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Association Ghrelin Level with Insulin Resistance in Type 2Diabetes mellitus Obese patients.

Methak, J.H.AL-Jboori[1]*, Ali, H.Al-Saadi[2] and Haider, K. Al-Saadi[2]

[1]College of Science, University of Mustansiriyah, [2]College of Science, University of Babylon.

ABSTRACT

The aim of this study was to investigate the relationship between serum ghrelin concentration with markers of insulin resistance and obesity in type 2 diabetes , Evaluation of the possible association of the obesity in type 2 diabetes and gene polymorphism. The results show that the Levels of ghrelin (mean **± S.E**) ghrelin was significantly lower in obese type 2 diabetes compared to control group (p< 0.0001) and Levels of ghrelin did not differ between the 49men and the 59 women . smoking history was significantly. ghrelin showed significant negative correlation with BMI (r= -0.62) , Waist/Hip ratio(r = -0.68), SBP (r= -0.53) and DBP(r= -0.43).A significant negative correlation between Ghrelin level and FBG (r= -0.55) , HbA1c (r= -0.60) , Insulin (r= -0.44) and insulin resistance index (HOMAIR HOMA-IR(r= -0.46). There was an inverse correlation between Ghrelin level and cholesterol(r= -0.15), triglycerides(r=-0.38) and VLDL(r= -0.38) respectively, while there is no significant correlation with LDL. As well as there were significant positive correlation between Ghrelin level and HDL. A significant negative correlation between Ghrelin level and Syctolic blood pressure and diastolic blood pressure (r= -0.53 andr=-0.43) was observed in the obese type 2 diabetes groups.

Keywords: Ghrelin, Diabetes mellitus Obese

Corresponding author

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INTRODUCTION

Obesity and associated diabetes mellitus are epidemic through the world; obese individuals characteristically manifest with insulin resistance and hyperinsulinemia, which predispose to glucose intolerance, diabetes and cardiovascular disease1 Type 2 diabetes mellitus is a heterogeneous and polygenic disease associated with abnormal insulin secretion or defects of insulin action(Smith,1996; Atabek and Pirgon ,2007).

Ghrelin an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHSR), was originally discovered in extracts of rat and human stomach, where it is localized in the endocrine X/A-like cells of the fundus mucosa representing about 20% of gastric mucosal cells in humans (Aydin, 2006a; Higgins *et al*.,2007).

Ghrelin is the first natural hormone to be identified in which the hydroxyl group of one of its serine residuesis acylated with an n-octanoyl. Approximately 70% of the ghrelin is produced by stomach and the rest is mainly produced by the small intestine. Minor amounts of ghrelin have been detected in the lungs, pancreatic islets, adrenal cortex, kidney and brain (Jeon *et al*., 2004; Lely *et al*.,2004; Hosoda*et al*., 2006).

Ghrelin plays a role in both short and long term regulation of energy balance, appetite, and weight gain. This hormone also increases gastric motility, gastric and pancreatic secretions, regulates glucose and lipid metabolism, stimulates cellular differentiation in adipose tissue, inhibits apoptosis in adipocytes, inhibits lypolysis and stimulates lipogenesis. In long term energy balance, ghrelin increases food intake, decreases the use of fat as ametabolic fuel and promotes fat deposition (Cummings *et al*.,2004; Gil-Campos *et al.,*2006).

There is a growing body of evidence indicating a suppressive role of ghrelin in the release of insulin from the pancreatic islets. Low ghrelin concentrations were shown to associate independently with Type 2 diabetes and insulin resistance. Circulating ghrelin concentrations are also reduced in the healthy offspring of type 2 diabetic patients and the compensatory hyper insulinaemia due to insulin resistance was associated with significantly reduced ghrelin concentrations (Ostergård *et al*., 2003).

Poykko *et al.,*(2003) showed that fasting plasma concentrations of total ghrelin were lower among subjects with Type 2 diabetes compared to those without Type 2.

The human ghrelin gene (GHRL) spans 5 kb of the genomic DNA on the short arm of chromosome 3 (3p25-26) and contains six exons (2 are noncoding) ,same as the mouse gene and 4 introns and encodes a 511 bp mRNA.The short first exon contains only 20 bp, which encode part of the 5-untranslated region(Tanaka *et al*., 2001a; Seim *et al*.,2007).

