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THE ENPP1 K121Q POLYMORPHISM PROTECTIVE IMPACT AGAINST HYPERTENSION IN IRAQI TYPE 2 DIABETIC PATIENTS

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ABSTRACT

Background

Patients with hypertension having diabetes mellitus or obesity are more likely predisposed to target organ damage. ENPP1is ubiquitously expressed, most importantly in insulin-sensitive tissues such as liver, skeletal muscle and adipose tissue.

Method

A case-control study conducted to find the association between SNP rs1044498 in T2DM with and without hypertension in AL-Najaf Governorate, Iraq. The study included 188 T2DM patients with hypertension randomly selected based on World Health Organization (WHO) guideline AND 148 T2DM patients without hypertension as a control group. DNA was extracted from blood and genotyped by PCR-RFLP by using (AvaII) enzyme. Multinominal logistic regression was applied to compare the proportions of genotypes and alleles. The odds ratio for risk of developing hypertension in T2DM was calculated with and without adjustment for age, sex and BMI.

Results

ENPP1 K121Q gene rs1044498 polymorphism (heterozygous KQ, and homozygous QQ genotype was found to have a protective impact against the development of hypertension in type 2 diabetic patients, after the adjustment for age, sex and BMI.

Conclusions

The SNP of ENPP1 (K121Q) rs10444989 gene have a protective role against the occurrence of hypertension in type 2 diabetic patients in Al-Najaf Governorate,Iraq, andchanges of the serum lipid concentration as well as Rseistin levels may be taken place independent on the types of the investigated genotypes. BMI is seemed to be independent on the genotype of the investigated gene (ENPP1 K121Q rs10444989).

KEYWORDS: Diabetes Mellitus (T2DM), World Health Organization (WHO), Multinomial Logistic Regression, VLDL Cholesterol and TG

INTRODUCTION

Background

Hypertension is a chronic medical condition in which the blood pressure is elevated (Anthea M.et al.1993)**.** Type 2 diabetes mellitus (T2DM) and hypertension are major health problems worldwide, associated with increased prevalence

of obesity and excess morbidity and mortality. Furthermore, patients with hypertension having diabetes mellitus or obesity are more likely predisposed to target organ damage (Chobanian AV.et al 2003, Whiteworth JA. et al. 2003, Olsen MH.et al 2010 and Ogihara T. et al 2009).

Ectonucleotide pyrophosphatase/phosphodiesterase1 (also known as plasma cell-1, *PC-1*) is a 25-exon long gene located on chromosome 6q22-23 (Buckley *et al*. 1990, Lee *at al.* 2010). ENPP1is ubiquitously expressed, most importantly in insulin-sensitive tissues such as liver, skeletal muscle and adipose tissue. The role of ENPP1in relation to insulin resistance and T2DM has been widely investigated.

Several studies have reported that the genomic region where ENPP1was mapped is linked to insulin resistance, highlighting its disease risk-associated effect (Goldfine I. D. *et al*. 2008). Thus, this study is important because it is the first one that investigated this SNP in Iraq Arabic type 2 diabetic patients with hypertension.

METHODS

Study Design

A case–control study of 336 individuals included two groups (188 type 2 diabetic patients T2DM with hypertensionand 148type 2 diabetic patients T2DM without hypertension) randomly selected was conducted to assess the association of SNP(rs1044498) of ENPP1 gene.

The study was performed on 336 type 2 diabetic patients (184 male and 152 female). The patients included two groups {188 type 2 diabetic patients with hypertension (98 male and 90 female), The patients ages ranged between 34-65 years with mean ± SD (48.25 ± 6.31)}and {148 type 2 diabetic patients T2DM without hypertension (86 male and 62 female), the patients ages ranged between 38-66 years with mean ± SD (48.58 ± 6.38) } were included as a control group. The patient population who attended the diabetes center at AL-Sader Medical City, Najaf, Iraq from September 2014 to January 2015. All patients were diagnosed by specialist physicians as having type 2 diabetes, and based on WHO guidelines.

