Iran J Public Health, Vol. 45, No.4, Apr 2016, pp.460-468

Original Article

Association of the −243A>G, +61450C>A Polymorphisms of the Glutamate Decarboxylase 2 (*GAD2*) Gene with Obesity and Insulin Level in North Indian Population

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(Received 18 Jan 2015; accepted 22 Oct 2015)

Abstract

Background: Obesity associated with type 2 diabetes, and hypertension increased mortality and morbidity. Glutamate decarboxylase 2 (GAD2) gene is associated with obesity and it regulate food intake and insulin level. We investigated the association of GAD-2gene −243A>G (rs2236418) and +61450C>A (rs992990) polymorphisms with obesity and related phenotypes.

Methods: Insulin, glucose and lipid levels were estimated using standard protocols. All subjects were genotyped (PCR-RFLP) method.

Results: The −243A>G polymorphism of the GAD-2 gene was significantly associated with higher risk of obesity ($P<0.05$).

Conclusion: GAD-*2* gene polymorphisms influence obesity and related phenotype in complex manner, probably by regulating the food intake, insulin and body weight.

Keywords: Obesity, Insulin, GAD-2, Food intake, Polymorphism

Introduction

Obesity often begins early in life and is one of the most common non-communicable diseases and public health issues in the world at every stage of development. The magnitudes of these conditions include increased risk for cardiovascular diseases, diabetes, and many other diseases. The recent assessments specify that around 1.46 billion people globally are overweight and 502 million of them are obese (1). Obesity is strongly associated with significantly higher all-cause mortality during adulthood due to its association with various complex diseases (2).

Genome-wide scans for obesity genes have repetitively detected linkage with a locus on chromosome 10p11.22-23 (3). The previous studies showed that glutamate decarboxylase 2 (*GAD2)* located on chromosome 10, has been linked to obesity (3,4). The first candidate gene for obesity identified through the genome wide approach was *GAD2* (5). It encodes the glutamic acid decarboxylase enzyme GAD65 connected to obesity via the hypothalamic regulation of food intake. *GAD2* regulates the formation of the γ- aminobutyric acid (GABA) from the glutamic acid. GABA functions together with neuropeptide Y in the paraventricular nucleus to stimulate food intake. In pancreatic islets, GABA modulates hormone secretion by pancreatic islets (6). Family-based genetic analyses showed association of obesity with other SNP, +61450C>A (rs992990),

which located in the *GAD2* gene. −243A>G polymorphism (rs2236418) located in the promoter region of the *GAD2* gene, has been associated with morbid obesity in various studies (5,7). The *GAD2* gene polymorphisms were associated with childhood obesity, birth weight and binge eating (7). The G allele of −243A>G polymorphism was modestly associated with lower body mass index (BMI) and lower fasting glucose concentrations (8). Inconsistent results have been obtained regarding the association of *GAD2* gene SNPs with obesity. In addition, other studies involving Europian and American population showed no association with obesity (3, 9, 10).

Now, obesity and obesity associated phenotypes becomes very common and both environmental as well as genetic factors significantly contribute toward susceptibility of obesity. As the association studies are influenced by populations and ethnicities, we ascertained the role of these SNPs in genetic susceptibility to obesity and obesity associated phenotypes in our north Indian population.

Materials and Methods

Subjects

The population studied included 642 apparently healthy individuals (347 men, 295 women) ranging in age from 19 to 60 yr. The samples were collected randomly from the outpatients department of King George's Medical University, Lucknow and from the general population of Lucknow (North India). 309 obese (BMI > 30 $k.g/m^{2)}$ and 333 non obese (BMI ≤ 30 $k.g/m^{2)}$ individual were participated in the study, selected based on the strict inclusion criteria. In all subjects' body height, body weight, waist circumferences and hip circumferences were measured for calculation of BMI and waist to hip ratio (WHR). The Subjects were free from serious and/or chronic illnesses, especially diabetes mellitus, coronary artery disease and pregnant women were excluded. All individuals were of north Indian origin and the population was homogeneous with regard to ethnic background (11). Subjects were classified as type 2 diabetes patients if they were on hypoglycemic medications or when their fasting glucose concentrations exceeded 126 mg/dL. Prior informed written consent was taken from each participant and the identity of all participants was kept confidential. All protocols and the study were carried out with the approval of local ethics committee (IRB number- XXXIV ECM/P6) at King George's Medical University Lucknow.

