

## Ghrelin Arg51Gln Polymorphism in Egyptian Patients with Type II Diabetes Mellitus

Amina R. El-Gezeery\*, Ebtesam M. Abdalla\*, Mohamed M. Mokhtar\*, Gihan Khalil\*\*

**ABSTRACT: BACKGROUND/OBJECTIVE:** Ghrelin is a peptide hormone known to play a role in glucose homeostasis; therefore, functional variants of the human ghrelin gene could contribute to the genetic susceptibility to diabetes or may modulate some aspects of the glucose intolerance phenotype. The study aimed at investigating the differences in the frequencies of Arg51Gln polymorphisms among Egyptian patients with type II diabetes and healthy control subjects and at verifying whether this polymorphism could influence the diabetes phenotype. **METHODS:** One-hundred-four Egyptian type II diabetic patients attending the Medical Research Institute were enrolled into the study. Clinical data concerning medical and family history were collected by a clinical interview. Another group of 100 non-diabetic apparently healthy subjects were included to compare the Arg51Gln genotypes frequencies. The ghrelin Arg51Gln polymorphism was studied by PCR restriction fragment length polymorphism method in the diabetic and control subjects. The metabolic profile of the diabetic patients was also analyzed. A  $\chi^2$  test was adopted to compare the ghrelin Arg51Gln genotype and allele frequencies among the two groups. Moreover, in order to test whether the differences in phenotypic variables between the patient groups were influenced by ghrelin genotype, ANOVA test was performed. **RESULTS:** The frequency of the 51gln heterozygotes and homozygotes were significantly higher in the patients' group than in the control sample ( $X^2 = 8.962$ ,  $p = 0.0113$ ). The 51gln allele frequency was higher in the patients than in the control group ( $q = 0.27$  and  $q = 0.14$ , respectively); a difference that was found statistically significant ( $X^2 = 5.185$ ,  $p = 0.022$ ). The fasting blood sugar and triglycerides levels were higher in patients carrying the ghrelin 51Gln allele than in those with the wild allele (statistically significant,  $p = 0.014$  and  $p = 0.004$ , respectively). No statistically significant difference was observed between the total cholesterol, HDL and LDL cholesterol concentrations among these two groups. **CONCLUSIONS:** There is a significant positive association between ghrelin 51Gln polymorphism and type II diabetes in the Egyptian population. Further studies are warranted to elucidate the role of ghrelin in the development of this disease.

### INTRODUCTION

It is well established that type II diabetes mellitus has a substantial genetic component.<sup>[1]</sup> Some type of diabetes which have been identified, include several loci for type I diabetes<sup>[2]</sup> and for maturity-onset diabetes

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\* Human Genetics Dept., Medical Research Institute, University of Alexandria, Egypt

\*\* Chemical Pathology Dept., Medical Research Institute, University of Alexandria, Egypt

of the young.<sup>[3,4]</sup> However, the genes that cause the most common forms of diabetes remain unknown, and it is therefore likely that additional important diabetes-susceptibility loci remain to be identified. Moreover, the specific risk factors through which such genes influence the development of type II diabetes are also unknown.<sup>[5]</sup>

Ghrelin is a peptide hormone predominantly produced by the stomach.<sup>[6]</sup> It has been suggested that ghrelin plays a role in glucose homeostasis as several studies have shown that ghrelin administration induces hyperglycemia and reduces serum insulin levels. Based on these recent studies, it appears that changes in the activity or concentration of the ghrelin hormone might constitute a risk factor for impaired glycemic control.<sup>[7-9]</sup>

The human ghrelin gene is located on the chromosomal locus 3p25-p26, encoding a 117 amino acid peptide termed preproghrelin. However, post-translational

modification of human preproghrelin produces a 28 amino acid peptide; the mature ghrelin. The amino acid Arg51 in the preproghrelin, which corresponds to amino acid residue 28, the last amino acid in mature ghrelin product, is the target site for proteolytic cleavage of the carboxy-terminal amino acids to produce mature ghrelin.<sup>[10]</sup> Different polymorphisms of this gene have been described<sup>[11-13]</sup>, including the single base substitutions at Arg51Gln, with glycine (Gln) replacing arginine (Arg).<sup>[14]</sup> As Arg51Gln mutation disrupts the recognition site in the last codon of the mature ghrelin hormone and it is associated with lower ghrelin concentrations,<sup>[15]</sup> it has been introduced as a candidate polymorphism for type II diabetes.

