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Relationship between *NPY* gene polymorphism in rs16147 region and some physiological variables in type 2 diabetic patients in Al-Anbar province – Iraq

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Abstract
This study was conducted to investigate the relationship between some physiological variables in type II diabetic patients and its relation to the genetic polymorphisms of the *NPY* gene at the region Promot -399 (rs16147).
Total of 150 samples of males from Anbar province, the samples were divided into two groups. The first group included 100 samples of patients with type 2 diabetes, and the second group included 50 samples of healthy people and was considered a control group. The present study included the study of some physiological and molecular variables. In the physiological study, some clinical parameters were measured in serum for both groups, which included measuring renal function (urea, creatinine and uric acid) and measuring liver enzymes (ALT, ALP, AST, AST and GGT), as well as measuring blood lipids (cholesterol and triglycerides) LDL and high density lipoprotein (HDL). The study also measured the level of NPY and the measurement of thyroid hormones (TSH, T3 and T4). While the molecular study include polymorphism of *NPY* gene at Prometric-399 (rs16147).
The physiological tests showed a significant increase (P <0.05) in the level of urea, creatinine and uric acid in patients with type 2 diabetes compared to the control group. as well as there is a significant increase (P <0.05) in liver enzymes ALP, ALT and GGT in patients compared to group control, while the AST enzyme despite its slight increase in the pateints but did not rise to the level of significant, the study also showed a significant increase (P <0.05) in the level of blood lipids cholesterol, triglycerides and LDL and a significant decrease in the level of HDL in patients compared to the control group,
NPY level in patients we found a significant increase (P <0.05) compared with healthy people, the study also showed a significant increase in the hormone TSH and a significant decrease in the level of the T3 hormone in patients when compared to the control group, the study did not show significant difference in the level of T4 hormone between the two groups. The results of the molecular study of promoter-399 (rs16147) site showed that the C allele appear a high frequency in the sample of patients and as a cause of the disease.
The present study showed significant differences in the level of renal function (urea, creatinine and uric acid) and liver enzymes (ALT, ALP and GGT) as well as blood lipid cholesterol, triglycerides, LDL and HDL, in addition to NPY and thyroid hormones TSH and T3, all these changes are related to susceptibility of Type 2 diabetes. The molecular study showed that polymorphism of *NPY* at promoter-399 (rs16147) was associated with the risk of type 2 diabetes and could be considered as an indicator of male susceptibility to this disease.

INTRODUCTION

Diabetes is a chronic metabolic disorder, usually is due to the pancreas gland cannot produce enough insulin, or defect in insulin action or both, leading to hyperglycemia (1) ,diabetes is an epidemic disease that is more prevalent over time in all parts of the world a study in 2014 indicated that there are approximately 380 million people with diabetes around the world (2).
Diabetes can be classified according to its causes to several types but the most common types are diabetes type I, which is dependent on insulin and diabetes type II is not dependent on insulin The high sugar level in both types caused by the lack of insulin secretion or imbalance in their receptors, which leads to disruption of metabolism of carbohydrates, proteins and fat (3).
There are numerus causes of diabetes, heredity, obesity, lack of physical activity, age, food type, exposure to chemicals, as well as psychological condition, were psychological shock as a result to certain sudden event, one of the stimuli is to increase blood sugar levels by releasing a number of the opposite hormones in its work for insulin such as glucagon, catecholamine hormones and growth hormone (4).
Type 2 diabetes usually affects adults and is estimated to have about 90% of diabetics, i.e. about 3 per 100 individuals in the community and thus more prevalent than the first type (5). Symptoms of this type are slow in relation to type 1 (6), resulting in multiple complications of disorder blood lipids and increased risk of heart disease were deaths are significantly higher in cardiovascular disease (7).
Type 2 diabetes is associated with chronic renal disease (8), the lack of insulin in diabetic patients leads to an imbalance in the hepatic tissue lead to damage in hepatocytes due to the basic role of the hormone insulin in the control of many metabolic processes within the liver cells (9), also noted Suhail (2014) that people with diabetes have increased thyroid disorders at a greater rate compared to natural persons(10).
Many genes contribute to an increased risk of developing type 2 diabetes, a strong correlation was found between the genetic factors and the risk of developing the disease. For example, in twin when a sibling is infected, the risk of developing the disease is about 90% whiles the susceptibility to diabetes in non-identical twins 25-50% (11).