At baseline all study participants were subjected to a thorough screening program that included assessment of a detailed personal and family history, physical examination, determination of anthropometric indices and measurement of various biochemical parameters were done.

Estimation of body fat composition

The Body fat analyzer (Bioelectrical impedance was obtained using a device, Tanita–TBF–310, Japan) was used for assessing the BMI, %body fat, fat mass (FM), fat free mass (FFM) and total body water (TBW).

Laboratory measurements

Venous blood was collected after an overnight fast, and was centrifuged within 2 h after collection. Plasma samples were immediately separated and stored in aliquots at −80 °C until they were analyzed. Commercial enzymatic tests were used for determining fasting glucose, lipid profile while low density lipoprotein (LDL) cholesterol was calculated by the formula of Friedewald. Fasting insulin level was determined by enzyme-linked immunosorbent assay RIA (Linco Research). The degree of insulin sensitivity/ resistance was calculated according to the homeostasis model assessment (HOMA) which is a good index for assessing insulin sensitivity/resistance. A description of each method was given by us elsewhere (12). *GAD2* SNPs were genotyped by PCR-based restriction fragment length polymorphism analysis or by tetra amplification refractory mutation system PCR (9).

Quality control

Quality control and assessment was done at every step of the study. The amount of isolated DNA was of good quality (Absorbance 260 nm/280 nm

ratio >1.75). One sample with known genotype and a reagent blank were included after every 20 samples in the PCR. A 50 base-pair marker was included during electrophoresis. Twenty percent of samples from patients and controls, including samples of each genotype were re-genotyped by other laboratory personnel. No discrepancy was found after sequencing the randomly selected 5% samples.

Statistical analysis

Statistical analyses were performed using SPSS 15 (Chicago, IL) statistical tools. The independent segregation of alleles was tested for the Hardy-Weinberg equilibrium (HWE), comparing the observed genotype frequencies with those expected (X^2 test). Genotype and allele distribution was compared between obese and non-obese subjects using X^2 test. Haplotype analysis was done by SNP analyzer version 1.2 by expectation–maximization algorithm (13). Most frequent homozygous genotype in the control group was used as reference for association analysis. Statistical significant was determined at $P<0.05$ for all tests. The hypothesis of different genetic models (Additive model, Co-dominant model, Dominant model, Recessive model, Over dominant model, and Multiplicative model) used in the study is described in Table 1. The differences among the three groups were assessed by one-way ANOVA for continuous variables and variables are given as the mean ± SD.

Table 1: Different genetic models used and associated hypothesis

SN	Type of models	Hypothesis
1	Additive model	r-Fold increased risk for genotype with one risk (minor) allele and 2rfold increased risk for homozygous variant or with two risk (minor) allele
2	Co-dominant model	Risk genotype with two minor allele and wild genotype with two major alleles
3	Dominant model	Risk genotypes with at least one minor allele and wild genotype with two major alleles
4	Recessive model	Risk genotype with two minor allele and wild genotype with at least one major allele
5	Over dominant model	Risk genotypes with two minor allele or two alleles and wild genotype with one minor allele and one major allele
6	Multiplicative model	r-Fold increased risk for hetero genotype and r2 increased risk for homozygous minor allele

Results

Clinical characteristics of the obese (BMI≥30) and non-obese (BMI<30) participants of the present study are shown in Table 2. Systolic blood pressure, diastolic blood pressure, FM (fat mass), % body fat, WHR, insulin, HOMA-IR, and lipid profile were significantly different between obese and non-obese subjects.

