/day) and oxygen consumption (ml/min) were calculated.¹⁵

Body weight was measured to an accuracy of 0.1 kg and body mass index computed as body weight/(height²). Waist (narrowest diameter between xiphoid process and iliac crest) and hip (widest diameter over greater trochanters) circumferences to derive waist-to-hip ratio (WHR) were measured, too. Tetrapolar body electrical bioimpedance was used to determine body composition with an accuracy of 5 g¹⁶ (Biodynamics Model 310e, Seattle, WA, USA).

Blood pressure was measured twice after a 10 minutes rest with a random zero mercury sphygmomanometer, and averaged.

Dietary intake and habits

Patients received prospective serial assessment of nutritional intake with 3 days written food records. All enrolled subjects received instruction to record their daily dietary intake for three days including a weekend day. Handling of the dietary data was by means of a personal computer equipped with personal software, incorporating use of food scales and models to enhance portion size accuracy. Records were reviewed by a dietitian and analysed with a computer-based data

evaluation system. National composition food tables were used as reference.¹⁷

Statistical analysis

Sample size was calculated to detect differences over 5% in insulin resistance with 90% power and 5% significance. The results were expressed as average \pm standard deviation. The distribution of variables was analyzed with Kolmogorov-Smirnov test. Quantitative variables with normal distribution were analyzed with a two-tailed Student's-t test. Non-parametric variables were analyzed with the U-Mann-Whitney test. Qualitative variables were analyzed with the chi-square test, with Yates correction as necessary, and Fisher's test. The statistical analysis was performed for the combined *G1359A* and *A1359A* as a group and wild type *G1359G* as second group, with a dominant model. A p-value under 0.05 was considered statistically significant.

Results

Sixty patients gave informed consent and were enrolled in the study. The mean age was 57.44 ± 11.7 years and the mean BMI 37.84 ± 6.4 , with 14 males and 46 females.

Thirty-five patients (58.3%) had genotype *G1359G* (wild type group) and 25 (42.7%) patients *G1359A* (mutant type group). Age was similar in both groups (wild type: 56.3 ± 11.8 years vs mutant group: 58.7 ± 10 years: ns). Sex distribution was similar in both groups (wild vs mutant type groups), males (22.9% vs 24%) and females (77.1% vs 76%).

Table I shows anthropometric variables, without statistical differences.

Table II shows cardiovascular risk factors. Insulin and HOMA levels were higher in wild type group than mutant group, without statistical differences.

Table I
Anthropometric variables

Characteristics	<i>G1359G</i> (n = 35)	<i>(G1359A)</i> (n = 25)
BMI	37.8 \pm 6.9	38.1 \pm 5.9
Weight (kg)	97.4 \pm 18.2	95.3 \pm 15.8
Fat free mass (kg)	53.4 \pm 14.8	51.4 \pm 12.8
Fat mass (kg)	43.3 \pm 16.1	43.1 \pm 13.9
WC (cm)	113.6 \pm 12.2	118.4 \pm 13.8
Waist to hip ratio	0.94 \pm 0.1	0.96 \pm 0.09
Systolic BP (mmHg)	137.1 \pm 14.1	136.5 \pm 17.5
Diastolic BP (mmHg)	85.1 \pm 8.9	86.2 \pm 10.3
RMR(kcal/day)	2,025 \pm 617	2,230 \pm 603

RMR: resting metabolic rate. WC: Waist circumference. No statistical differences between groups. (*) p < 0.05, in each group with basal values.

Table II <i>Classical cardiovascular risk factors</i>		
<i>Characteristics</i>	<i>G1359G</i> (<i>n</i> = 35)	<i>(G1359A)</i> (<i>n</i> = 25)
Glucose (mg/dl)	127.7 ± 26.5	135.4 ± 21.3
Total ch. (mg/dl)	212.1 ± 36.1	215.1 ± 44.3
LDL-ch. (mg/dl)	140.5 ± 34.1	138.5 ± 43.2
HDL-ch. (mg/dl)	51.6 ± 12.4	52.6 ± 9.3
TG (mg/dl)	143.1 ± 75.9	154.2 ± 67.8
Insulin (mUI/L)	26.7 ± 21.4	23.5 ± 19.9
HOMA	8.6 ± 8.9	7.9 ± 7.1
CRP (mg/dl)	7.2 ± 5.5	8.4 ± 7.4

Ch: Cholesterol. TG: Triglycerides CRP: c reactive protein. HOMA: Homeostasis model assessment. (*) $p < 0.05$, in each group with basal values.

Table III shows nutritional intake with 3 days written food records. Caloric, carbohydrate, fat, and protein intakes were similar in both groups, without differences between allelic groups.

Table IV shows levels of adipocytokines. No differences were detected between genotypes.