The aim of the present study was to evaluate the potential role of ghrelin Arg51Gln polymorphism as a risk factor for type II diabetes in the Egyptian population and to characterize a number of metabolic

profiles in the diabetic patients carrying different ghrelin Arg51Gln alleles.

## **MATERIAL AND METHODS**

The study included 104 patients with type II diabetes mellitus referred from the Chemical Pathology Lab. (Medical Research Institute). Type II diabetes was determined according to World Health Organization (WHO) criteria.<sup>[16]</sup> Another 100 age-matched Egyptian non-diabetic subjects were included as control group. The control population was used for comparing the ghrelin Arg51Gln genotypes distribution with that of the diabetic patients.

All the studied patients were subjected to the following:

1. Detailed history: including the age of onset of diabetes and past medical history of hypertension, obesity, and coronary heart disease (CHD). Thorough genetic family history was also obtained stressing on history of type II diabetes, obesity, and

cardiovascular diseases.

2. Laboratory investigation including the measurement of fasting blood glucose (FBG), triglycerides (Tg), total cholesterol (Ch), high density lipoprotein (HDL), and low-density lipoprotein (LDL) levels. The analysis was performed after 12 hours fasting using Konelab 30i system autoanalyzer.

3. Molecular study (patients & controls): The ghrelin Arg51Gln polymorphism was detected by the polymerase chain reactions restriction fragment length polymorphism method (PCR-RFLP). The DNA fragment covering exons 1 and 2, which encompass the entire ghrelin product, was amplified using the PCR technique, then the Arg51Gln mutation was identified using the restriction endonuclease *SacI*, which retains the mutated site (guanine replaced by adenine) at base 346 of the ghrelin gene undigested.

- Genomic DNA was extracted from peripheral venous blood using the salting-out method.<sup>[17]</sup>
  - PCR was performed in a final volume of 25 µl containing ~150 ng of genomic DNA, 2 mM MgCl<sub>2</sub>, 0.2 mM of each of the dNTPs, 1X reaction buffer [75 mM Tris HCl (pH 8.8), 20 mM (NH<sub>4</sub>) SO<sub>4</sub> and 0.01% Tween 20], 0.3 µM of each primer and 0.75 µTaq polymerase (MBI Fermentas). The primers used were those described by Ukkola *et al.*,<sup>[15]</sup>; forward primer: 5'-GCTGGGCTCCTACCTGAGC-3' and reverse primer: 5'-GGACCCTGTTCCTACTGCCAC-3'. PCR was performed in a 'ThermoHybaid PCR Express Thermocycler as follows: initial denaturation for 5 minutes, followed by thirty cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, elongation at 72°C for 30 seconds and a final elongation step at 72°C for 5. A fragment of 618-bp was obtained after electrophoresis of the 5 µl of the PCR product on 2% agarose gel stained with ethidium bromide.
  - Restriction enzyme digestion was carried out in 25 reaction volume containing 5 µl of the PCR product, -- µl 10X Fermentas buffer [10 mM Tris HCl (pH 8.5), 10 mM MgCl<sub>2</sub>, 100 mM KCl and 0.1 mg BSA] and 5 units of the enzyme *SacI*. The incubation was done according to the manufacturer's directions.
  - The fragments were separated on a 2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. The wild allele shows fragments of 455-bp and 163-bp, while the 51Gln allele shows a fragment of 618 bp (undigested).
4. Statistical analysis: Allele frequencies were estimated by direct gene counting. Chi square goodness-of-fit test was used to assess the Hardy-

Weinberg equilibrium in the studied groups. Statistical significance for the differences in the genotype and allele frequencies between the diabetic cases and the controls were determined by the  $\chi^2$ -test (significance set at 0.05). ANOVA test was used to compare the means of the measured variables. Difference in phenotypes between patients with different genotypes was expressed as odds ratios (Ors) with 95% confidence intervals (95% Cis).

## RESULTS

The subjects studied were 104 Egyptian type II diabetic patients. The mean age of the patients was  $44.67 \pm 5.32$  years, while the mean age of onset of diabetes was  $46.53 \pm 8.96$ . Also, 100 healthy control subjects were included to compare the Arg51Gln genotypes frequencies.