Genetic association with obesity

The GAD2 gene polymorphisms (rs2236418 and rs992990) were genotyped in all the subjects. There was no significant deviation from the Hardy-Weinberg equilibrium among the subjects with respect to both the SNPs ($P<0.05$). The distributions of allele and genotype and haplotype frequencies along with their odds ratios among obese and non-obese subjects are presented in Table 3. Overall -243 A>G polymorphism shows significantly higher minor allele and genotype frequencies in obese cases, when compared with non-obese subjects (odd ratio (OR), 4.01; 95% confidence interval (CI), 1.91- 8.42; $P=<0.001$). The genotype and allele frequencies of SNP +61450 C>A was not different in obese subjects compared with non-obese subjects and showed no association with obesity.

A positive significant association has also been observed in the additive, dominant and recessive model for SNP (rs2236418: OR 1.52; $P=0.016$; OR 1.75; $P=0.001$, OR 3.47; $P=0.001$), respectively, while no significant association has been seen in over dominant genetic model. However, no association has been seen in any genetic model for the SNP rs992990. The haplotype consisting of wild type alleles of two-studied variable (A-C) were represented more often in the non-obese group (61.0%) than in the obese group (49.8%). The frequency of combined haplotypes CG+AG was observed significantly higher in obese than non-obese (OR 1.94, $P=0.001$), Table 4.

Table 2: Demographic characteristic of the study subjects

Variable	Non-obese (*n*=333)	Obese (*n*=309)	*P*-value
Waist-to-hip ratio	0.95 (0.08)	0.96±0.10	0.025
SBP (mm Hg)	120.51 ±12.00	128.40 ±15.20	<0.001
DBP (mm Hg)	81.00±8.00	86.23 ±8.05	0.027
Adiposity			
Weight (kg)	69.00 ±6.00	78.40 ±7.42	0.032
% body fat	27.86±6.12	37.28±6.16	<0.001
FM (kg)	20.60±8.16	30.60±8.33	<0.001
Insulin sensitivity			
Fasting glucose (mg/dl)	109.22±5.93	109.63±8.61	NS
Fasting insulin (µU/ml)	10.27±3.01	14.99±4.73	<0.001
Homa Index	2.83±1.83	4.15±2.86	<0.001
Lipid profile			
T Cholesterol (mg/dl)	161.71±34.70	213.53±25.71	0.003
HDL Cholesterol (mg/dl)	46.30±10.16	42.82±7.13	<0.001
VLDl Cholesterol (mg/dl)	21.42±3.91	26.05±5.77	0.008
Triglyceride (mg/dl)	107.12±12.57	130.27±21.88	0.008
LDL Cholesterol (mg/dl)	99.68±27.07	151.28±22.44	<0.001

Values are expressed in Mean±SD; The bold values represent statistically significant values less than 0.05.

Table 3: Distribution of GAD2 gene among non-obese (*n*=333) and obese subjects (*n*=309)

Genotype	Non-obese *n* (%)	Obese *n* (%)	OR (95% CI)	*P* value
GAD2 (rs2236418;-243 A>G)				
AA	227 (68.2)	170 (55.0)	1	
AG (additive model)	96 (28.8)	109 (35.3)	1.52 (1.08- 2.13)	0 .016
GG (co-dominant model)	10 (3.0)	30 (9.7)	4.01 (1.91- 8.42)	<0 .001
AG+GG (dominant model)	106 (31.8)	139 (45.0)	1.75 (1.27-2.42)	<0.001
GG versus AG+AA (recessive model)	323 (97.0)	299 (90.3)	3.47 (1.67-7.23)	0.001
AA+GG versus AG (over dominant model)	237 (71.2)	200 (64.7)	0.74 (0.53-1.04)	0.080
Allele frequency				
A (multiplicative model)	550 (82.6)	449 (72.7)	0.56 (0.43-0.73)	<0 .001
G (multiplicative model)	116 (17.4)	169 (27.3)	1.79 (1.37- 2.33)	<0 .001
GAD2 (rs992990;+61450 C>A)				
CC	204 (61.3)	190 (61.5)	1	1
CA (additive model)	77 (23.1)	75 (24.3)	1.05 (0.72-1.52)	0.815
AA (co-dominant model)	52 (15.6)	44 (14.2)	0.91 (0.58-1.42)	0.674
CA+AA (dominant model)	129 (38.7)	119 (38.5)	0.990 (0.721-1.361)	0.953
AA versus CA+CC (recessive model)	281 (84.4)	265 (85.8)	0.897 (0.581-1.386)	0.625
CC+AA versus CA (over dominant model)	256 (76.9)	234 (75.7)	1.066 (0.760-1.534)	0.732
Allele frequency				
C (multiplicative model)	485 (72.8)	455 (73.6)	1.042 (0.814-1.334)	0 .726
A (multiplicative model)	181 (27.2)	163 (26.4)	0.960 (0.750-0.1.229)	0 .746

