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Prognostic Value of Leptin Gene Polymorphisms in Type 2 Diabetes Mellitus Patients in Egypt

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The work wasto study polymorphisms in the LEP gene in type 2 diabetics in Minia, Egypt and determined the relationship between the leptin and c-peptide levels in different genotypes and insulin resistance in obese patients. The study also has evaluated the role of leptin gene polymorphism in prediction of diabetes mellitus prognosis and its prevention.
Study Design: Investigative.
Place and Duration of Study: Samples were analyzed at Biochemistry Department, Faculty of Medicine- Minia University, Egypt, between August 2012 and April 2014.
Methodology: This study was performed in 80 patients with type 2 diabetes mellitus (54 men and 26 women) and 15 normal controls (12 men and 3 women). In our thesis we measured HbAc, fasting blood glucose, leptin hormone and c-peptide. DNA extracted and the human leptin gene (for product 242 bp) was amplified by PCR, Restriction analysis of the PCR products was performed with restriction enzyme HhaI and genotyped at the restriction site located -2549 bp from the

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transcription initiation site of leptin gene. Presence of allele (C at -2549 bp) and absence of allele (A at the same position) were identified through the GCGC sequence.
Results: Our study showed C^{-2549} A variant is associated with the fasting leptin levels, the results which are in agreement with previous studies. The polymorphism in leptin gene has an effect on the level of plasma leptin in different genotypes, and individuals with AA genotype have the lower plasma levels of leptin and also c-peptide than AC and CC variants.
Conclusion: Our study reveals that both diabetic patients and their non-diabetic relatives have different basal leptin and c-peptide specific to different leptin genotypes. This suggests that the association between leptin and insulin in members of diabetic families may be controlled by inheritance.

Keywords: C-peptide; genotypes; leptin; polymorphisms; type 2 diabetes mellitus.

1. INTRODUCTION

Diabetes mellitus is a group of metabolic diseases in which a person has high blood sugar that results from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism [1].

Leptin is known as the adipose peptide hormone. It plays an important role in the regulation of body fat and inhibits food intake by its action. Moreover, it is believed that leptin level might be the cause of obesity and may play an important role in the development of Type 2 diabetes mellitus (T2DM), as well as in cardiovascular diseases [2]. Leptin may also regulate glucose homeostasis independently of its effects on adiposity; leptin regulates glycaemia at least partly via the CNS, but it may also directly regulate pancreatic β- cells and insulin-sensitive tissues [3]. The idea that leptin may directly act on pancreatic β-cells to control insulin secretion was derived from observations in genetically leptin-deficient *ob*/*ob*mice and leptin receptor–defective *db*/*db*mice. First, it was noted that defective leptin signaling in these mice strains leads to initial hyperinsulinemia in young animals, even before the development of the obese and diabetic phenotype [4].

Leptin is produced in proportion to fat stores. Circulating leptin serves to communicate the state of body energy repletion to the central nervous system (CNS) in order to suppress food intake and permit energy expenditure [5]. Leptin action on these hypothalamic neurons also regulates the activity of the autonomic nervous system, although direct effects of leptin on LRb-containing neurons in the brainstem and elsewhere probably also have an important role. The effects of leptin on the immune system and vasculature appear to result from direct action on hematogenous cells that contain LRb [6].

Lack of leptin signaling due to mutation of leptin (e.g., *ob/ob*mice) or the leptin receptor (LR) (e.g., *db/db*mice) in rodents and humans results in increased food intake in combination with reduced energy expenditure and a phenotype reminiscent of the neuroendocrine starvation response (including hypothyroidism, decreased growth, infertility, and decreased immune function) in spite of obesity [7].

Leptin resistance is believed to play an important role in the development of obesity and this is due to the fact that an increased amount of leptin cannot perform its role in controlling food intake [8]. A number of mechanisms have been proposed to explain leptin resistance; these include alterations in the transport of leptin across the blood-brain barrier (BBB), alterations in cellular long-form leptin receptor ($LEPR_b$) signaling, perturbations in developmental programming, and others [9]. Indeed, each of these mechanisms may contribute to the totality of leptin resistance.

The aim of the work was to study polymorphisms in the LEP gene in type 2 diabetics in Minia, Egypt and determined the relationship between the leptin and c-peptide levels in different genotypes and insulin resistance in obese patients. The study also has evaluated the role of leptin gene polymorphism in prediction of diabetes mellitus prognosis and its prevention.