MATERIALS AND METHODS

Samples of people with type 2 diabetic patients were collected from Ramadi General Hospital and private laboratories for the period from January 2018 to March 2018, the study included 100 samples of type 2 diabetic patients, all of whom were male, compared with 50 healthy men, their age between 35 and 65 years.
Seven mL of venous blood was withdrawn and 4 mL of the tubes were placed in plastic tubes and the blood was allowed to coagulate for 15 minutes at laboratory temperature, the blood serum was then centrifuged (3000 cycles / min) for 10 minutes to obtain serum and placed 3 ml of blood In tubes containing an EDTA inhibitor and kept at a temperature of -20 to be used on the molecular side.
Renal function tests, which included measurement of Uric acid, Urea, creatinine, were measured as described by Instructions of the manufacturer the Spanish company (Linear Chemical).
Blood lipid measurements included cholesterol level, triglyceride, LDL and HDL, which were measured according Instructions of the manufacturer the Spanish company (Spinreact).
Measuring the liver enzymes: Aspartate aminotransferas (AST), Alanine amino transferase (ALT), Alkaline phosphatase (ALP) and Gamma-glutamyl transpeptidase (GGT), according to the Instructions of the manufacturer which described in the German prepared kit (Rouche).
Measurement of thyroid hormone level: include TSH, T3 and T4 were measured according to Instructions of the manufacturer which is described in the French BioMerieux analysis kit.

Molecular study: DNA was extracted from the blood of the samples studied according to the instructions of the Korean company (genaeid), the electrophoresis was performed to detect the DNA fragments, the DNA were amplified in PCR at the promoter-399 region (rs16147) by using the primer F: 5'-TTCCTACTCCGGCACCCAGTGAG -3 'and the primer R: 5'-GGGCTTTTATGGAGCTTCCTCGC -3', PCR PreMix was used according to the instructions of the Korean manufacturer Bionner and the reaction solution was prepared using 3 µl (50 ng) of Genomic DNA with 1 ul for both the primer forward and the reverse and 2 ul for PCR buffer at X10 concentration. 2 ul of dNTPs and 0.3 ul of Taq polymerase were added and finally 11 µl of The Nucleases free H2O, the above materials were mixed and transferred to the Thormocycler device for the purpose of amplifying the piece. The device was set and the PCR conditions for the reaction mixture were all: Initial Denaturation at 95 °C for 5 minutes, Denaturation at 95 °C for 30 seconds, The annealing phase at 61 °C for 30 seconds, the extension phase at 72 ° C for 30 seconds, the final extension phase at 72 °C for 10 minutes, the first step was one cycle, then the three steps (39 cycles) and the last step It was one cycle as well.

The electrophoresis of DNA samples were carried out after the reaction on the 2% gel, the *Alu* 1 enzymes pieces were used from Promega (USA) and using the PCR-RFLP technique, which include the reaction mixture 1 u/l PCR reaction product and 2 u/l 10X RE Buffer, 0.2 ul Acetylated BSA and 0.5 u/l of *Alu* 1 and 16.3 u/l deionized water, to become the final size of the blend 20 u/l, mix the above materials and transfer them to the thermocycler tubes and adjust the special reaction conditions At 37 °C for 1-4 hours. The electrophoresis is then performed on the 2% agarose gel, the colored bands were stained with an ethidium bromide fluorescence and Photographed by the Photo documentation system with a special camera.

Statistical analysis: All test results were statistically analyzed using Statistical Packages for Social Sciences-version 22 (SPSS-22). The difference between independent averages was tested by using the f-test for all independent averages below the probability level P <0.05, the frequency of genotype and their alleles, Odd ratio and confidence interval were analyzed using the program calc/odds ratio.php / WWW. medcalc.org and using the Fishers Test and the Hardy-Weinberg equilibrium. The differences between the values of the studied data were tested using Duncan's test where possible under different levels of significance (P≤0.05).

RESULTS AND DISCUSSION

The results of electrophoresis of *NPY* gene and PCR amplification showed the appearance of a 402 bp band for the promoter region -399 rs16147 as shown in Fig.1

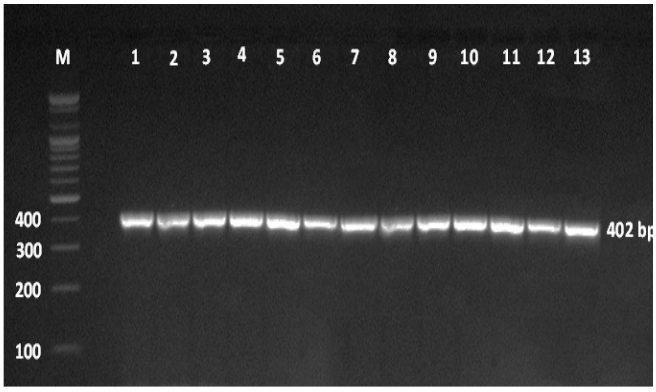

Figure 1: Electrophoresis of the PCR product to detect amplification of the *NPY* gene (*NPY* gene promoter) which apear band with 402 bp on the prepared 2% agarose

The results of the PCR-RFLP replication of the diabetic type II samples and the control samples using the primers and in the presence of the *Alu* I enzyme that targeted the *NPY* sequence at the promoter-339 region which showed three genotypes: the CC genotype (365 bp) and genotypeTC (365, 388 bp) and the TT genotype are represented in the band (388 bp) as in Fig. (2).