There are two different transcriptional initiation sites, resulting in two distinct mRNA transcripts, of which one is the main form of human ghrelin mRNA *in vivo* ,one occurs at- 80 and the other at - 555 relative to the ATG initiation codon , resulting in two distinct mRNA transcripts (transcript-A and transcript- B) .In humans, parts of exons 1 and 2 of the ghrelin gene code for the ghrelin peptide and exon 3 encodes the peptide hormone obestatin figure (2-4) (Kanamoto *et al*.,2004).

METHODS

A total of 108 obese type 2 patient aged 30–75years; 49 men and 54women were enrolled atthe diabetic centre /Merjan Teaching Hospital in Babylon province/Iraq. While collecting data, those(93) apparently healthy that included 46 male and female 47 with matched age range 30-75 years.

All subjects were subjected to:

1- Full history taking with particular emphasis on age, family history, history of any systemic diseases e.g. diabetes, hypertension, dyslipidemia or history of any associated diseases and any drug intake.
2- Thorough clinical examination with special on:

Blood pressure was measured under standard conditions. Measurements were obtained for each patient three times by the auscultation method, measured in the sitting position by the clinic nurse and the mean of 3 readings separated by a 1 minute interval is recorded in the file as the patient's blood pressure. Hypertension was defined as a systolic blood pressure ≥ 135 mmHg and/or a diastolic blood pressure ≥ 85 mmHg , repeated 2–3 times over a 6-week interval or if the patient was already on antihypertensive medication.

- **Body weight** was measured to the nearest 0.5 Kilograms (kg) with subjects barefooted and wearing light indoor clothes. Body height was recorded to the nearest 0.5 centimeter (cm).

- **BMI** was calculated as the ratio of body weight to body height squared expressed as Kg/m2.

- **Waist circumference** (WC) was measured at the distal third of theline from the xyphoid process to the umbilicus.

Blood samples were taken with subjects having fasted for at least 12 hours.The blood sample was analyzed for lipid profile including: total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), and high density lipoprotein-cholesterol (HDL). Serum lipids were assayed by standard enzymatic methods(Jeffery *et al*.,2012).

LDL is not directly measured in the routine lipid panel; instead it is calculated by the Friedewald equation. This equation is: LDL Cholesterol = Total Cholesterol_ HDL cholesterol-(Triglycerides/5.

Insulin resistance was calculated as proposed by Matthewes et al. for homeostasis model assessment for insulin resistance (HOMA-IR) index. HOMA-IR was computed as follows: [fasting insulin (mU/L) · fasting glucose (mmol/L)]/22.5(Khoury *et al.*,2013)**.**

Fasting serum glucose:

Serum glucose was measureda single reagent glucose method based on a technique described by *Trinder, (1969)*.

Fasting serum insulin using ELISA technique:

The BioSource INS-EASIA (manufactured by Monobind inc. USA) is a solid phase Enzyme Amplified Sensitivityimmunoassay performed on microtiterplates. It is an immunoenzymetric assay for the in vitro quantitativemeasurement of human insulin (INS) in serum .. Results of the samples are determined using thestandard curves.

Serum Acylated Ghrelin ELISA technique:

Acylated Ghrelin was measured by an enzyme linked immunoassay

Human A-GHRL ELISA Kit obtained from the Elabscience (China).

Genomic DNA extraction

DNA was isolated and purified from whole blood (EDTA) according to the protocol provided by the manufacturer (Geneaid / Taiwan).DNA was stored at −20°C till the time of use.

Determination of Ghrelin gene

Amplification target sequence ofGhrelin gene using the **Forward primer** 5'-AGCCTCCTGCTCCTCGGCAT -3' and that of the R**everse primer** 5'- TGTGGGCGATCACTTGTCGGCT-3'. Was used master A master premix of Bioneer was used with a components . PCR optimization was done as a first step by using a gradient temperature ranging from 53 C° to 64 C°. After the determination of optimum annealing temperature (59C°).Amplification reactions were carried out by using GTC Series thermocycler (Cleaver Scientific /UK) apparatus .After Determination of the optimum annealing temperature the following program

was set in the thermocycler to amplify the target DNA fragments. Detection of PCR amplification products was performed by size fractionation on 1% agarose gel electrophoresis. SSCP analysis is generally considered to be most suitable for the detection of mutations in short stretches of DNA. Hence, the size of PCR fragments investigated are usually in the range of 175-250 bp. It is important to optimise the PCR reaction to minimise unwanted products which may interfere with gel analysis. The PCR products should be evaluated for purity by agarose gel electrophoresis before being loaded onto an SSCP gel.