The bold values represent statistically significant values < 0.05.

Table 4: Haplotype frequency of gene variants and its association with risk of obesity

Haplotype	Non-obese *n* (%)	Obese *n* (%)	OR (95% CI)	*P*-value
CA	203 (61.0%)	154 (49.8%)	1	Reference
AA	73 (21.9%)	71 (23.0%)	1.28 (0.87-31.89)	0 .210
CG+AG	57 (17.1%)	84 (27.2%)	1.94 (1.31- 2.89)	**0 .001**

The bold values represent statistically significant values < 0.05; The bold values represent statistically significant values less than 0.05.

Association of different metabolic parameters with homozygous variant genotype

GAD-2 rs2236418 showed association with different obesity associated phenotypes like weight, percentage body fat, FM, fasting blood glucose, and insulin (Table 5). The strongest association was found with weight and insulin. The GAD-2 rs992990 polymorphism also showed association with weight, percentage body fat, FM and insulin (Table 6).

Table 5: Association of GAD2 -243 A>G, (rs2236418) genotypes with obesity related phenotypes

	AA (*n*=397)	AG (*n*=205)	GG (*n*=40)	*P*-value
Waist-to-hip ratio	0.95(0.95-0.96)	0.96 (0.95-0.98)	0.98 (0.96-0.99)	0.855
SBP (mm Hg)	123.36 (122.21-125.15)	125.03 (123.32-127.49)	125.92 (122.69-127.56)	0.159
DBP (mm Hg)	82.83 (82.21-83.88)	83.07 (81.90-84.98)	84.49 (82.90-85.63)	0.09
Adiposity				
Weight (kg)	71.49 (70.55-72.39)	76.32 (75.42-77.65)	79.31 (78.53-80.87)	0.001
% body fat	31.35 (30.87-32.39)	33.28 (32.65-34.99)	33.31 (31.57-34.97)	<0.001
FM (kg)	24.15 (23.44-25.30)	26.56 (24.88-29.11)	27.12 (25.59-28.64)	0.018
Insulin sensitivity				
Fasting sugar (mg/dl)	106.54 (105.72-107.87)	114.10 (112.83-115.34)	114.68 (113.40-115.65)	0.039
Fasting insulin (µU/ml)	22.22 (19.70-24.53)	17.19 (16.80-18.51)	8.19 (7.82-8.66)	<0.001

Data represents mean and its 95% confidence interval from ANOVA;**P* values were derived from multiple linear regression analysis using the best genetic model; The bold values represent statistically significant values less than 0.05.

Table 6: Association of GAD2 +61450 C>A, (rs992990) genotypes with obesity related phenotypes

	CC (*n*=394)	CA (*n*=152)	AA (*n*=96)	*P*-value
WHR	**0.95 (0.95-0.97)**	**0.96(0.95-0.98)**	**0.97(0.94-0.99)**	**0.905**
SBP (mm Hg)	123.63 (122.33-124.93)	125.38 (123.47-127.30)	125.45 (118.58-132.32)	0.216
DBP (mm Hg)	82.87 (82.08-83.67)	83.88 (82.69-85.08)	86.05 (83.33-88.77)	0.004
Adiposity				
Weight (kg)	72.75 (71.33-74.17)	74.39 (69.90-78.87)	75.68 (73.65-77.70)	0.001
% body fat	31.19 (30.49-31.90)	33.85 (32.72-34.97)	36.85 (34.14-39.56)	<0.001
FM (kg)	24.05 (23.19-24.91)	27.27 (25.84-28.70)	29.41 (25.72-33.09)	0.015
Insulin sensitivity				
Fasting sugar (mg/dl)	108.71 (106.43-110.98)	109.58 (107.87-111.28)	111.58 (104.88-118.29)	0.019
Fasting insulin (µU/ml)	12.85 (12.04-13.65)	12.41 (11.19-13.64)	10.21 (7.88-12.54)	<0.001