The genotypes distribution and allele frequencies of ghrelin Arg51Gln polymorphism in type II diabetic patients

and in the control subjects are shown in Table 1. The genotype frequencies among patients and control groups were both consistent with the expected genotype distribution for a population in Hardy-Weinberg equilibrium. The frequency of the 51gln heterozygotes and homozygotes were significantly higher in the patients group than in the control sample ( $\chi^2 = 8.962$ ,  $P = 0.0113$ ). Also, the 51gln allele frequency was higher in the patients than in the control group ( $q=0.27$  and  $q=0.14$ , respectively); a difference that was found statistically significant ( $\chi^2 = 5.185$ ,  $P = 0.022$ ).

The clinical characteristics and family history data were distributed according the ghrelin Arg51Gln genotypes, (Table 2) There was no significant association between the Arg51Gln polymorphism and the frequency of obesity, hypertension, cardiovascular disease, or the family history of type II diabetes. The onset of diabetes occurred at an earlier age in the

diabetic patients who had the 51Gln allele than in those who did not. (47.17 years and 44.83, respectively) However, this difference was not statistically significant.

The main laboratory findings of the patients with type II diabetes mellitus classified according to their ghrelin Arg51Gln genotypes are shown in table 3. The fasting blood sugar and triglycerides levels were higher in patients carrying the ghrelin 51Gln allele than in those with the wild allele (statistically significant,  $P = 0.014$  and  $p = 0.004$ , respectively). No statistically significant difference was observed between the total cholesterol, HDL, and LDL cholesterol concentrations among these two groups.

#### **DISCUSSION:**

The present study shows that the Gln51 allele frequency is significantly higher in diabetic type II patients than in controls, indicating that this polymorphism increases the risk for type II diabetes. This is in accordance with Poykko *et al.*,<sup>[14]</sup> and

Krzyzanowska-Swiniarska, *et al.*<sup>[18]</sup> while it was inconsistent with others.<sup>[19,20]</sup> Larsen *et al.*,<sup>[20]</sup> reported that although the allele frequency was slightly higher in diabetic patients than in controls, they can not rule out that the allele might be a risk factor for the development of type II diabetes.

The amino acid Arg51 of the ghrelin is a target site for endoprotease action which leads to proteolytic cleavage of the COOH-terminal amino acids to produce mature ghrelin. The Arg51Gln mutation disrupts the recognition site in the last codon of the mature ghrelin product.<sup>[15]</sup> Previous studies have shown that subjects with the Gln51 allele have low ghrelin concentrations compared to the Arg51 allele subjects.<sup>[14,21]</sup> Other studies have reported a clear inverse association between plasma ghrelin and fasting insulin levels. In particular, low ghrelin levels were independently associated with higher levels of fasting plasma insulin, which is an indicator of underlying insulin resistance

and a characteristic of type II diabetes.<sup>[7,9,11,22]</sup>

It is difficult to explain the mechanism behind the association between low ghrelin concentrations and the increased prevalence of type II diabetes. However, the ghrelin deficiency may be itself and/or the decreased somatotrophic effects associated with ghrelin deficiency decreased insulin insensitivity and eventually lead to type II diabetes.

Ghrelin has been implicated in body weight and energy balance regulation.<sup>[6]</sup> Therefore, the ghrelin gene was considered as a candidate gene for obesity.<sup>[23-25]</sup> Several studies have identified various polymorphisms in the ghrelin gene and found that they are associated with obesity and obesity related phenotypes.<sup>[15,21,26]</sup> Although the ghrelin Arg51Gln polymorphism was among the variants that were reported to increase the risk of obesity,<sup>[13,15]</sup> in the present study, no association between the ghrelin Gln51

allele and obesity could be detected. This finding is in agreement with previous studies.<sup>[11,12,20]</sup> The inconsistency of findings, however, could be attributed to the differences between populations regarding the environmental factors that influence the phenotypic expression of the gene variants.

