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Adiponectin Single Nucleotide Polymorphism 45T/G and its Relationship to Adiponectin Level in Egyptian Patients with Type 2 Diabetes Mellitus

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ABSTRACT: ***Background:*** Adiponectin, is a circulating 30-kDa protein. ***Aim*** is to investigate the association between SNP45 T/G of the adiponectin gene with serum adiponectin levels, insulin resistance and risk of T2DM. ***Subjects and methods:*** The study conducted 64 subjects divided into; group I, 32 diabetic patients & group II, 32 normal glucose tolerance subjects. FBG, lipid profile, fasting insulin & adiponectin by ELISA were done. PCR-RFLP was used to determine the frequency of the SNP45 T/G in the adiponectin gene. ***Results:*** Group I exhibited a higher distribution of TG/GG genotype compared with group II. Logistic regression analysis shows that subjects with TG/GG genotype were at increased risk for T2DM compared with those having TT genotype. Group I, TG/GG genotype had significant higher levels of HOMA-IR and lower plasma adiponectin concentrations compared with TT genotype. ***Conclusion:*** The G allele carriers who have reduced plasma concentrations of adiponectin may have associated insulin resistance.

Key Words: adiponectin; gene ; polymorphism; diabetes mellitus; (HOMA-IR) homeostasis model assessment of insulin resistance; PCR-RFLP (Polymerase chain reaction–restriction fragment length polymorphism); FBG (Fasting blood glucose).

INTRODUCTION

Adiponectin is a protein secreted from adipocytes released in the circulation of human healthy subjects at relatively high levels. Plasma adiponectin levels have been reported as decreased in states of obesity, type 2 diabetes and coronary artery disease (1). Adiponectin exerts its insulin-sensitising effects in the liver by suppressing gluconeogenesis and in the skeletal muscle by enhancing fatty acid oxidation (2 and 3). Adiponectin gene is localized on chromosome 3q27 within the region which was identified as susceptibility locus for type 2 diabetes and metabolic syndrome (4). Genetic associations of SNP in exon 2 (45T/G) of adiponectin gene with type 2 diabetes and adiponectin level were reported in Japanese population and with insulin resistance in some Caucasian populations (Italy, Germany) (5).

The aim of this study was to investigate the association between SNPs 45 T/G of the adiponectin gene with serum adiponectin level, insulin resistance and risk of T2DM.

MATERIALS AND METHODS

The present study was carried out at Clinical Pathology Department in collaboration with Medical Biochemistery & Internal Medicine Departments, Faculty of Medicine, Menufiya University in the period between March, 2009 & March, 2010. Sixty four patients were included in this study and divided into two groups: Group I, thirty tow diabetic patients (twelve males and twenty females) with a mean ±SD of 56.21±7.05 year & group II: thirty two subjects with normal glucose tolerance (fourteen males and eighteen females) with a mean ±SD of 55.90±7.08.none of normal glucose tolerance subjects had previous history of hypertension; family history of T2DM. Patients with impaired glucose tolerance (IGT) were excluded following an oral glucose tolerance test. For all the subjects the followings were done: history and clinical examination, body mass index (BMI), and laboratory investigations including fasting blood sugar, lipid profile (total cholesterol, triglyceride, LDL-C& HDL-C). Fasting insulin levels, plasma adiponectin & the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method was used to determine the distribution of allele and genotype frequencies of the SNP45 T/G in adiponectin gene. Written informed-consents were provided by all participants.

Sampling:

Under complete aseptic conditions, 5 ml of venous blood were collected after 12 hour fasting & divided as follows: Tube A, 1ml of blood collected in citrate (to prevent clotting and DNA degradation) for DNA extraction and kept

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immediately at -20 C°. Tube B, 2 ml were collected, left to clot serum was separated and used for immediate assay of lipid profile& fasting blood glucose. Tube C, 2 ml were collected on citrate, plasma was separated and kept at -20 C° for assay of adiponectin & insulin.

Laboratory Methods:

FPG, total cholesterol, triglyceride, and HDL-C concentrations were determined by using an enzymatic colorimetric assay on Synchron *Cx9. LDL-C concentration was calculated according to the Friedewald formula (6), insulin was measured by use of** DIA source INS-EASIA Kit. The homeostasis model assessment for insulin resistance (HOMA-IR) value was calculated by use of the following formula to estimate the level of insulin sensitivity: HOMA-IR = fasting serum insulin ($\mu U\ ml^{-1}$) × fasting blood glucose ($mg\ dl^{-1}$)/405 (7). Serum adiponectin concentration was measured by Kit was supplied by AviBion Human Adiponectin ELISA ***(Orgenium Laboratories).