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters . Chi-square test was used to significant compare between percentage and Least significant difference –LSD test was used to significant compare between meansin this study. Genetic analysis was performed using Chi-square test. P values less than (0.05) is considered significant and less than (0.01) is considered highly significant.

RESULTS

This study included 108 obese type 2 diabetic: 49 males (45.3%),59 females (54.7%) with a mean age of 52.46 ±0.82 years and Non obese control : 46 males (49.4%), 47 females(50.6%) with a mean age of 46.60 ± 1.22 years. There was no significant statistical difference in between cases and controls as regards sex. 61.4% of the total samples have a family history for diabetes mellitus and 49.46%in controls (*P*=0.231).

A comparison among cases according to different anthropometric, clinical and laboratory parametersis displayed in Table (1),the subjects in obese type 2 diabetes patients exhibit higher significant in Body mass index (BMI), waist circumference (WC) and Waist/hip ratio (WHR) compared to those in control groups (all P<0.0001).A statistically highly significant difference were found between obese type 2 diabetic and control as regards Hemodynamic variables (Systolic blood pressure and Diastolic blood pressure). Physical activity In adequate and smoking habit were documented in (88.46%) and 26.15% of the Obese subjects.

Insulin level was significantly higher in obese type 2 patients compared to control group (p< 0.0001) , while ghrelin was significantly lower in obese type 2 diabetes patients compared to control group (p< 0.0001).There was no significant statistical difference in between Male and female as regards ghrelin levels.

Table (1) Anthropometric, Clinical and Laboratory Data among Cases and Controls

Variables	Control groups No.(93)	Obese type 2 diabetes groupsNo.(108)	T-test value	P-value
Age (years)	46.60 ± 1.22	52.46 ±0.82	3.923 *	0.0491
Male (%)	49.46%	45.3%	2.072 NS	0.3662
Female (%)	50.57%	54.7%	1.71 NS	0.513
BMI kg/(m^2)	23.10 ± 0.13	34.03 ± 0.45	1.072 **	0.0001
Waist (cm)	83.47 ± 0.83	111.67 ± 2.59	9.312 **	0.0028
Waist/hip ratio	0.877 ± 0.003	1.04 ±0.004	0.0108 **	0.0001
History of diabetes (%)	28.57%	61.43%	10.317 **	0.0001
Hemodynamic variables				
Systolic blood pressure(mm Hg)	115.74 ± 1.82	151.09 ± 1.71	5.021 **	0.0001
Diastolic blood pressure (mm Hg)	78.90 ± 1.25	93.76 ± 1.17	3.451 **	0.0001
Life style variables				
Physical activityIn adequate	78.49%	88.46%	5.84 *	0.0482
Smoking (%)	14.69%	26.15%	5.942 *	0.439
Insulin (µIU/mL)	7.33 ± 0.42	30.54 ± 1.99	4.719 **	0.0001
Ghrelin (pg/ml)	1244.59 ± 39.71	469.92 ±20.96	82.116 **	0.0001

*P value is significant ≤ 0 .0001 level.

This study revealed that Ghrelin and insulin resistance was dependent of age.

Insulin as well as HOMA-IR was significantly different in obese type 2 diabetes patients.. HOMA-IR was positively correlated with major obese type 2 diabetes patients related parameters. The correlation was strongest with BMI, WC/Hip ratio, FBG,HbA1c,TG ,Atherogenic Index ,SBP,DBP and Insulin and with negative correlation with Ghrelin Table (2).

The correlation between the different parameters incases revealed a significant negative correlation between Ghrelin level and each of FBG (r= -0.55), HbA1c (r= -0.60), Insulin (r= -0.44) and HOMA-IR(r= -0.46) as well as between BMI and each of waist circumference andsystolic blood pressure (Table 2).

Our result demonstrate that there was an inverse correlation between Ghrelin level and cholesterol(r= -0.15), triglycerides(r=-0.38), VLDL(r= -0.38) and Atherogenic Index(r= -0.50) respectively, while there is no significant correlation with LDL. As well as there were significant positive correlation between Ghrelin level and HDL.Negative significant correlation between Ghrelin and insulin and HOMA-IR (r= -0.44 and r= -0.46) respectively**.**

Table (2) Correlation between Ghrelin hormone level with some parameters in obese type 2 diabetes group