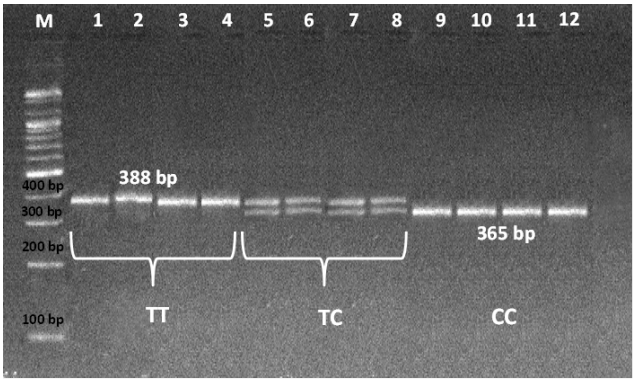

Figure 2: PCR-RFLP electrophoresis for the promoter-399 region (rs16147)

Samples (1, 2, 3, 4) represent the TT genotype; Samples (5, 6, 7, 8) represent the genotype TC; Samples (9, 10, 11, 12) represent the genotype CC; M represents the 100 bp DNA Ladder

Results of the frequency distribution of T and C alleles for the promoter region (rs16147) and using the equilibrium law Hardy-Weinberg equilibrium showed that there was a difference between type 2 diabetes and control sample, the allele T in the sample of 64 patients compared with allele C, which recorded 136 frequency, while the allele T In the control sample 74 compared with the allele C, which was recorded 26. Table 1 shows that the frequency distribution of the T allele was not significant in p <0.05 in the patients samples and the control samples. In Fisher's test, the critical ratio of T allele was 0.864 with a confidence interval of 95% Ranged between 0.577-1.295 while the frequency distribution of the allele C was significantly P <0.05 with the Oods ratio 5.230 with a confidence interval of 95%, ranging between 3.247-8.425.

The high frequency of the allele C in the patients samples and the reduction in the control group confirms the importance of this allele in the disease, a previous study of Iranian society indicated that the SNP (rs16147) is one of the factors that contribute to the susceptibility to metabolic syndrome (hyperglycemia, Insulin, obesity, high blood pressure, high blood lipids), the study showed that the allele T was the highest frequency among the infected, while the allele C was recorded as a protective agent to reduce the risk of metabolic syndrome (12).

The results also showed that there was a change in the frequency of the genotypes of this region. The CC showed a significant high frequency P <0.05 in patients with type 2 diabetes in relation to the control samples and the rates were 54 and 7, respectively, using Fisher's test. The Odds ratio was 3.857 with Confidence interval ranged from 1.636-9.091, while the TT genotype differed significantly less in the sample of patients compared to the control group at P <0.05 with a value of 0.0003. It was frequented in the control samples 31 versus 18 for the patients samples.

The Odds ratio was 0.290 and confidence interval was ranged between 0.148-0.568, while the genotype TC showed a higher frequency in people with type 2 diabetes compared to control group at 28 and 12, respectively, and there was no significant difference. The Odds ratio was 1.166 and the confidence interval ranged between 0.547-2.486. The above results show that the CC genotype is associated with the risk of type 2 diabetes. The present results agree with the findings (13).

Table 1: The observed genotypes of the promoter-399 (rs16147) region in the patients samples with Type II diabetes and control sample.

Genotype	Control - Frequency (No. 50)	Patients – Frequency (No. 100)	P for Association	Odds ratio	(95%CI)
TT	31	18	0.0003	0.2903	0.1482- 0.5689
TC	12	28	0.6897	1.1667	0.5474- 2.4864
CC	7	54	0.0020	3.8571	1.6364- 9.0917
Allele					
T	74 (0.74)	64 (0.32)	0.4811	0.8649	0.5775- 1.2953
C	26 (0.26)	136 (0.68)	p<0.0001	5.2308	3.2475-8.4253

Table 2: Comparison of genotypes at region rs16147 for the gene *NPY* when measuring the level of some biochemical and hormonal variables for pateints with type 2 diabetes

Variables	CC	TC	TT
NPY(pg/mL)	805.28	745.88	630.45
S.Urea (mg/dl)	56.520	49.068	36.690
S.Creatinine (mg/dl)	1.4333	1.3100	1.0627
S.Uric acid (mg/dl)	4.948	4.693	4.153
ALT (U/L)	55.70	59.95	47.16
ALP (U/L)	134.28	129.23	109.08
GGT (U/L)	86.41	78.93	63.61
AST (U/L)	26.51	27.85	23.84
Cholesterol(mg/dl)	272.46	257.20	203.22
TG (mg/dl)	252.54	248.88	175.67
HDL (mg/dl)	35.03	40.95	48.71
LDL (mg/dl)	138.98	130.10	100.04
TSH (mlu/L)	4.351	3.845	2.633
T3 (ng/dl)	0.852	0.863	0.986
T4 (ng/dl)	7.411	7.690	7.749