2. SUBJECTS AND METHODS

2.1 Subjects

Eighty patients with type 2 diabetes mellitus (54 males and 26 females) and 15 normal controls (12 males and 3 females) were participated in

the study. All ethical issues are taken on patients, written informed consent obtained from patients or legal guardians for all cases.

All patients were chosen to be elder than 40 years old and all of them have discovered the disease after 30 years old and they were either treated by orally drugs or injecting by insulin. They all were chosen of BMI 25-30. All of normal controls had normal glucose tolerance as shown from their fasting blood glucose (FBG) test.

Samples collection

A 5 ml blood samples were drawn from all fasting cases used for:

- 500 µl in EDTA was taken as whole blood for HbA1c.
- 1000 µl in Na florid, then centrifuged, plasma was taken for FBG determination.
- 3500 µl serum was taken for leptin and c-peptide determination and cells were taken for DNA extraction.

2.2 Biochemical Assays

2.2.1 Fasting Blood Glucose (FBG)

Using spectrum glucose- Liquizyme (single reagent).kit. GOD- PAP enzymatic colorimetric method.

2.2.2 Hemoglobin A1c

Using Hemoglobin A1c Chromatographic-Spectrophotometric Ion Exchange kit. (COD 11045). According to manufacture instruction.

2.2.3 Measurement of human C-peptide

Using quantitative C-peptide ELISA kit (WKEA MED SUPPLIES COR, Changchun, China). According to manufacture instruction.

2.2.4 Measurement of human leptin

Using quantitative leptin ELISA kit (WKEA MED SUPPLIES COR, Changchun, China). According to manufacture instruction.

2.2.5 DNA extraction and PCR amplification

DNA extracted manually according to Maniatis 1989 [10], DNA was used immediately or stored at –20ºC.The human leptin gene (for product 242 bp) was amplified by PCR using the following primer set. Gustincich et al 1991 [11]: Sense: 5\ TTTCCTGAATTTTCCCGTGAG 3\

Antisense: 5\ AAAGCAAAGACAGGCATAAAAA 3\ (Eurofins, Germany). PCR was performed in a 25 µL reaction mixture, containing 1µL of template DNA (approximately 100ng/µL), 12.5 µL of PCR master mix (DreamTaq TM Green Master Mix (2X), Fermentas). It is a ready to use solution containing Dream Taq TM DNA polymerase, optimized Dream Taq TM Green buffer (2X), 4mM MgCl and dNTPs (dATP, dCTP, dGTP and 2dTTP, 0.4 mM each), and 1 µL (10 pmol) of each primer and 9.5 µL of nuclease free water. The reaction was carried out in a thermocycler (TECHINE TC 512, UK) as follows:Initial denaturation 94ºC, for 3 minutes 1 cycle, denaturation 94ºC, for 45 seconds, annealing 56ºC, for 45 seconds, extension 72ºC, for 30 seconds (for 35 cycles). Final extension 72ºC, for 5 minutes 1 cycle. The PCR product was analyzed by using (2%) agarose gel electrophoresis using 50 bp ladder (QIAGEN Gelpilot). A single product of 242 bp was produced.

2.2.6 Analysis of Restriction Fragment Length Polymorphism (RFLP)

Restriction analysis of the PCR products was performed with restriction enzyme HhaI, (Thermo Sientific Fast digest), according to the manufacturer's instructions as follows; 10 µL of PCR products,1µL of Fast Digest enzyme, 2µLof green buffer and 17µL of nuclease free water at 37°C for 5minutes. RFLP fragments were identified by electrophoresis through 3% agarose gel using 50bp ladder (QIAGEN Gelpilot).

All subjects were genotyped at the restriction site located -2549 bp from the transcription initiation site of leptin gene. Presence of allele (C at -2549 bp) and absence of allele (A at the same position) were identified through the GCGC sequence by the fast digest HhaI, as follows:

5\.....GC G C…….3\

3\……..C G CG……..5\

2.2.7 Reproducibility testing

RFLP reproducibility was determined by analyzing 3 PCR products 3 times by HhaI digestion.

2.3 Statistical Analysis

The data were expressed with SPSS version 13 soft ware as the mean ± standard error (SE), Independent t-test, for parametric quantitative data. Chi- square x2-test, for categorical data* Significant if p value <0.05.