Phenotype Measurements

We collected clinical data, such as weight, height, and other data. The BMI was calculated as weight (in kg) divided by the square of height (in m). Serum chloestrol, triglyceride, high-density lipoprotein cholesterol and low- density lipoprotein cholesterol were measured. Total serum Resistin was estimated using an enzyme-linked immune sorbent assay (ELISA).

Genetic Analysis

Blood samples of T2DM with and without hypertension were collected in EDTA-anticoagulant tubes, and then DNA was extracted from whole-blood samples using DNA extracted kit(Favorgen, Tawan). Then DNA concentration and purity were measured by UV absorption at 260 and 280 nm (Bio Drop, U. K.).

Genotyping was performed by polymerase chain reaction- restriction fragments length polymorphism (PCR-RFLP) for ENPP1K121Q(rs1044498) gene using thermocycler (Biometra,Germany). The primer sequences were obtained from (Simonetta Bacci et al 2005): forward 5’GCAATTCTGTGTTCACTTTGGA3’ and reverse 5’GAGCACCTGACCTTGACACA3’. Amplification was performed in a total volume of 25 µl which contained 12.5 µl of

GoTaq Hot start Green Master Mix,(promega corporation, Madison, W1), 1.5 µl of each primer (1Mm final concentration) (One Alpha, U.S.A.), 3.5 µl Nuclease free water, and 6 µl of DNA template. Cycling condition were 94˚c for 2 min followed by 30 cycles of 94˚c for 1 min, 55˚c for 40$_s$, 72˚c for 40 $_s$ and final extention of 72˚c for 10 min. Amplification product of ENPP1 K121Q (rs1044498) gene was 208 bp. The product was digested with 10 U of restriction enzyme (AvaII)(Promega) and ran on 2% agarose gels.

Statistical Analysis

Student T test and ANOVA test were used to compare phenotypic data between with and without hypertension in T2DM patients using SPSS windows software (spss Inc.,Chicago,IL). Genotype frequencies were tested for Hardy-Weinberg Equilibrium by x^2 test using online software web-Assotest (www.ekstoem.com). Genetic power was calculated using the online software OSSE (OSSE.bii.a-star.edu.sg). Genotype and allele frequencies in with and without hypertension in T2DM patients were tested by multinomial logistic regression analysis with and without adjustment for age, sex and BMI using SPSS.

Results

Anthropometric andbiochemical characteristics of study individuals are presented in table 1. Results of digestion with restriction enzyme (AvaII) for ENPP1 K121Q gene rs1044498 included 208bp band for wild type (KK) genotype, for heterozygous genotype (KQ) three bands 208,155, and 53 bp and for homozygous genotype(QQ) two bands155 and 53bp as shown in figure 1. Genotype and allele frequencies of ENPP1 K121Q gene are shown in table 2.

Genotype frequencies of ENPP1 (K121Q) (rs1044498) gene polymorphisms were consistent with Hardy–Weinberg equilibrium (HWE) in T2DM without hypertension (P=0.317). The power of this study to detect a significant difference at level of 0.05 was 100%.

The heterozygous genotype (KQ) was found to have a protective impact (OR=0.176, CI95%=0.108-0.287, P= 0.001) against the development of hypertension in type 2 diabetic patients, after the adjustment for age, sex and BMI. Also no significant variation was obtained when the analysis was carried out without adjustment. Similarly the homozygous genotype (QQ) was evident to have a somewhat less protective effect (OR= 0.295, CI 95% 0.101-0.864, P= 0.02) against the occurrence of hypertension in the investigated patients with respect to those with wild type. Further analysis of the genotype distribution with use of the dominant and recessive models pointed out a protective role of the rs1044498 SNP of the ENPP1 gene against the development of hypertension in T2DM. The assessment of the minor allele (Q) frequency indicated significant (P=0.0001) elevation in type 2 diabetic patients without hypertension with respect to those without hypertension, strongly suggested the protective impact of the analyzed genotype.