DNA analysis:

PCR-RFLP method was used to determine the distribution of allele and genotype frequencies of the SNP45 T/G polymorphism in exon 2 of the adiponectin gene. The DNA was isolated and purified by genomic DNA purification kit****(Fermentas Science Life). The PCR was performed on 30 ng DNA in 20 mL containing 10 mmol/L, Tris HCl, 50 mmol/L KCl, 1.5 mmol/L MgCl2, pH 8.3, 0.2 mmol/L dNTP, 0.035 U/mL Taq polymerase and 0.4 mmol/L forward and reverse primers: forward primers 5'-GCA GCT CCT AGA AGTAGA CTC TGC TG-3' and the reverse primers 5'-GCA GGT CTG TGATGA AAG AGG CC-3'. for 35 cycles (30 s at 94 C°, 30 s at 60 C°, 30 s at 72 C°). The PCR fragments (372 bp) were digested using the restriction enzyme FastDigest®SmaI **** (recognition site: CCC → GGG). The digested samples were separated by electrophoresis on 3% agarose gel stained with ethidium bromide and visualized on a UV trans-illuminator. This resulted in three genotypes identified: (i) wild type TT (372 bp); heterozygous TG (restriction fragments 370, 209 and 160 bp); and homozygous mutant GG (restriction fragments 209 and 163 bp) (fig. 1).

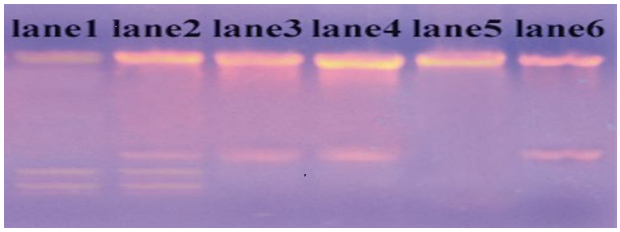

Fig. (1): The three genotypes identified: lane 3, 4 & 6 are the wild type TT (372 bp); lane 2 is the heterozygous TG (restriction fragments 370 , 209 and 160 bp); lane 1 is the homozygous mutant GG (restriction fragments 209 and 163 bp) & lane 5 is the negative control.

Statistical analysis:

Proportions of genotypes of alleles were compared by Chi-square test ($\chi 2$) which used for two qualitative variables analysis. Odd ratios (ORs) and 95% confidence intervals (CI) were calculated by logistic regression analysis. Differences in anthropometric and biological indices in normal glucose tolerance and type 2 diabetic individuals with different genotypes were tested by Mann Whitney U test & the significance level was set at 0.05 or less. All data analysis was performed using SPSS 11.0 software (8).

RESULTS

The results were presented in figures and tables**.**

DISCUSSION

Adiponectin is an adipocytokine which a circulating 30-kDa protein solely expressed in white adipose tissue. Inspite of this fact, its concentration is paradoxically reduced in obesity in contrast with other adipocytokines & is associated with insulin resistance and type 2 diabetes (9).

In the present study; it was found that adiponectin level showed high statistical significant lower level comparing with the normal glucose tolerance group (fig 2: 9.31, 19.78 ug/ml respectively, t=8.18, P<0.001). These findings were in accordance with the results reported by Wasim et al., who found a significant decrease in the plasma adiponectin levels in diabetics compared with individuals with normal glucose tolerance ($p < 0.05$) (10). Also, in a study done by Rizk et al., found that patients with DM had significantly lower levels of adiponectin compared to controls (11) These results support the suggestion that adiponectin may play a crucial role in the regulation of insulin sensitivity and glucose metabolism and that reduced plasma adiponectin levels caused by genetic and environmental factors may lead to the development of insulin resistance, type 2 diabetes and metabolic syndrome (12).

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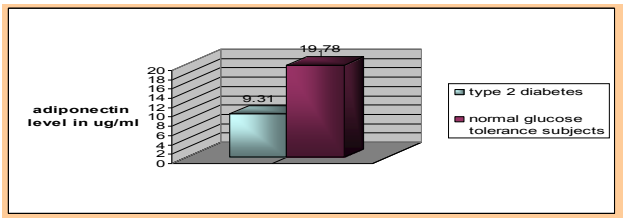

Fig. (2): Comparison between T2DM patients and subjects with normal glucose tolerance regarding plasma adiponectin level.

