ASSESSMENT OAF -243A>G POLYMORPHISM OF GLUTAMATE DECARBOXYLASE 2 GENE IN OBESE PATIENTS

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ABSTRACT

Background: *Obesity is one of the most common non-communicable diseases and public health issues in the world at every stage of development. The World Health Organization (WHO) estimates that 13% of adults worldwide are obese.*

Aim of the Work: *To investigate the relationship between -243A>G polymorphism of GAD2 gene and the presence of obesity.*

Subjects and Methods: *The present case-control study included thirty (30) obese patients with BMI ≥30 kg/m². In addition, twenty (20) age- and sex- matched healthy subjects served as a healthy control group with BMI <25 kg/m². All subjects were genotyped for the GAD2 gene SNP -243 A>G (rs2236418) using PCR-RFLP technique. The study was approved by the Research Ethics Committee of Ain Shams University.*

Results: *The present study revealed that the (AA) genotype and the (A) allele frequencies were significantly higher in healthy controls when compared to obese patients. Meanwhile, the frequencies of the (AG) and (GG) genotypes as well as the (G) allele were significantly higher among obese patients as compared to healthy controls.*

Conclusion: *The present study demonstrated the presence of a significant association between the -243A>G polymorphism and obesity.*

Keywords: *Obesity- Food intake- -243A>G Polymorphism- GAD2*

INTRODUCTION:

Obesity is associated with an increased risk of comorbid diseases such as cardiovascular diseases, diabetes, hyper-tension, musculoskeletal disorders especially osteoarthritis and some cancers as breast, endometrial, ovarian, prostate, liver and colon. Moreover; a higher mortality rate was recorded among obese adults as a con-sequence of the associated co-morbidities[(1)].

Genome-wide scans for obesity-associated genes have repetitively detected a linkage with a locus on chromosome 10p11.22-23. The previous studies showed that glutamic acid decarboxylase 2 *(GAD2)* gene is one of the genes located in this locus, where it encodes for the GAD2 (GAD65) enzyme[(2)].

The enzyme GAD is involved in the hypothalamic regulation of food intake by controlling the formation of the γ-aminobutyric acid (GABA) from the glutamic acid, where the former functions together with the neuropeptide Y in the paraventricular nucleus to stimulate food intake[(3)].

Some researchers hypothesize that single nucleotide polymorphisms (SNPs) in

GAD2 gene may enhance the over expression of the GAD2 resulting in overeating and obesity. One of the suggested SNPs in *GAD2* gene is the -243A>G polymorphism (rs2236418) in which the adenine (A) is substituted by guanine (G) at the 243 position in the 5-prime promoter region of the *GAD2* gene. Some recent studies started to explore the association between this polymorphism and the enhanced transcriptional activity of *GAD2* gene[(2)]**.**

AIM OF THE WORK:

The aim of the present study is to investigate the relationship between -243A>G polymorphism of *GAD2* gene and the presence of obesity.

SUBJECTS AND METHODS:

I) Subjects:

The present case-control study was carried out at the Clinical Pathology Department of Ain Shams University Hospitals. The study included thirty (30) obese patients recruited from the Bariatric Surgery Department in Al- Demerdash Hospital between May 2017 and May 2018. In addition, twenty (20) age- and sex-matched healthy subjects served as a healthy control group. An informed oral consent was obtained from each participant before enrolment in the study. Moreover, the study was approved by the Research Ethics Committee of Ain Shams University.

A) <u>Obese Patients' Group (Group I; n=30)</u>:

This group included thirty (30) obese patients with BMI ≥30 kg/m²[(4)]**.** They were eight (8) males and twenty-two (22) females, ranging in age between 25 and 45 years (mean age = 35 ±5 years).

Exclusion criteria:

1. Patients with psychiatric disorders as unipolar depression, obsessive compulsive or anxiety disorders.
2. Patients suffering from neurological disorders such as Parkinson disease, ataxia, progressive encephalomyelitis and epilepsy.
3. Patients suffering from type 1 diabetes mellitus.
4. Patients with endocrinal disorders such as hypothyroidism and Cushing disease.

B) <u>Healthy Control Group (Group II; n=20)</u>:

This group included twenty (20) age- and sex-matched apparently healthy adults (BMI <25 kg/m²) serving as a control group. There were six (6) males and fourteen (14) females, ranging in age between 20 and 47 years (mean age= 33.5 ± 6.75 years).