Biochemical characteristics of T2DM with hypertension individuals according to ENPP1 gene polymorphism (rs1044498) genotypes co-dominant and dominant model are shown in table 3 and 4. It demonstrated insignificant association of changes of the investigated parameters with respect to the co-dominant and dominant models

Table 1: Anthropometric Andbiochemical Characteristics of Study Individuals

	T2DM Withhypertension Subjects N=188	T2DM Without Hypertension Subjects	P- Value
No (M/F)	188 (98/90)	148 (86/62)	0.275
Age (y)	48.25 ± 6.31	48.58 ± 6.38	0.727
BMI (kg/m$^{2)}$)	30.29 ± 5.53	29.76 ± 4.92	0.352
Cholesterol (mg/dl)	233.65 ± 36.20	232.82 ± 33.80	0.882
Triglycerides(mg/dl)	229.84 ± 41.00	227.32 ± 38.70	0.565
VLDL (mg/dl)	45.96 ± 8.218	45.46 ± 7.74	0.565
LDL(mg/dl)	140.12 ± 37.40	138.84 ± 35.12	0.748
HDL(mg/dl)	47.56 ± 6.71	48.51 ± 6.58	0.195
Resistin (ng/ml)	10.45 ± 2.16	8.20 ± 2.09	0.020
SBP(mmHg)	126.27 ± 17.25	123.54 ± 11.74	0.086
DBP(mmHg)	80.95 ± 9.65	79.12 ± 8.85	0.071

*****phenotypic data expressed as mean ± standard deviation

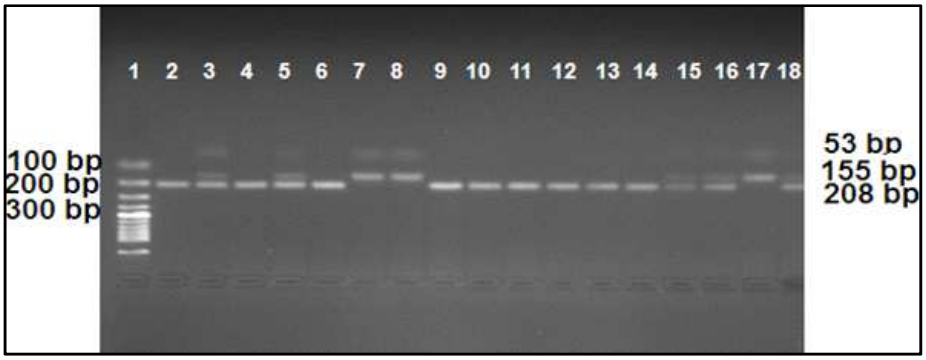

Figure 1: Genotyping Result for ENPP1 K121Q Gene (rs1044498). line 1: Marker Line: For individuals have the Wild Type (KK) of 208bp Fragment (Lines 2,4,6,9-13 and 14): For Individuals have the Heterozygous (KQ)Genotype that Showed Three Fragments with Size of 208 bp, 155 bp, and 53 bp.(Lines 3,5,15-16 and 18):For Individuals have the Homozygous (QQ) Genotype Exhibited Two Fragments of 155 bp and 53bp Size

Table 2: Genotype and Allele Frequencies of Rs1044498 Polymorphism of ENPP1 K121Q Gene and Association of This Variant In T2DM With and Without Hypertension in the Study Individuals

rs1044498 (K/Q)	T2DM With HT* n=188 (%)	T2DM Without HT n=148 (%)	Unadjusted or (CI 95%)	P-value	Adjusted or (CI 95%)	P- value
Co-dominant						
KK(Ref)	133(70.74)	47(31.75)				
KQ	48(25.53)	93(62.83)	0.182 0.113-0.295	0.001	0.176 0.108-0.287	0.001
QQ	7(3.72)	8(5.40)	0.309 0.106-0.899	0.031	0.295 0.101-0.864	0.026
Dominant						
QQ+KQ	55	101	0.192 0.121-0.307	0.001	0.186 0.116-0.298	0.001
Recessive						
KK+KQ(Ref)	181	140				
QQ	7	8	0.677 0.240-1.911	0.461	0.671 0.237-1.896	0.451
Frequency of Q allele	62	109	0.176 0.109-0.283	0.0001		