In fact, several single nucleotide polymorphisms of the adiponectin gene have been associated with insulin resistance, T2DM, and hypoadiponectinemia (13 and 14). However, previous work on ADIPOQ single nucleotide polymorphisms have shown that the SNPs associated with type 2 diabetes or circulating adiponectin levels differ according to both the study cited and the ethnic population studied (15).

The results obtained in this study (table 1) revealed that T2DM group had a statistical significant lower distribution of the TT genotype and T allele frequency than the NGT group (53.1%, 76.60% 81.3%, 90.60 respectively) and a significant statistically higher distribution of the TG/GG genotype and G allele frequency than the NGT group (46.9 %, 23.40%, 18.7 % &9.40 respectively) where $\mathbf{X^2}$ =5.741, 4.61& p=0.017, 0.031 respectively. These results were in accordance with a study done in Uygurs of the Xinjiang region, China by Li et al., who found that comparing with the normal glucose tolerance group, the T2DM group exhibited a higher distribution of the TG + GG genotype, G allele frequency ($\mathbf{X^2}$ =5.03 , P = 0.025) (16).

Table (1): Differences in allele distribution and genotype frequency of adiponectin gene single nucleotide polymorphism (SNP) 45 between studied groups

	Studied groups		χ^2 (P value)
	Group I (T2DM) N =32	Group II (NGT) N =32	
genotype TT TG & GG	 17(53.1%) 15(46.9 %)	 26(81.3%) 6 (18.7 %)	5.741 (0.017) S
Alleles T G	N = 64 49 (76.60) 15 (23.40)	N = 64 58 (90.60) 6 (9.40)	4.61 (0.031) S

P: Probability of error P < 0.05 significant NGT: normal glucose tolerance subjects T2DM: type 2 diabetes patient's mellitus.

Also, another study done in China by Sun et al., found that in patients with T2D, the allelic frequency of *45T/G* was 28.8%, which was close to the frequencies in the Japanese population. While, the allelic frequency of 45T/G was 21.3% in healthy volunteers with a significant difference in allelic frequency of patients with T2D and healthy controls ($P < 0.05$) (17). Another study done in Iran by Mohammad Zadeh and Zarghami, found that the G allele and TG/GG genotype of SNP 45 occurred more frequently than the T allele and TT genotype in T2DM patients comparing with the controls (p<0.05) (14).

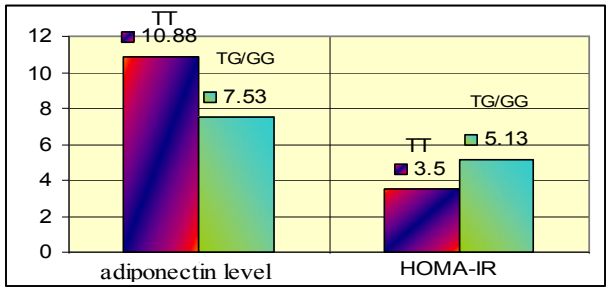

Figure (3): Comparison between TT&TG/GG genotypes of T2DM patient group regarding to adiponectin level and HOMA-IR

The current study addressed the question of whether plasma adiponectin is related to SNP45 T/G polymorphism and. The results (table 2) revealed that plasma level of adiponectin in individuals with the TG/GG genotype in the T2DM group were significantly lower than those with the TT genotype (7.53±2.99, 10.88±5.06, ug/ml respectively p= 0.020) (fig.3). Meanwhile, the plasma adiponectin concentration of TG /GG genotype carriers was not significantly different from that of the TT genotype in the NGT group (21.0±4.85, 19.50±5.90 ug/ml respectively p=0.438). By regression analysis (table 3) (fig.4), low plasma level of adiponectin carry the risk of T2DM (OR= -4.006, CI 95%). In the study done by Li et al., plasma levels of adiponectin of the TG / GG genotype in the T2DM group, were significantly lower than those of the TT genotype while adiponectin concentration of TG/ GG genotype carriers was not significantly different from that of the TT genotype in the NGT group (16). Also, the study done by Rizk et al., revealed a significant association between the SNP 45TG in the adiponectin gene and low serum adiponectin levels (p<0.001) (11).