3. RESULTS

A total of 77 patients with type 2 diabetes mellitus, 53 (69%) were males and 24 (31%) were females that was statistically non-significant (*p*=0.39), and 15 normal controls, 12 males (80%) and 3 (20%)females with (p=0.1).The mean age of the total study patients was (40-67) 49.7±6.8 while that of controls was (40-55) 46.7±4.4 years but that was not statistically significant (*p*=0.1).

3.1 The Laboratory Parameters of the Study Groups

Comparison between the laboratory parameters of the study groups, which include fasting blood glucose (FBG), hemoglobin A1c (HbA1c), leptin and c-peptide was determined in Table 1. It is obvious that the level of FBG and HbA1c is lower in normal controls than in diabetic type 2 patients.

3.2 Frequency of Different Genotypes among the Diabetic and Normal Groups

After amplification of the leptin gene, all subjects were genotyped at the restriction site located -2549 bp from the transcription initiation site of leptin gene. Presence of allele (C at -2549 bp) and absence of allele (A at the same position) were identified through the GCGC sequence by the fast digest HhaI on the PCR products, after digestion 3 genotypes profiles AA, AC, CC appeared Fig. 1. The frequencies of the polymorphism $C^{-2549}\longrightarrow A$ in leptin gene were studied in both diabetic type 2 patients and in normal control. The genotypes frequencies were different among the two groups. The presence of AA genotype in diabetic type 2 group is 10.4% which is a higher percent than that of the normal control group which is 6.6% as shown in Table 2.

3.3 Distribution of Demographic Dataand Laboratory Parameters in Genotyping Groups of Diabetic Type II Patients

The diabetic patients of AA genotype had lower fasting leptin and c-peptide than other genotypes. Diabetic patients with AC genotype had a decreased fasting leptin and c-peptide values as compared with these of CC genotype. Also FBG and HbA1c levels in the homozygote AA were lower than genotypes as shown in Table 3 and Figs. 2, 3, 4 and 5.

4. DISCUSSION

Leptin is a product of obese (ob) gene expression that plays a role in energy metabolism and co-modulation of body weight. In recent years, it has been shown that the presence of leptin is associated with diabetes, glucose metabolism and insulin metabolism [12]. Mammes discovered that there is a $C\longrightarrow A$ polymorphism at locus 2549 in the promoter of leptin gene, which is related to serum leptin levels in western populations [13].

Table 1. The laboratory parameters of the study groups

Laboratory Parameters	Control No = 15	Diabetic patients No = 77	p
FBG mg%			
Range:	72-100	86-384	<0.001*
Mean ± SD:	84.9±8.6	171±69.4	
HbA1c %			
Range:	4-5.3	5-14.5	<0.001*
Mean ± SD:	4.5±0.4	8±2.5	
Leptin ng/ml			
Range	0.24-1.75	0.25-3.33	0.91
Mean ± SD:	1.1±0.5	1.08±0.62	
C-peptide ng/ml			
Range:	0.39-2.25	0.31-3.76	0.69
Mean ± SD:	1.43±0.52	1.51±0.77	

*FBG: Fasting Blood glucose, Independent t-test, for parametric quantitative data * Significant if p value<0.05*

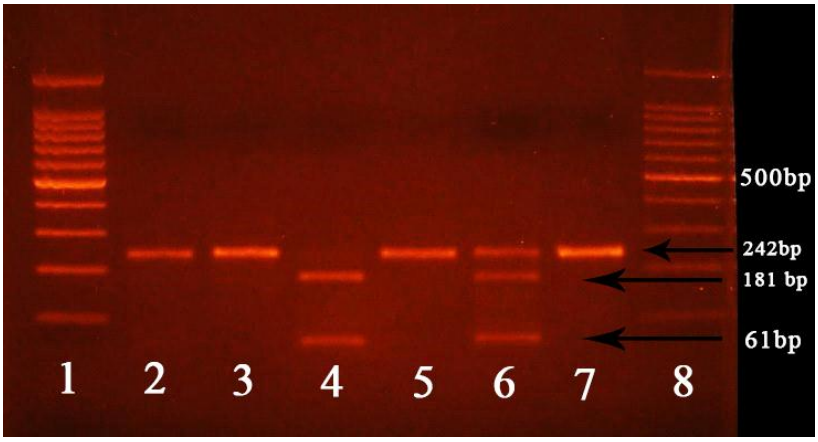

Fig. 1. Agarose gel showing PCR products at 242 pb and restriction digests of leptin at 181 and 61. Lanes (1, 8) represent 100bp ladder; lanes (3, 5, 7) represent PCR products (242bp); lanes (2, 4, 6) represent AA (242bp), CC (61 and181bp), AC (61,181 and 242bp) variants respectively