*HT: hypertension

Table 3: Biochemical Characteristics of T2DM with Hypertension Individuals According to ENPP1 (K121Q) Gene Polymorphism (Rs1044498) Genotype (Co-Dominant Model)

Biochemical Characteristics	KK (n =133) Mean ± SD	KG (n =48) Mean ± SD	QQ (n =7) Mean ± SD	P-value
BMI (kg/m^2)	30.21 ± 5.83	29.99 ± 4.28	33.87 ± 6.04	0.216
Cholesterol (mg/dl)	235.88 ± 37.86	228.46 ± 31.81	226.94 ± 26.49	0.425
Triglycerides(mg/dl)	225.84 ± 40.20	239.08 ± 40.46	242.57 ± 48.47	0.114
VLDL (mg/dl)	45.16 ± 8.04	47.81 ± 8.09	48.51 ± 9.69	0.114
LDL(mg/dl)	143.20 ± 37.68	133.35 ± 35.16	127.90 ± 38.31	0.202
HDL(mg/dl)	47.50 ± 6.32	47.29 ± 7.42	50.52 ± 7.88	0.489
Resistin (ng/ml)	8.31 ± 2.14	7.96 ± 2.02	7.81 ± 1.08	0.547

Table 4: Biochemical Characteristics of T2DM with Hypertension Individuals According to ENPP1 (K121Q) Gene Polymorphism (rs1044498) Genotype (Dominant Model)

Biochemical Characteristics	KK (n =133) Mean ± SD	KG +QQ (n =55) Mean ± SD	P-value
BMI (kg/m^2)	30.21 ± 5.83	30.49 ± 4.73	0.759
Cholesterol (mg/dl)	235.88 ± 37.86	228.27 ± 31.19	0.192
Triglycerides(mg/dl)	225.84 ± 40.20	239.52 ± 41.58	0.078
VLDL (mg/dl)	45.16 ± 8.04	47.90 ± 8.31	0.063
LDL(mg/dl)	143.20 ± 37.68	132.65 ± 35.62	0.079
HDL(mg/dl)	47.50 ± 6.32	47.70 ± 7.56	0.854
Resistin (ng/ml)	8.31 ± 2.14	7.94 ± 1.93	0.277

DISCUSSIONS

The associations of common variants in ENPP1 (rs1044498) with BMI were examined in this study and there were insignificance differences were observed among comparison of the three groups of genotypes. However, in the current study, rs1044498 minor K121 allele seemed to be associated with high BMI values among the three groups of genotypes in T2DM with hypertension.

Several authors have investigated the impact of ENPP1 polymorphism on obesity. However, conflicting data have been reported; Barroso *et al*. 2003, Meyre *et al.* 2005, Bottcher *et al.* 2006 and Santoro N *et al*. 2009 have reported that the Q121 genotype is associated with higher BMI and this finding is similar to our results. Other authors, Matsuoka *et al.* 2006 and Prudente *et al.* 2007 have shown that the Q121 genotype is associated with lower BMI in non-diabetic populations of European and African American populations. In contrast, Grarup N *et al.* 2006, Lyon HN *et al.* 2006, Weedon MN *et al.* 2006 Keshavarz P *et al.* 2006, Kubaszek A *et al.* 2003 and Chen MP *et al.* 2006 found no effect of ENPP1 gene variants, including minor K121Q allele, on body weight.