Variable	Correlation coefficient (r) with HOMA-IR	Correlation coefficient (r) with Ghrelin
BMI (kg/m^2)	**0.39 ****	**-0.62 ****
Waist/Hip ratio	**0.50 ****	**-0.68 ****
FBG (g/dl)	**0.76 ****	**-0.55****
HbA1c(%)	**0.73 ****	**-0.60 ****
Triglyceride (mg/dl)	**0.37 ****	**0.38 ****
Cholesterol (mg/dl)	**0.04 NS**	**-0.15 ***
LDL (mg/dl)	**-0.001 NS**	**-0.11 NS**
HDL (mg/dl)	**-0.17 NS**	**0.26 ****
VLDL (mg/dl)	**0.37 ****	**-0.38 ****
Atherogenic Index	**0.43 ****	**0.50 ****
SBP	**0.42 ****	**-0.53 ****
DBP	**0.31 ****	
Insulin	**0.89****	-0.44 **
HOMA-IR	**-----**	**-0.46 ****
Ghrelin	**-0.46 ****	**--------**

NS: Non-significant ,Correlation coefficient (r), ** Correlation is significant ≤ 0.0001 level **.**BMI = Body Mass Index ,. BP = Blood pressure , HOMA= Homeostasis Model Assessment.

Genetic Study

DNA extraction

The first steps in genetic study were DNA extracted from whole blood, then concentration and purity estimated, the results of these steps show in the Figure (1), and the concentration ranged (45-170) ng and the purity ranged (1.8-2.2).

The Electrophoresis pattern of DNA extracted from blood for obese type 2 diabetes patients and control show in Figure (1).

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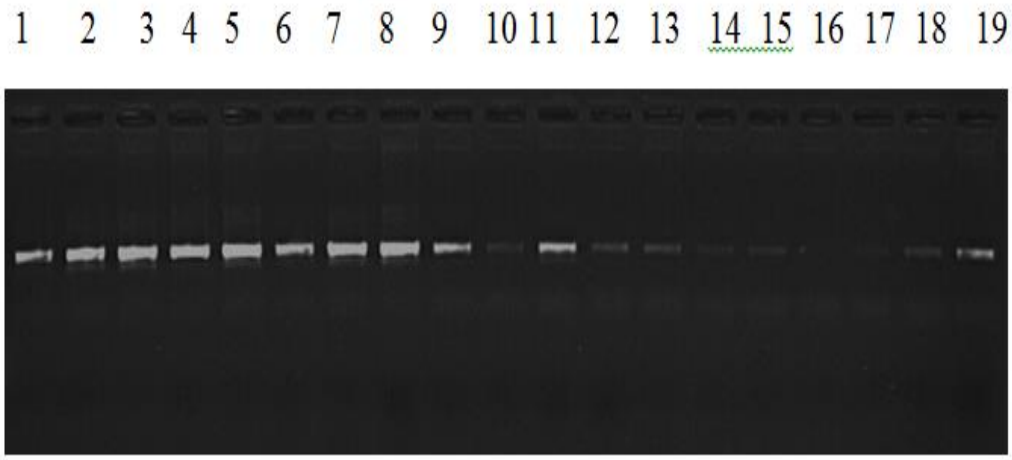

Figure (1):Electrophoresis pattern of DNA extracted from blood for patients and control, 1% agarose,75 V, 20 mA for 1h. lane 1-10 DNA from patient , lane 11-19 DNA from control.

Ghrelin Gene polymorphisms

The amplification of Ghrelin gene using specific primer was 90bp and as show in Table (3) and Figure (2).

Table (3) Virtual Descriptive of ghrelin gene sequence

PCR Products results	Descriptive
GGGCAGAGGATGAACTGGAAGTCCGGTTCAACGCCCCC TTTGATGTTGGAATCAAGCTGT CAGGGGTTCAGTACCAGCAGCACAGCCAGG	>90 bp product from linear template Homo sapiens chromosome 3, GRCh38.p2 Primary Assembly, base 335 to base 424 .