Discussion

The finding of this study is the lack of association of the G1359A and A1359A CB1 phenotypes with anthropometric and biochemical parameters in naïve patients with diabetes mellitus type 2.

The literature supports the notion that endocannabinoid system is positioned for regulation of endocannabinoid levels that could influence craving and reward behaviors through the relevant neuronal circuitry and metabolic parameters.¹⁸ This provide a link between the consequences of this polymorphism and the present epidemiological study indicating that the

Table III <i>Dietary intake</i>		
<i>Characteristics</i>	<i>G1359G</i> (<i>n</i> = 159)	<i>(G1359A)</i> (<i>n</i> = 131)
Energy (kcal/day)	1,728.7 ± 717	1,781 ± 658
CH (g/day)	178.4 ± 81	176.2 ± 65
Fat (g/day)	74.8 ± 31	72.6 ± 35
S-fat (g/day)	21.3 ± 12.1	21.7 ± 11.2
M-fat (g/day)	36.2 ± 11.1	35.8 ± 14.2
P-fat (g/day)	7.3 ± 4.2	7.1 ± 3.1
Protein (g/day)	84.4 ± 28	85.6 ± 24
Exercise (hs./week)	1.71 ± 2.4	1.59 ± 2.2
Dietary fiber	14.47 ± 7.2	13.94 ± 7.1

CH: Carbohydrate. S-fat: saturated fat. M-fat: monounsaturated fat. P-fat: polyunsaturated fat. No statistical differences

Table IV <i>Circulating adipocytokines</i>		
<i>Characteristics</i>	<i>G1359G</i> (<i>n</i> = 35)	<i>(G1359A)</i> (<i>n</i> = 25)
BMI	37.8 ± 6.9	38.1 ± 5.9
IL 6 (pg/ml)	1.82 ± 1.9	1.77 ± 2.8
TNF- α (pg/ml)	6.33 ± 4.1	6.71 ± 3.5
Adiponectin (ng/ml)	31.6 ± 26.8	31.7 ± 26.4
Resistin (ng/ml)	3.13 ± 1.1	3.81 ± 2.3
Leptin (ng/ml)	82.9 ± 90.1	90.1 ± 89.1

IL-6: interleukin 6. No statistical differences between groups.

CB1 receptor G1359A polymorphism may be one risk factor for susceptibility to obesity. Also, the CB1 receptor is expressed in some peripheral human tissue studied in relation to the pathogenesis of obesity and obesity-associated metabolic disorders and marked down-regulation of the fatty acid amide hydrolase (FAAH) gene expression was found in the adipose tissue of obese women, suggesting that adipose tissue may be an important contributor to endocannabinoid inactivation.¹⁹ However in our study, obesity was not related with this polymorphism. The type of patients (naïve diabetics) could explain this lack of association.

The percentage of GA genotype was (41.6%), similar that other studies; 43.5%,²⁰ but higher than others; 19.6%²¹ and 33.1%.²² The lack of association between body mass index and this polymorphism is in contrast with the association detected by Gazerro et al.²⁰ with SNP G1359A of CB1 receptor, A3813A and A4895A SNPs of CB1 receptor²³ and with (G1422A) SNP of CB1 receptor.²⁴ The inconsistencies between association studies may reflect the complex interactions between multiple population-specific genetic and environmental factors. Perhaps, these different results could be explained by bias in previously studies of the literature. These previous studies would require composition analysis of the diet to determine whether dietary components could be responsible for the lipid profile modifications. In our study dietary intake did not show statistical differences between groups, in this way our dates have been controlled by dietary intake and previous discrepancies could be explain by this uncontrolled factor.

Other possibility to explain these discrepancies is the presence of other polymorphism in CBR receptor gene. In other study,²⁴ the A1422A homozygotes patients are more abdominally obese. Carriers of 3813G allele had higher level of total body fat and central fat deposition,²³ no association was observed with A4895G variant. A positive correlation of the A10908G and T5489C polymorphisms with obesity in two obese populations has been described.²⁵

Our study did not detected metabolic differences between genotypes. In the literature, differences have been detected after an intervention no in basal state.

First, Ravinet et al.⁵ found that CB-1 gene-deficient mice were lean and resistant to diet-induced obesity and showed reduced plasma insulin and leptin levels. Second, Alberle et al.²¹ have shown that carriers of at least one A allele in CB1 lost more weight and reduced LDL cholesterol than wild type patients.

The theoretical explanation of this published association could be due by the adipose tissue. Cannabinoids modulate the expression of several cellular target genes via the CB1 receptor dependent pathway.²⁶ Perhaps, a direct role of the endocannabinoid system in lipid and glucose homeostasis could be speculated in an independent way of body mass index in non diabetic patients.

In conclusion, the finding of this study is the lack of association of G1359A polymorphism of CB receptor 1 gene with obesity, cardiovascular risk factors and adipocytokines.

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