Discordant results in genotype-phenotype association studies are common in the evaluation of complex disorders (Lyon HN *et al*. 2006). Having established the association between ENPP1 K121Q and hyperglycemia, we explored the interaction between K121Q and BMI because of preliminary evidence that the effect of the Q allele on glycemic traits is mediated by an increase in adiposity (Abate N *et al.* 2005, Meyre *et al.* 2005, Bochenski J *et al.* 2006, McAteer JB *et a*l. 2008, Bacci S *et al.* 2007, Cauchi S *et al.* 2008, Stolerman ES *et al.* 2008 and Meyre *et al.* 2007).

The question here is how ENPP1/PC1 influence BMI? The mechanisms by which it might modulate BMI are unkown (Prudente S *et al*. 2006). It is possible that Q121 allele carries develop insulin resistance via hypothalamic

neurons, where insulin has potent anorectic actions (Plum L *et al*. 2005)

The genetic powers of SNP (rs1044498) was calculated. The genetic power obtained seemed to be higher than the optimal level (80%).

Genotype frequencies of ENPP1 (rs1044498) gene were consistent with HWE in type 2 diabetic patients. Thesefindings were also reported by Michael N *et al.* 2006 and Roberto B *et al*. 2008.

The understanding of common genetic variants influencing T2DM and of the genetic/non-genetic factors with which they interact is a major focus of research to perceive the mechanisms underlying the pathogenesis of the disease as well as related pathological consequences. Combining these genetic variations with new developments in the fields of bioinformatics, genomics, and proteomics will lead to new information on diagnostics, treatment and eventual prevention of the disease of Iraqi society. Advances such as the development of genome-wide association studies (GWAS) have enabled the identification of a number of genes associated with T2DM risk. In this scenario, common genetic variant in ENPP1 (K121Q) has been associated with insulin resistance and its related comorbidities including T2DM by many studies (Lyon *et al*., 2006; Stolerman E. S *et al.*, 2008; Müssig K. *et al*., 2010

The results of genetic frequency of ENPP1 (rs1044498) gene for KK, KQ and QQ were 133(70.74%), 48(25.53%) and 7(3.72%) respectively in T2DM with hypertension, whereas in T2DM without hypertension were 47(31.75), 93(62.83) and 8(5.40) were observed respectively. The derived allele frequency for K allele of K121Q polymorphism of ENPP1 gene was 83.51%, 63.17% in T2DM with and without hypertension respectively, whereas the derived allele frequency for Q allele of K121Q polymorphism of ENPP1 gene was 16.48%, 36.82% in T2DM with and without hypertension respectively. Results of ENPP1 gene polymorphism demonstrated a protective impact against the occurrence of hypertension in T2DM after adjustment for age, sex and BMI. Also, insignificant variation was obtained when the analysis was carried out without adjustment. Similarly the KQ genotype highlited comparable protective influence against the occurrence of hypertension in T2DM.

Previous studies dealt with the association of the investigated polymorphisms with T2DM have revealed positive results for such association. A study on Chinese population by Chen et al 2006, on German Caucasians population by Gouni et al 2006, on Caucasian populations in Sweden and Denmark (Gu HF et al 2000, Rasmussen SK et al 2000), on North Indian Sikh populations (Bhatti JS et al 2010), on British population (Weedon *et al*., 2006), African- and European-Americans, on Polish (Lyon *et al*., 2006) and Tunisian Arabs (Ezzidi *et al.*, 2009) have demonstrated insignificant relationships. Moreover, a cohort study in Spain has shown that the ENPP1 K121Q polymorphism is insignificantly associated with T2D (Gonzalez-Sanchez et al 2003).

Conversslly, some reports have indicated significant variation. A report on South Asian population (Abat *et al* 2005) and some studies suggested a significant association of *ENPP1* (rs1044498) with T2DM in different ethnic population groups in European (McAteer et al 2008) Chinese population (Li *et al*., 2012). Moroccan population (El Achhab *et al*., 2009) and Italy (Bacci et al 2005).