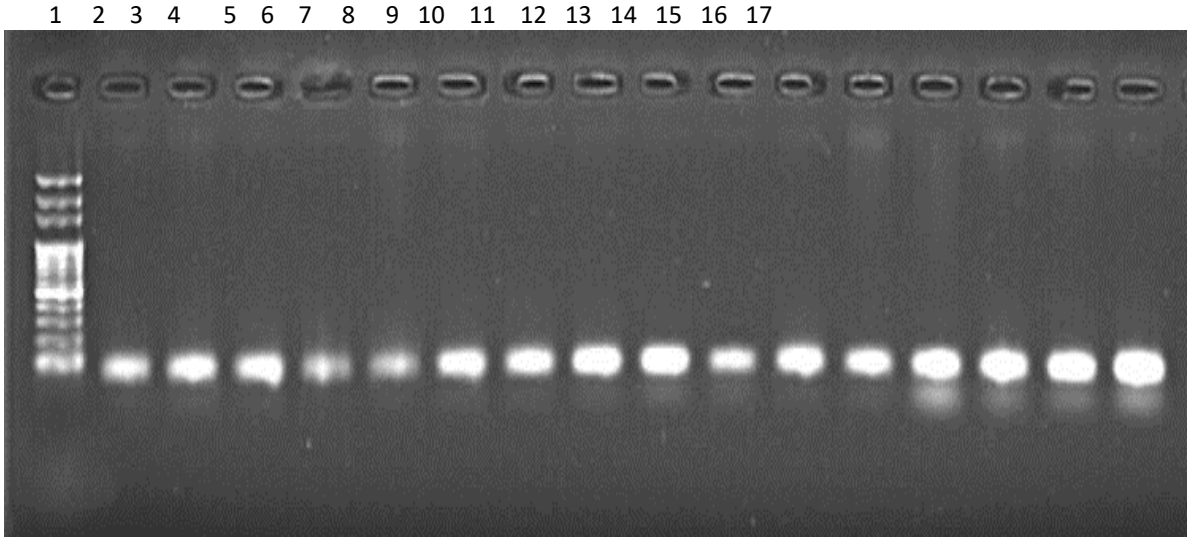

Figure (2)Electrophoresis pattern of PCR product forGhrelin gene, the amplification product was one band 90bp lane 2-9 PCR product for obese type 2 diabetes patients , lane 10-17 PCR products for control ,1% agarose , 75v, 20 Am for 120 min.(10µl in each well).

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Genotype of Ghrelin gene polymorphism by PCR-SSCP technique

The results of ghrelin gene polymorphism using PCR-SSCP technique show significant differences between patient and control, there are two patterns appeared in present study , **Two** bands and **Four**bands , **Two**bands appeared in the (22.35)% and **Four** bands in (77.64)% ofobese type 2 diabetes patients while in control **Two** bands don't appeared while**four** bands appeared in (100)% as show in Figure (3) and Table (4).

Table (4) Ghrelin gene polymorphisms characterization in obese type 2 diabetes patients **and control group.**

Variable	Study Group		χ^2	*P* values	Odds Ratio (C.I 95%)
	Patient (%)	Control (%)			
4 bands	77.64%	100%	**12.518**	**0.0004***	28.4436(1.6761 - 482.6957)*
2 bands	22.35%	0.0			

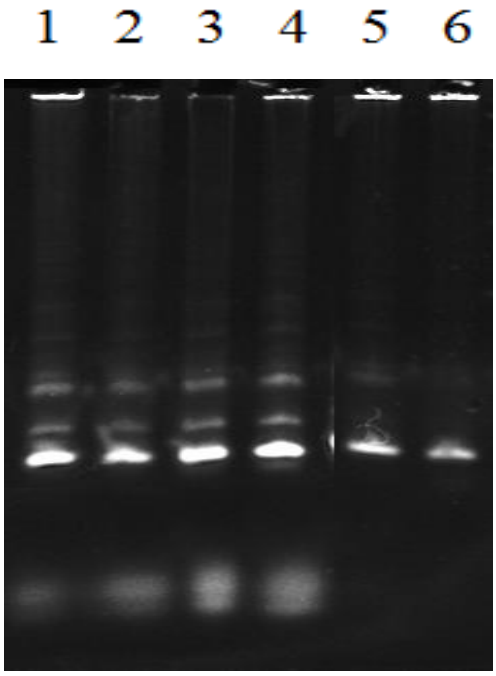

Figure (3):Electrophoresis pattern of PCR-SSCP (90bp) for patient and control.

DISCUSSION

Although obesity, defined as excess body fat, is frequently accompanied by insulin resistance, the molecular basis forthe link between obesity and insulin resistance has not yet been clarified *(Zou et al. 2007)*.

The present study confirmed that the serum Ghrelin concentration was significant negative correlation with BMI, WC, SBP and DBP. Some studies revealed that a negative relation between ghrelin concentration and BMI and waist circumference has been detected (*Ikezaki et al.,2002;Park et al*.,2005).