Table 2. Frequency of different genotypes among the study groups

Genotyping	Control No = 15	Diabetic patients No = 77	P
AA: No (%)	1 (6.6%)	8 (10.4%)	0.33
AC: No (%)	7 (46.7%)	39 (50.6%)	0.39
CC: No (%)	7 (46.7%)	30 (39%)	0.29

Z-test (test of proportions), for categorical data,
** Significant if p value<0.05*

Table 3. Distribution of demographic data and Laboratory parameters in genotyping groups

Demographic and Laboratory dat	AA group No = 8	AC group No = 39	CC group No = 30	P1	P2	P3
Age in years						
Range:	40-58	40-67	40-66	0.58	0.21	0.24
Mean ± SD:	47.6±6.8	49.1±6.1	51±7.6			
Gender						
Male: No (%)	6 (75%)	30 (77%)	17 (57%)	0.99	0.44	0.12
Female: No (%)	2(25%)	9 (23%)	13 (43%)			
FBG mg%						
Range:	86-184	90-184	92-384	0.075	0.082	0.99
Mean ± SD:	128±38.5	176±72	176.1±69.6			
HbA1c %						
Range:	5.1-9.2	5-14.5	5-13.5	0.13	0.32	0.42
Mean ± SD:	6.9±1.6	8.4±2.7	7.86±2.36			
Leptin ng/ml						
Range	0.25-0.44	0.49-1.23	1.22-3.33	0.005*	<0.001*	<0.001*
Mean ± SD:	0.38±0.07	0.7±0.15	1.76±0.42			
C-peptide ng/ml						
Range	0.31.0.69	0.8-2.17	1.54-3.76	<0.001*	<0.001*	<0.001*
Mean ± SD:	0.41±0.12	1.15±0.22	2.28±0.61			

*Independent t-test, for parametric quantitative data; Chi- square x2-test, for categorical data; P1: AA group Vs AC group; P2: AA group Vs CC group; P3: AC group Vs CC group; * Significant if p value<0.05*

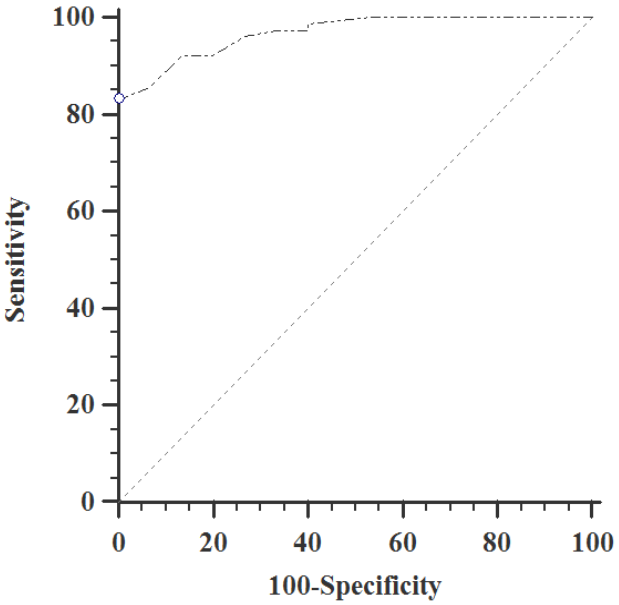

Fig. 2. Receiver operating characteristic (ROC) plots curve for fasting blood glucose level of study groups. This figure shows that mean of area under ROC curve for fasting blood glucose level was 0.968 with confidence interval (0.909 – 0.994) and p-value < 0.0001