One of the very important observations is the protective effect of the investigated polymorphism against the occurrence of hypertension in T2DM. Under the codominant model, the KQ allele was found to have a significant impact. Simillarly, the QQ genotype was evident to have the same effect. The overall results indicated that the allele K was found

at a high frequency in T2DM with hypertension than those without hypertension, whereas allele Q was present at a slightly high frequency in T2DM without hypertension than T2DM with hypertension.

In our study, the minor allele frequency (MAF) of rs1044498 in T2DM with hypertension is slightly lower (16.48%) than MAF in Malaysian study (17.27%) by (R. Vasudevan et al 2009) while MAF in T2DM without hypertension in our study is higher (36.82%) than T2DM without hypertension in Malaysian study (22.00%). The higher MAF in our population than in Malaysian population might be due to large sample size of our study (148 subjects) compared with (50 subjects) in Malaysian study i.e. our finding is similar to Malaysian study in that mutant allele was found in higher frequency in T2DM without hypertension than T2DM with hypertension that suggest our finding that Q allele is productive role

Furthermore, MAF in our study is lower than Q allele frequency in Central Indian population (18.94%) in diabetic group (Tripathie et al 2013) and in Indian study the MAF was found to be higher in diabetic subjects than the control group (15.71%). Also in our study, the K allele frequency was higher in T2DM with hypertension, reverse results in Indian population were found in which K allele frequency is higher in control group (84.29%) than in diabetic group (81.05%) that suggest a protective role of K allele in Central Indian population

MAF in our study is slightly lower than Q allele frequency in Moroccan population (37.6%) by El Achhab *et al*., 2009 who evaluated the association of the mutant Q allele with T2DM and with obesity and found the MAF is higher in diabetic and obese group than normoglycemic and non-obese group suggest a causative role of Q allele.

The dyslipidaemia that is often present in individuals with T2DM is characterized by hypertriglyceridaemia, raised LDL-cholesterol and a low HDL cholesterol profile (Krauss RM et al 2004). The overstimulation of lipogenesis at the liver due to hyperinsulinaemic conditions is thought to be a critical component of the overproduction of VLDL particles seen in T2DM (Adeli K et al 2001). Adipose tissue can influence glucose homeostasis by release of metabolites (FFA), hormones (leptin, adiponectin, resistin, visfatin) and proinflammatory cytokines (IL-1, TNFα, MCP-1).(Klein S et al 2011). Our findings are consistent with many studies that showed abnormal lipid profile associated with T2DM likeAnne Sofie Andreasen et al. 2010

It is reasonable to speculate on the changes of the serum lipid concentrations with the variations of the genotypes in diabetics with hypertension. Unfortunatlly, such changes seemed to be taken place independent on the genotypes. Really, such phenomenon seemed to be surprising since the SNPwas evident to have a protective impact on the occurrence of hypertension in type 2 diabetics. The situation is to complex and further studies are essential for clarification.

The resistin measurement pointed out significant elevation in the group of T2DM with hypertension when they were compared with those without hypertension. This finding is consistent with some studies that found increased circulating resistin levels and its mRNA expression in adipose tissue in patients with obesity and T2DM (Degawa-Yamauchi et al., 2003) Again levels of resistin concentration in type 2 diabetics with hypertension seemed to be changed independently on the types of genotypes and further studies are critical to achieve the exact mechanism by which resistin is involved in the pathogenesis of hypertension in type 2 diabetes mellitus.

CONCLUSIONS

The SNP of ENPP1 (K121Q) rs10444989 gene have a protective role against the occurrence of hypertension in type 2 diabetic patients in Al-Najaf Governorate,Iraq, andchanges of the serum lipid concentration as well as Rseistin levels may be taken place independent on the types of the investigated genotypes. BMI is seemed to be independent on the genotype of the investigated gene (ENPP1 K121Q rs10444989).

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