Regarding ghrelin in this study, it was significantly lower in diabetic patients compared to healthy individuals. Moreover, itwas negatively correlated to FBG, HbA1c, insulin resistance. Despite the significant positive correlation that found between ghrelin and HDL and the significant negative correlation with blood pressure, TG and LDL.Other observational studies have reported that low ghrelin levels are associated with

insulin resistance and type II diabetes (Chen *et al*.,2007; Farajallah *et al*.,2014).This is mostly explained by higher BMI in subjects with lower ghrelin levels because adiposity influences all other features of the metabolic syndrome(Murray *et al*.,2005).

The best possible explanation for the ghrelin level in diabetic patients group supposes a competition between the factors that increase ghrelin level (insulin deficiency)and factors that decrease ghrelin level(obesity, glucose, and hyperinsulinemia). The high percentage of insulin resistance in diabetic patients may support this explanation (Yaturu *et al*.,2008).

Gender difference showed no significant data among our investigated parameter . In a study conducted by Farajallah *et al*. (2014) who reported that higher ghrelin level was negatively associated with measures of obesity, HbA1c, and blood pressure in females and positively associated with increased insulin resistance in Arab males.

Insulin resistance is commonly associated with obesity, and HOMA-IR is a sensitive and specific method for its determination This distinction will be useful in studies of population known to have high genetic predisposition for diabetes in whom the range of HOMA-IR values is likely to be higher than other populations with lower genetic susceptibility. The importance of HOMA-IR index as an adequate tool for determination of IR in obese children was further supported by Makni *et al*. (2012) who reported that HOMA-IR correlated better with the majority of MS components in both sexes.

Regarding the exact relationship between ghrelin and insulin, conflicting results have been reported. In a study conducted by Adeghate *et al*.(2002) ghrelin was found to stimulate insulin secretion from the pancreas of normal and diabetic rats, whereas Egido *et al*.(2002) reported an inhibitory effect of ghrelin on insulin and somatostatin secretion. Schaller *et al*.(2003).On the other hand, reported that plasma ghrelin concentrations are not regulated by glucose or insulin. According to this study, hyperinsulinemia at concentrations typically seen in IR did not affect plasma ghrelin levels. In the same study, it was observed that insulin at pharmacological concentrations caused a dose-dependent decrease in circulating plasma ghrelin. Glucose in combination with supraphysiological insulin concentrations might cross the blood-brain barrier more easily and influence the central regulation of gastric ghrelin release. Saad *et al*.(2002) reported that insulinemia possibly mediates the effect of nutritional status and energy balance on plasma ghrelin. Notably, insulin could play a pivotal role in regulating body weight through its down-regulating effects on plasma ghrelin concentrations. Erdman *et al*.(2005) have reached the conclusion that in obese subjects with associated hyperinsulinemia, ghrelin suppression is due to insulin, whereas leptin can be important for reduction of ghrelin release during moderate increases of body weight.

The results of ghrelin gene polymorphisms using PCR- SSCP technique show significant variation between patient and control, many study investigated association risk allele with obesity. Several genome-wide scans have suggested that certain areas of the chromosome 3, the same chromosome where ghrelin and ghrelin receptor genes are located, might be linked with obesity or metabolic syndrome (Ukkola *et al* ., 2002; Ukkola *et al* ., 2006). Polymorphisms in the human GHRL gene and the 5 flanking region have been intensively studied. The most studied exonic SNPs include the Leu72Met located in exon 3 and Arg51Gln, which is located in exon 3 within the last codon of the mature ghrelin protein and disrupts the recognition site of the endoprotease, leading to proteolytic cleavage of the carboxy-terminal 66 amino acids to produce mature ghrelin (Poykko *et al.,*2003), Table 1. Most of the association studies are focused on metabolic syndrome and T2DM, which are summarized in Table 3. A number of studies have shown associations between GHRL SNPs and obesity or related traits, although the results are contradictory The Met72 allele of GHRL has been associated with earlier age at onset of obesity and higher BMI (Bing *et al* ., 2005; Miraglia *et al* ., 2004) but negative findings have also been reported (Vivenza *et al* ., 2004; Ando *et al* ., 2007) The − 5 0 1 A>C in the promoter region of the GHRL gene and the intronic + 3 0 5 6 T>C polymorphisms has been shown to associate with obesity and related conditions (Steinle *et al* ., 2005; Kim *et al*., 2005) while some studies have failed to find association with these SNPs (Vartiainen *et al* 2008; Mager *et al* ., 2006) .

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