All individuals included in this study were subjected to the following:

1. Full medical and family history taking.
2. Thorough general examination with measurement of body weight and height for the calculation of BMI and measurement of waist circumference.
3. Detection of -243A>G polymorphism of *GAD2* gene in peripheral blood by PCR-RFLP technique.

II) Sampling:

Two milliliters (2mL) of blood were collected in a sterile K3 EDTA vacutainer and were stored at -20˚C to be used for the detection of -243A>G polymorphism of *GAD2* gene. Repeated freezing and thawing were avoided.

III) Methods:

A) Analytical Methods: 243A>G polymerphism of *GAD2* gene detection

The process was performed through several steps including DNA extraction,

DNA amplification by PCR technique, and DNA digestion by a restriction endonuclease enzyme (DraI). Visualization of the bands was done after agarose gel electrophoresis.

1) Genomic DNA extraction:

The -243A>G *GAD2* gene polymorphism DNA extraction was performed using whole blood genomic DNA Purification Mini Kit supplied by. Thermo Scientific Gene JET and kept at -20˚C until analysis.

2) PCR amplification

Amplification of the extracted DNA was done by polymerase chain reaction using 3Prime thermal cycler. (Thermoscientific, 168 Third Avenue, Waltham, MA, USA.)

The test primers were prepared by Applied Biosystems. (Applied Biosystems, Headquarters, Waltham, Massachusetts, USA.) The forward primer was 5'-CCTCATTCATCCCCACTG-3' and the reverse primer was 5'-CACGCAGGAACAGAAAACG -3'. The reaction mixture consisted of 10 uL DNA extract, 1 uL forward primer, 1 uL reverse primer, 0.5 uL nuclease-free water and 12.5 uL hot start master mix (ready to use) formed of hot start Thermus aquaticus (Taq) DNA polymerase, optimized hot start PCR buffer, Mg^2, and deoxy-ribonucleotide triphosphates (dNTPs). The master mix was supplied by Thermo Scientific. The PCR conditions were as follows: an initial 4 minutes' activation step at 94^{o}C, followed by 35 cycles of denaturation at 95^{o}C for 35 seconds, annealing at 60^{o}C for 30 seconds and extension at 72^{o}C for 60 seconds, with a final extension at 72^{o}C for 10 minutes.

3) Restriction fragment length polymerphism (RFLP):

The analysis by PCR-RFLP is associated with the creation or abolishment of a restriction enzyme recognition site **(5).** In the present study, the PCR product was digested with the restriction enzyme specific for the recognition site (DraI) supplied by Fast Digest, Thermo Scientific. The components were mixed gently, spun down and incubated at 37° C for 20 minutes. Finally, the reaction mixture and the DNA ladder (100 bp) were loaded on 2% agarose gel and the DNA fragments were separated by an electrophoretic system supplied by Cleaver Scientific Limited (100 volts for 30 minutes)**.**

Interpretation of the electrophoretic separation:

The (A) allele of *GAD2* gene has one restriction site and produces two fragments while the G allele has no restriction site and hence produces a single undigested band. According to the band length of restriction fragment (Figure 1), genotyping is as follows:

- Homozygous wild (AA) genotype: 2 bands at 98 and 105 bp.
- Heterozygous (AG) genotype: 3 bands at 98, 105 and 203 bp.
- Homozygous mutant (GG) genotype: a single undigested fragment at 203 bp.

Figure (1): 2% agarose gel electrophoresis of *GAD2* gene -243 A>G polymorphism genotypes.
Lane 1: Molecular weight marker (100 bp ladder). Lane 2: Homozygous mutant (GG) (203 bp).
Lane 3 and 4: Homozygous wild (AA) (98 and 105 bp).
Lane 5: Heterozygous (AG) (98, 105 and 203 bp).

B) Statistical Methods:

- **Sample size determination:** the required sample size was calculated at the Community Department of Faculty of Medicine, Ain shams University using the G* power software version 3.1 (Universität Düsseldorf, Germany). Based on a previous study **(2),** it was estimated that a sample size of thirty (30) patients and twenty (20) controls would achieve a power of 80% (i.e: type II error, 0.20) to detect a statistically significant difference among the 2 groups with a confidence of 95% (i.e: type I error, 0.05).
- The collected data were revised, coded, tabulated and introduced to Statistical Package for the Social Sciences soft-ware programme (SPSS, version 25.0, IBM Corp., USA, 2017-2018). Data were presented and suitable statistical analysis was done according to the type of data obtained for each parameter. Qualitative data were expressed as number and percent (n; %); parametric quantitative data were expressed as mean and standard deviation (SD). Comparative statistics for qualitative data was done by the Chi squared test (X^2) between two independent groups and the ANOVA test between three independent groups for qualitative data. As for quantitative parametric data, and Student's t test was performed.