Recently, ghrelin has been shown to exert beneficial hemodynamic effects in humans by reducing cardiac afterload and increasing cardiac output.<sup>[27]</sup> It also has a vasodilator effect as intravenous injection of ghrelin into human volunteers induced a decrease in blood pressure.<sup>[28]</sup> Low plasma ghrelin levels were found inversely correlated with systolic and diastolic blood pressure and associated with hypertension.<sup>[22]</sup> Several genetic studies have investigated the role of ghrelin Arg51Gln polymorphism in hypertension and showed inconsistent results. Whereas some studies have demonstrated that Gln51 allele increases the risk of

hypertension,<sup>[14,18,29]</sup> other studies have reported negative associations.<sup>[19,30]</sup> In the present work, no association was found between hypertension and the ghrelin gln51 allele.

To date, few studies have been performed to investigate the relationship between the ghrelin Arg51Gln polymorphism and the biochemical phenotypes. In the present study, the subjects carrying the ghrelin Gln51 allele had significantly higher levels of FBS and TG than those homozygous for the wild allele (Arg51). They also had lower, although statistically not significant, HDL levels. This finding, which is consistent with Poykko *et al.*,<sup>[14]</sup> indicate that subjects with the ghrelin Gln51 allele might manifest a subset of phenotypes tending towards the metabolic syndrome (high FBS, high

TG, and low HDL). Other studies, however, have reported a negative association.<sup>[19,20]</sup> Therefore, the interaction of ghrelin polymorphism and metabolic processes remain controversial and more detailed phenotypic characterization as well as functional studies of these variants will be necessary to define the role of ghrelin polymorphisms in metabolic syndrome and to delineate the molecular and physiological mechanisms of their effects .

In conclusion, a positive association was found between the Arg51Gln polymorphisms of the ghrelin gene and type II diabetes in the Egyptian population. Further studies are warranted to elucidate the role of ghrelin in the development of this disease.

**Table 1: Distribution of genotypes and allele frequencies (percentages in parenthesis) of ghrelin Arg51Gln in patients with type II diabetes and control subjects**

	Frequencies (%)	
	Patients	Control
<u>Genotype<sup>a</sup></u>		
Arg51Arg	59 (56.7%)	76 (76%)
Arg51Gln	33 (31.7%)	20 (20%)
Gln51Gln	12 (11.5%)	4 (4%)
<u>Allele<sup>b</sup></u>		
51Arg	151 (72.6%)	172 (86%)
51Gln	57 (27.4%)	28 (14%)

<sup>a</sup> Genotype frequencies,  $\chi^2=8.962$ ,  $P = 0.0113$  (Statistically significant;  $P < 0.05$ )

<sup>b</sup> Allele frequencies,  $\chi^2=5.185$ ,  $P = 0.022$  (Statistically significant;  $P < 0.05$ )

**Table 2: Clinical characteristics of the patients with type II diabetes mellitus according to ghrelin Arg51Gln genotype**

	Ghrelin Arg51Gln genotype				OR	95% CI	P
	Arg51Arg (N=59)		Arg51Gln & Gln51Gln (N=45)				
	No.	%	No.	%			
• Prevalence of obesity	17	28.8	8	17.8	0.534	0.207-1.380	NS
• Prevalence of hypertension	31	52.5	19	42.2	0.660	0.302-1.442	NS
• Prevalence of CHD	16	27.1	17	37.8	1.632	0.710-3.750	NS
• Family history of diabetes	39	66.1	28	63.6	0.845	0.376-1.896	NS

P = probability for the difference between the genotypes in ANOVA. Gln51Gln subjects were pooled to the heterozygotes in the analysis.

**Table 3: Main biochemical characteristics of the patients with type II diabetes mellitus by ghrelin Arg51Gln genotype**

	Ghrelin Arg51Gln genotype		P
	Arg51Arg (N=59)	Arg51Gln & Gln51Gln (N=45)	
Fasting blood sugar (mg/dl) *	180.75 ± 73.43	217.29 ± 81.56	0.014 *
Triglycerides (mg/dl) <sup>9</sup>	125.80 ± 48.73	168.71 ± 74.33	0.004 *
Total cholesterol (mg/dl)	204.17 ± 56.18	197.76 ± 50.99	0.581
HDL cholesterol (mg/dl)	36.12 ± 12.81	33.07 ± 10.84	0.444
LDL cholesterol (mg/dl)	144.27 ± 52.01	132.02 ± 42.14	10.255

Data are means ± SD. p = probability for the difference between the genotypes. Gln51Gln subjects were pooled to the heterozygotes in the analysis.

\* Statistically significant; p<0.05

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