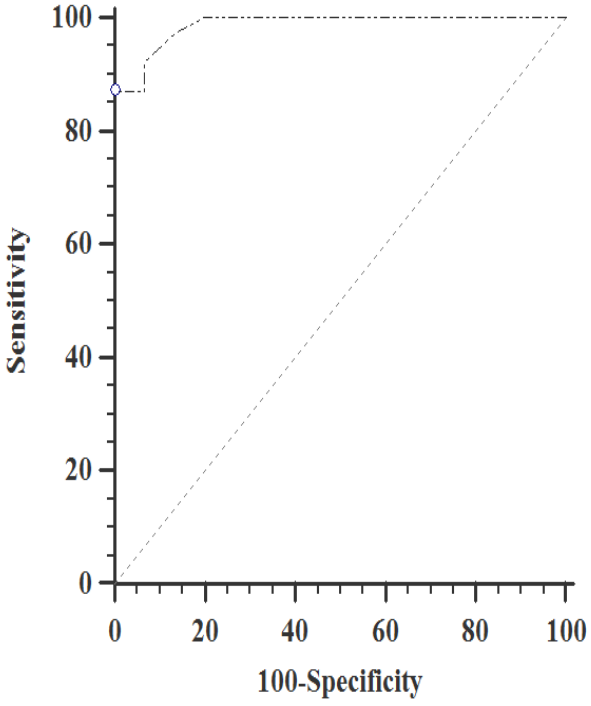

Fig. 3. Receiver operating characteristic (ROC) plots curve for hemoglobin A1c level of study groups. This fig. shows that mean of area under ROC curve for Hemoglobin A1c level was 0.987 with confidence interval (0.937 – 0.999) and p-value < 0.0001

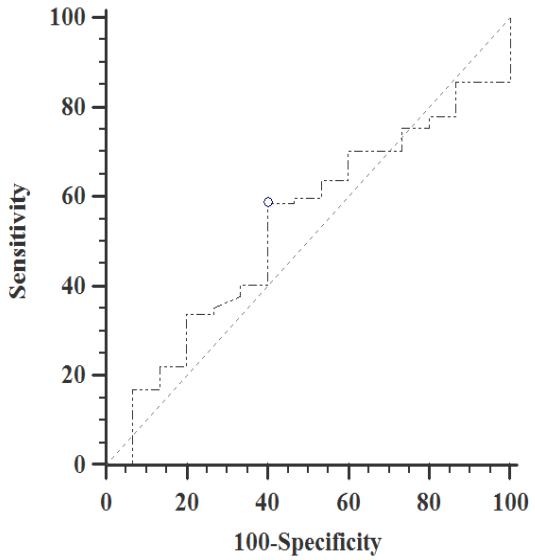

Fig. 4. Receiver operating characteristic (ROC) plots curve for leptin level of study groups. This figure shows that mean of area under ROC curve for leptin level was 0.53 with confidence interval (0.42 – 0.64) and p-value = 0.69

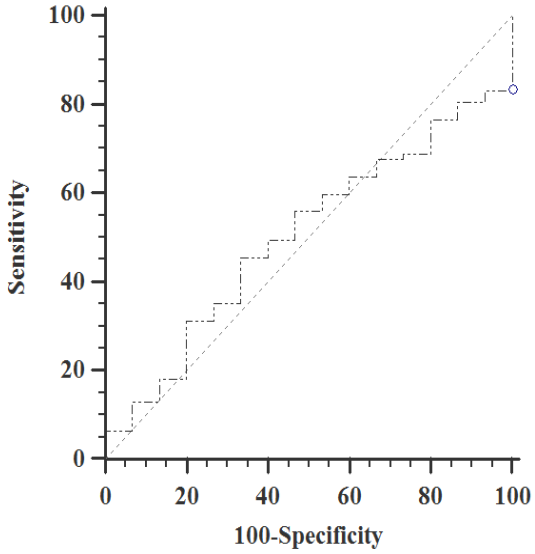

Fig. 5. Receiver operating characteristic (ROC) plots curve for c-peptide level of study groups. This figure shows that mean of area under ROC curve for c-peptide level was 0.5 with confidence interval (0.4 – 0.61) and p-value = 0.97

There are many genes that interact with the environment that lead to diabetes and these genes have been investigated to be involved in determining the whole range of BMI in the population [14].

Obesity is now a global epidemic. It is one of the most significant causes of ill-health worldwide. Anti-adiposity efforts to reverse the increasing trend are somewhat disappointing. However, all overweight or obese people do not have the same risk of developing insulin resistance, resulting in adiposity-related health risks such as type 2 diabetes, metabolic syndrome and cardiovascular diseases [15].

Besides the leptin gene receptor, polymorphism possibly has role in regulation of body weight,

obesity, fat mass distribution, serum leptin levels, glucose homeostasis, and diabetes, among others [16].

Circulating leptin level was found to be proportional to adipose tissue mass, and body fat percentage was possibly the best adiposity-related predictor of serum leptin concentrations in human, which may be due to leptin resistance. However, it is not always easily accessible to directly measure the percentage of body fat, especially in epidemiological studies [17].

Several lines of evidence suggest that most of the polymorphisms associated with signaling impairment of leptin action in the central level would correspond to Gln223Arg polymorphism and in turn be associated with high circulating of leptin levels, and this may play a role in the pathophysiology of diabetes [18].