RESULTS

The results of the present study are illustrated in Tables (1) and (2).

The descriptive and comparative statistics of demographic and clinical data of all participants in the study are included in Table (1). Controls and patients were age matched (p >0.05). Moreover, both groups were sex matched with the female sex forming the majority of both groups (70% in the control group versus 73.3% in the patients' group; p > 0.05). A highly significant difference was found between both groups as regards the BMI and the waist circumference being both higher in obese patients as compared to healthy controls (p <0.01 respectively).

The *GAD2* gene SNP -243 A>G (rs2236418) was genotyped in all subjects.

Testing the deviation from Hardy-Weinberg equilibrium showed no significant deviation from Hardy-Weinberg Equilibrium among the subjects. The descriptive and comparative statistics of the genotype and allele frequencies of the studied polymorphism among healthy controls and obese patients are presented in Table (2)**.** The (AA) genotype and the (A) allele showed significantly higher frequencies in healthy controls as compared to obese patients. On the other hand, the genotypes (AG) and (GG), as well as the (G) allele, showed significantly higher frequencies in obese patients as compared to healthy controls (*p* <0.01 respectively).

Table (1): Descriptive and Comparative Statistics of Demographic and Clinical Data of All Participants Using Student's (t) Test for Continuous Parametric Data and Chi-Square Test for Categorical data

Parameters	Controls (n=20)	Patients (n=30)	*p*-value
	n (%) / $\bar{X}$±SD*		
Age (years)	33.5 ± 6.8*	35 ±5.0*	> 0.05
Male gender Female gender	6.0 (30.0%) 14.0 (70.0%)	8.0 (26.7%) 22.0 (73.3%)	> 0.05
Adiposity			
Body mass index (kg/m²)	23.3 ± 1.3*	40.2 ± 3.5*	< 0.01
Waist circumference (cm) Males Females	 94.4 ± 4.5* 77.9 ± 7.1*	 138.2 ±10.7* 109.4 ±20.1*	< 0.01

BMI: body mass index. Values are expressed as mean ±SD for continuous data; and as number and percent (%) for categorical data. *p* >0.05: non-significant; *p* <0.05: significant, *p* <0.01: highly significant (the bold values).

Table (2): Descriptive and Comparative Statistics of Genotype and Allele Frequencies of *GAD2* Gene -243 A>G Polymorphism in Patients and Controls Using Chi-Square Test

Parameters	Controls n (%)	Patients n (%)	X2	*p*-value
Genotype				
AA	14.0 (70.0%)	2.0 (6.7%)		
AG	4.0 (20.0%)	8.0 (26.7%)	8.8	<0.01
GG	2.0 (10.0%)	20.0 (66.7%)	23.4	<0.01
Allele frequency				
A	32.0 (80.0%)	12.0 (20.0%)		
G	8.0 (20.0%)	48.0 (80.0%)	35.1	<0.01

GAD2: glutamic acid decarboxylase 2; A: adenine; G: guanine. *p* <0.01: highly significant (the bold values).

DISCUSSION:

Obesity is one of the most common non-communicable diseases and a public health issue affecting more than 13% of adults all over the world[4]**.** The disease is highly prevalent in Egypt with an estimated prevalence of approximately 61–70% of the adult population with a higher percentage in females more than males. Obesity is also considered as one of the top contributors to mortality among Egyptians due to the associated comorbid diseases as hypertension and diabetes[6].