NPY level

Results showed that there was a difference in the frequency of the genotype of promoter-399 (rs16147) region when measuring the level of NPY in patients with type 2 diabetes, the highest level of NPY in the CC genotype followed by the TC genotype and the lowest level of NPY in the TT genotype as shown in Table (2). The results of the present study showed significant differences P <0.05 between the pateints and the control group. The average level of neurobiptide in patients (Pg / mL 850.43 ± 21.15), while in the healthy people (pg/mL 496.12 ± 28.76) This increase may be attributed to the fact that neurobiptide is a powerful dietary stimulant and thus weight gain is one of the important reasons for the susceptibility to the type 2 diabetes. The results of this study were agreement with many studies that showed a high level of NPY in type 2 diabetic patients (13,14,15).

The level of kidney function

The results of the genetic analysis showed a difference in the level of renal function in the three genotypes of rs16147 where the highest level of urea, creatinine and uric acid was found in the CC genotypes followed by the TC genotype and the lowest of the variables mentioned in the TT genotype were shown in Table 2, The results of the present study showed a significant increase P <0.05 in the level of urea, creatinine and uric acid in patients with type 2 diabetes (59.29 ± 12.12, 1.50 ± 0.21, 5.01 ± 0.91 mg / dl) respectively, compared to its control group (25.58±1.64, 0.83 ± 0.12, 3.82 ± 0.52 mg / dl) respectively, these results were agree with the results of (16).

Level of liver enzymes

The results of phenotypic variability of rs16147 showed a difference in the frequency of genotypes when measuring the level of liver enzymes. The highest level of ALP and GGT was observed in the CC genotype followed by the TC genotype and the lowest level of these two enzymes was found in the TT genotype. while the AST and ALT were highest in the TC followed by CC with a small difference as well as the lowest level of these two enzymes were recorded in TT genotype (table 2), The results of the current study showed a significant increase P <0.05 in the level of liver enzymes ALT, ALP, and GGT in type 2 diabetic patients (63.05 ± 11.6, 139.24 ± 11.4, 91 ± 8.37 U / L), respectively, compared to its control group (36.04 ± 13.62, 95.62 ± 14.78, 48.88 ± 11.68 U / L) respectively, and these results were agree with previous studies that confirm an increase in the level of liver enzymes in type 2 diabetes patients (17,18,19), the results of statistical analysis did not show any significant differences in the concentration of AST enzyme in people with type 2 diabetes compared with the control group, this result was similar to that of (20).

Blood lipid level

In Table 6, there is a variation in the frequency of the genotype of the promoter region rs16147 in patients with type 2 diabetes when measuring blood lipid level. The highest concentration of cholesterol, triglycerides and low-density lipoprotein (LDL) was observed in the CC genotype followed by TC and the lowest level of blood lipid mentioned in the genotype TT, while the highest level of high-density lipoprotein HDL in the genotype TT and recorded the lowest level in the genotype CC, and the results showed a significant increase in the level of probability (p <0.05) in the concentration of cholesterol and triglycerides and LDL in type II diabetics patients (293.5 ± 45.60, 284.7 ± 53.209, 143.68 ± 16.43 mg / dl), respectively, compared with control group (150.32 ± 27.08, 109.96 ± 23.43, 84.32 ± 9 mg / dl) respectively, whereas HDL concentrations was decreased significantly in type two diabetic patients (33. 9 ± 7. 2 mg/dl) compared to control group (55.44 ± 7.37 mg / dl). These results were agreement with previous studies (21,22,23), where these studies showed an imbalance in blood lipids due to type 2 diabetes.

Level of thyroid hormones

The results of phenotypic variability of the promoter-399 (rs16147) region of the *NPY* gene in type 2 diabetic patients showed the highest level of TSH in CC genotype followed by the TC genotype and the lowest level of this hormone in the TT genotype. The T3 and T4 hormone showed the highest level in the genotype CC (table 2) The results of the study showed a significant increase in the concentration of TSH (4.59 ± 12.12 mlu / L) in the plasma type II diabetic patients compared to control group (1.78 ± 0.74 mlu / L), while a significant reduction in serum concentration of T3 (0.8012 ± 0.12 ng / ml) was found in patients serum, the results showed no significant difference in the concentration of T4 in patients with type 2 diabetes compared with the control group. Our results were agreed with (24,25).

The current study showed that the polymorphisms of single nucleotide of *NPY* at site promoter-399 (rs16147) were associated with the risk of type 2 diabetes and that allele C appeared to be associated with the risk of developing the disease.

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