Data represents mean and its 95% confidence interval from ANOVA;**P* values were derived from multiple linear regression analysis using the best genetic model; The bold values represent statistically significant values less than 0.05.

Discussion

In the present study, we found association of SNPs in *GAD2* (genotype, allele and haplotype) with the predisposition to human obesity and its association with insulin secretion. We observed significant association of *GAD-2* -243 A>G polymorphism at genotype and allele level with risk of obesity while *GAD-2* +61450 C>A polymorphism do not associated with obesity. The variant genotypes of -243 A>G and +61450 C>A polymorphism also associated with weight, % body fat and fat mass in obese and non-obese subjects both (Table 5 & 6). Haplotypes were estimated from -243 A>G and +61450 C>A for each subject and tested for differences between the obese and non-obese subjects by logistic regression. There were two common haplotypes: CA, and AA, with the remaining haplotypes being combined into a single haplotype (CG+AG). The

combined haplotype showed significant association with obesity in the present study (*P* value = 0.001, Table 4).

Table 7 shows the list of various studies on the *GAD-2* polymorphisms related to obesity. The (−243) A>G polymorphism of the *GAD2* gene showed a strong association with obesity (5), while other studies report the contradictory results showing no association with obesity (3,9,10). The contribution of *GAD2* promoter SNP -243 A<G to the genetic risk for childhood obesity and with lower BMI, fasting glucose concentrations were observed (8). In addition, investigations into the functional consequences of the (−243) A→G SNP in the promoter region of the *GAD2* gene have reported contradictory results (5,9).

+61450 C>A polymorphism was not associated with obesity, so we did (9). The *GAD-2* +61450 C>A polymorphism did not show association with obesity in Utah population (10). However, a family-based genetic study, reported significant association of +61450 C>A polymorphism with obesity (5).

GAD2 encodes the 65-kDa isoform of the enzyme glutamate decarboxylase, which enhance catalyses of the production of c-aminobutyric acid (GABA) from glutamic acid that interact with neuropeptide Y in neurons of the hypothalamic arcuate nucleus to stimulate food intake (14). These acute neuropeptide Y neurons also slow down the similarities and opposite effects of neighbouring pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript neurons via GABA-ergic collateral inputs (15). Food intake suppressed after the administration of the antisense GAD-65 and GAD-67 oligonucleotides by inhibiting the GABA synthesis in the ventromedial hypothalamus (16).

Table 7: Association of GAD2 -243 A>G, (rs2236418), +61450 C>A (rs992990) gene polymorphisms with obesity in previous studies

GAD2 -243 A>G polymorphism (rs2236418)	
Association	**Reference**
Association with obesity	(5)
Association with obesity	(7)
No association with obesity	(9)
No association with obesity	(10)
No association with obesity	(8)
Association with resting energy expenditure	(23)
Association with obesity	Present study
GAD2 +61450 C>A polymorphism (rs992990)	
Association with obesity	(5)
No association with obesity	(9)
No association with obesity	(10)
No association with obesity	(24)
Susceptibility to hunger& weight gain	(25)
No association with obesity	Present study

In addition, *GAD2* gene polymorphisms showed significant association with the insulin level in obese and non-obese subjects. GABA pool in the hypothalamus may enhance by an increased activity of the *GAD2* gene and improved orexigenic effect of GABA resulting in changed feeding behaviour. Denbow et al. (17) observed the effect of varying doses of muscimol (GABA agonist) on food intake. *GAD2* SNP-related increase of the GABA pool in pancreatic islets also contributes in food intake (18).