Obesity is a chronic multifunctional disorder resulting from disruption of the balance between energy intake and energy expenditure[1] **.** The disease has a complex etiology involving behavioral, hormonal and genetic factors[7]**.** Tremendous progress has been made over the past 2

decades to elucidate the genetic impact on obesity[8]**.** Genome-wide scans for obesity-associated genes have repetitively detected a linkage with a locus on chromosome 10p11.22-23. Previous studies have identified *GAD2* gene as one of the genes located in this locus, where it encodes for the 65-kDa isoform of the enzyme GAD2 (GAD65)[2]**.**

The GAD2 enzyme is abundantly expressed in the hypothalamus. It is the rate limiting enzyme responsible for the production of the neurotransmitter GABA from glutamic acid, which in turn interacts with orexigenic neuropeptides (NPY and AgRP) in the ARC nucleus to stimulate food intake either directly or through the leptin pathway[3& 9]**.** Moreover, GABA can decrease the action of the POMC neurons which in turn decreases the energy expenditure and increases the body weight[10]. In addition to the central effects of GAD2, a peripheral contribution was also suggested where the GAD2 enzyme was found to be highly expressed in the pancreatic beta-cells. Accordingly, the released GABA from the beta-cells inhibits insulin exocytosis by activation of $GABA_B$Rs (G protein-coupled receptors) and suppresses glucagon release from the alpha-cells by activation of the $GABA_A$Rs chloride channels[11]**.**

Some researchers hypothesize that the presence of SNPs in the *GAD2* gene may enhance the over-expression of the *GAD2* with subsequent increase in the GABA formation, resulting in overeating and obesity. One of the suggested SNPs in *GAD2* gene is the -243A>G polymorphism (rs2236418). In this SNP, the adenine (A) nucleotide is substituted by guanine (G) nucleotide at the 243rd position in the non-coding 5'prime promoter region of the *GAD2* gene[2]**.** Accordingly, the present study aimed to study the relationship between -243A>G polymorphism of *GAD2* gene and the presence of obesity.

The population included in the present study was genotyped for the *GAD2* gene SNP -243 A>G (rs2236418). The present study demonstrated the presence of the homozygous (AA), homozygous (GG) and the heterozygous (AG) in all participants and he application of Hardy-Weinberg law proved the genetic equilibrium of the studied population regarding the distribution of the studied genotypes.

Data of the present study revealed that the frequencies of (AA) genotype and the (A) allele were significantly higher in healthy controls when compared to obese patients (AA: 70% vs 6.7%; A allele: 80% vs 20%). On the other hand, the frequencies of the genotypes (AG) and (GG), as well as the (G) allele, were significantly higher among obese patients as compared to healthy controls (AG: 26% vs. 20%; GG: 66.7% vs 10%; G allele: 80% vs 20%). These results were in accordance with that reported by Boutin et al.[11] on the French population, and Prakash et al.[2] on the North-Indian population, where the former noticed that the presence of the (G) allele increases the *GAD2* promoter activity six times and induces a 6-fold higher affinity for transcription factors; this in turn increases the GABA pool in the hypothalamus. Moreover, both authors found that the homozygous (GG) obese patients were characterized by overeating features with a higher sensitivity to the sensorial stimulation of food that may lead to a decreased ability to control food intake**.** These findings support the hypothesis that the wild-type (A) allele is protective against obesity, while the variant (G) allele increases the risk for the disease.

On the other hand, the results reported by the present study is in contrast to that reported by Swarbrick et al**.**[12] on the US and the Canadian populations, and Hunt et al.[13] on the Utah population. Both studies revealed that the distribution of genotypes

and alleles of the *GAD2* gene did not vary significantly between healthy controls and obese patients. Moreover, the study conducted by Boesgaard et al.[14] on the Danish population revealed that the (G) allele is not associated with higher BMI and that the (G) allele frequency decreased with increasing levels of BMI. The afore-mentioned discrepancies between the different studies could be related to several factors. One of these factors is the complexity of the disease. Obesity is a multifactorial disease, and the role of gene polymorphism in the pathogenesis of the disease is largely dependent on other factors that influence the impact of any altered gene phenotype and determines the disease severity[7& 15]**.** Population stratification may also account for some of the inconsistencies observed between the different studies. Considering the marked differences in allele frequency that we observed between the different ethnic groups for the *GAD2* −243 A>G polymorphism, as well as the known differences in the prevalence of obesity between the different populations, it is plausible that a small difference in ancestry between cases and controls could lead to spurious claims of association[12]**.**

Limitations of the Present Study:

The present study has few limitations: first, the lack of data such as lifestyle factors including education, social background and daily activity patterns. Secondly, the small sample size of the study which reduced the capability to identify certain associations.

Conclusion:

The present study demonstrated the presence of a significant association between the -243A>G polymorphism and obesity.

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