It was found that participants with insulin resistance had significantly higher leptin levels compared to those without the condition. The leptin level was almost double among the insulin resistance. Furthermore, data suggest that among the overweight/obese population those with insulin resistance had a significant higher level of leptin concentration [19].

Taken together, all effects of leptin in pancreatic β-cells that are known to date act concomitantly to exert a physiological long-term control of insulin secretion from the pancreatic β-cell, which adapts the amount of insulin secretion to the amount of body fat stores. It is important to note that this tonic restriction of insulin secretion by leptin generally does not seem to interfere with the short-term stimulatory actions of nutrients and hormones, such as glucose and incretion-dependent insulin secretion [20].

Mutations in the leptin gene and the leptin gene receptor may lead to extreme obesity, this was found to be true in mice, and in humans. They found, that the presence of a homozygous mutations in the leptin gene (homozygotes ob/ob) and also homozygous mutations in the leptin gene receptor (homozygotes db/db) were associated with early onset of extreme obesity due to hyperphagia, poor energy expenditure and severe insulin-resistance [21].

Taken together, all effects of leptin in pancreatic β-cells that are known to date act concomitantly to exert a physiological long-term control of insulin secretion from the pancreatic β-cell, which adapts the amount of insulin secretion to the amount of body fat stores. It is important to note that this tonic restriction of insulin secretion by leptin generally does not seem to interfere with the short-term stimulatory actions of nutrients and hormones, such as glucose and incretion-dependent insulin secretion [20].

The results presented in our study confirm the presence of the $C \xrightarrow{-2549} A$ polymorphism in the Egyptian population. The AA genotype frequency in the Egyptian diabetics is statistically lower than that reported in Chinese population [22]. This polymorphism is related to fasting plasma and fasting c-peptide levels.

Our study also has important clinical implications. It suggests the $C \xrightarrow{-2549} A$ variant is associated with the fasting leptin levels, the results which are in agreement with previous studies [23].

This polymorphism of leptin gene is related to the change in the leptin product, as different genotypes AA, AC and CC are obtained. The ANOVA analysis based on three genotypes revealed an association between leptin polymorphism and metabolism in Egyptian diabetics.

We also found that the AA genotype frequency in diabetes group was higher than that of normal controls. The presence of AA genotype in diabetic type 2 group is 10.4% which is a higher percent than that of the normal control group which is 6.6% which suggests that the polymorphism of leptin gene is higher in diabetics than normal controls.

The polymorphism in leptin gene has an effect on the level of plasma leptin in different genotypes and individuals with AA genotype have the lower plasma levels of leptin and also c-peptide than AC and CC variants.

Many studies have shown that insulin is an efficient in production of leptin in animals and humans. Such an effect has been seen in patients with type 2 diabetes [24]. There is an association between plasma leptin level and insulin among patients with type 2 diabetes and insulin may be an important modulator of plasma leptin concentration in type 2 diabetics [25].

The hyperinsulinemia of patients with type 2 diabetes may increase the release of leptin, which may explain the direct relationship between the leptin plasma levels and c-peptide

levels. This finding may explain the deviation of the adipo-insular axis in obese type 2 diabetic individuals who have leptin resistance on the level of pancreatic β –cell [20].

Our study reveals that both diabetic patients and their non-diabetic relatives have different basal leptin and c-peptide specific to different leptin genotypes. This suggests that the association between leptin and insulin in members of diabetic families may be controlled by inheritance.

Our study shows association between the levels of leptin, c-peptide and leptin genotypes may give an incidence that people carrying C allele have more severe insulin resistance. Therefore, ascertaining leptin genotype in individuals earlier may help to identify the high-risk individuals for diabetes with high basal level of leptin and c-peptide.

Our study reveals that the presence of AA and AC genotypes in women is markedly lower than the presence of CC genotype, as AC and AA genotypes represent 23% and 25% respectively in Egyptian women, while CC genotype represents 43%. The finding which recommend the greater prevalence of A allele in Egyptian women.

5. CONCLUSION

In conclusion, the distribution of the leptin $C \xrightarrow{-2549} A$ polymorphism in the Egyptian population differs from Western and Chinese populations. The DNA polymorphism in the leptin gene is associated with fasting leptin in Egyptian people. The distribution of genotypes among members belonging to diabetic pedigrees differs from that of normal controls. The A allele frequency in diabetic patients is higher than that of normal controls.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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