Heritability of obesity with improved body weight and fat deposition showed in transgenic mice with increased expression of GABA trans-

porter (19). The *GAD2* gene is highly expressed in pancreatic b cells. GABA-containing micro vesicles are released by Ca2þ-dependent exocytosis and may have an autocrine/paracrine function within the islet (20). GABA released from the b cell inhibits insulin exocytosis by activation of GABA-B receptors (G protein-coupled receptors) (21).

The significant association between *GAD2* promoter SNP (SNP+83897 T>A) and lower level of insulin in non-obese subjects were observed while no association was reported in obese subjects (5). The association of variant allele of -243 A<G SNP with 6-fold increased *GAD2* gene promoter activity (5). Increased GABA level in the central nervous system encouraged enhanced food intake and obesity (17, 19). In the present study we found the association between variant genotype of −243A>G and +61450 C>A and lower fasting insulin levels and HOMA-IR indexes which suggest the deleterious effect of *GAD2* SNPs on insulin secretion. Insulin secretion is strongly inhibited by GABA, released from b cells. An increase of the GABA pool in pancreatic b cells mediated by GAD65 higher enzymatic activity may be responsible for above explained association (22).

-243 A>G SNP and +61450 C>A SNP of the *GAD2*gene in linkage disequilibrium, variant allele, genotype of -243 A>G SNP and combined haplotypes of -243 A>G SNP and +61450 C>A SNP (CG+AG) showed the strongest association with obesity. Functional studies were performed to analyse its effects on transcription and nuclear protein binding. The (−243A>G) SNP increased transcriptional activity and promoter activity by which GABA production stimulated, may possibly have effect on feeding and insulin (5). Lack of data, in particular, lifestyle factors such as education, occupation, per capita income, population social background, and daily activity patterns, in the present analysis are an important limitation of this study. An additional limitation of the study is multiple comparisons; however, the majority of literature to date would indicate that the *GAD-2* gene variant is associated with measures of insulin secretion. Other influencing factors small sample size of the study which may reduce the capability to identify certain associations. Lastly, as our study nature is cross-sectional, so we are limited to identifying associations only and longitudinal studies required to identify and understand more clearly the role of *GAD-2* gene. However, this study also has a number of significant advantages: the present sample was collected from an ethnically and socioeconomically homogeneous population, with phenotype measurements not modified by medication. The study's participants were evaluated simultaneously for all the studied variables.

Conclusion

We reported that -243 A>G SNP, and the combined haplotypes of *GAD-2* gene significantly associated with obesity in north Indian population, while +61450 C>A SNP did not show association. However, an association between variant genotype of -243 A>G, +61450 C>A SNP with body weight and insulin level were observed. It is evident that GAD2 variants have a detectable influence on obesity and obesity related phenotype like insulin secretion in a diverse range of populations. Future studies of the association of these SNPs with obesity and obesity related abnormalities may more rigorously prove the suggested statistical association.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgments

The authors acknowledge the Indian Council of Medical Research, New Delhi, for the financial support to carry out this research work (Project number - 45/14/2008-HUM-BMS). No compet-

ing financial interests exist. The authors declare that there is no conflict of interests.

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List of abbreviations (in alphabetical order)

#	Abbreviation	Explanation	Units
1	ANOVA	Analysis of covariance	
2	BMI	Body mass index	kg/m²
3	DBP	Diastolic blood pressure	mm Hg
4	FM	Fat mass	kg/m²
5	GABA	γ- aminobutyric acid	
6	HDL-cholesterol	High density lipoprotein – cholesterol	mg/dl
7	HOMA	Homeostasis model assessment	
8	HOMA-IR Index	Homeostasis model assessment of insulin resistance index	
9	LDL cholesterol	Low density lipoprotein – cholesterol	mg/dl
10	PCR-RFLP	Polymerase chain reaction restriction fragment length polymorphism	
11	SBP	Systolic blood pressure	mm Hg
12	T cholesterol	Total – cholesterol	mg/dl
13	VLDL cholesterol	Very low density lipoprotein – cholesterol	mg/dl
14	WHR	Waist to hip ratio	
		Human Gene	
15	*GAD2*	Glutamate decarboxylase 2	