The results obtained by this study (table 2) revealed that in the T2DM group, compared with SNP45 T carriers, G carriers had higher HOMA-IR (5.13±1.90, 3.50±2.10, P= 0.014) while no significant differences in the other indices between these two genotype subgroups (p > 0.05) (fig.3). These results were in accordance with Li et al., where higher HOMA-IR was found in G carriers versus T carriers in the T2DM group (P < 0.05) (16). Logistic regression analysis revealed that, subjects with increased HOMA-IR were at increased risk for T2DM (OR= 3.227, CI 95% 1.770-8.021). The obtained results point to a significant role of the TG/GG genotype in plasma adiponectin levels and in the risk of T2DM. It was established recently that replenishment of

adiponectin may ameliorate insulin resistance in animal models of T2DM resulting from a high-fat diet (21) Administration of physiological doses of adiponectin lessened triglyceride accumulation in skeletal muscle and liver by facilitating fatty acid combustion and energy dissipation (22).Therefore, deficiencies in adiponectin would presumably trigger insulin resistance, en route to the development of T2DM. Also, the SNP45 polymorphism may affect insulin resistance, possibly through changes in mRNA stability, levels of adiponectin and eventually reduced plasma adiponectin concentrations (16).

TABLE 2: Relationship between anthropometric & biological indices in the studied groups stratified by adiponectin SNP 45

PARAMETER	STUDIED GROUPS				MANN WHITNEY U TEST	P VALUE
	Group I (T2DM) N =32		Group II(NGT) N =32			
	TT N = 17	TG/GG N = 15	TT N = 26	TG/GG N = 6		
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD		
BMI	30.76±2.58	30.53±2.32	24.30±1.37	24.33±1.94	0.244* 0.512**	0.731 0.609
FPG (mg/dl)	152.35±22.38	151.20±27.79	81.03±5.23	86.0±5.69	0.378* 1.555**	0.706 0.120
Cholesterol (mg/dl)	210.76±18.67	209.40±18.79	170.73±9.07	179.50±4.50	0.302* 2.156**	0.762 0.031
TG (mg/dl)	232.29±12.64	237.40±12.32	92.53±4.89	89.33±5.71	757* 1.139**	0.449 0.255
HDL-c (mg/dl)	34.35±3.18	35.63±2.70	43.38±1.60	42.83±1.83	1.521* 0.671**	0.128 0.502
LDL-c (mg/dl)	184.17±32.64	168.66±17.14	113.96±8.58	121.83±4.62	1.209* 2.082**	0.227 0.037
Systoic blood pressure	138.23±11.58	136.0±9.29	117.69±5.87	123.33±4.08	0.497* 2.217**	0.619 0.027
Diastolic blood pressure	80.88±6.18	79.33±7.28	77.50±7.10	85.0±4.47	0.852* 2.279**	0.394 0.023
Heart rate	82.0±5.19	81.26±4.47	74.96±3.79	73.50±3.56	0.417* 0.970**	0.677 0.332
Fasting plasma adiponectin (µg/ml)	10.88±5.06	7.53±2.99	19.50±5.90	21.0±4.85	2.332* 0.775**	0.020 0.438
HOMA-IR	**3.50±2.10**	**5.13±1.90**	**1.82±0.11**	**1.83±0.11**	**2.448* 0.291****	**0.014 0.771**

* = Comparison between TT and G carriers among T2DM group ** = Comparison between TT and G carriers among NGT group P: Probability of error
P < 0.05 significant P > 0.05 non significant SD= standard deviation

In contrast to the present results, Menzaghi et al., stated that no difference was observed in serum adiponectin levels at baseline among the SNP45 genotypes in their study (23). Moreover, in the study done by Lee et al., insulin resistance and plasma levels of adiponectin were not statistically different according to T45G, in both control and T2DM subjects (18). These Conflicts amongst association studies may be explained by differences in sample size or even in the linkage disequilibrium structure at this locus in different populations.

The study of the association between SNP45 and anthropometric and other biological indices in the NGT group (table 2), revealed that the comparison between SNP45 T carriers, G carriers show higher levels of SBP,

LDL-C & total cholesterol (P < 0.05 for all).While, no significant differences in HR, BMI, HOMA-IR, TG and HDL between these two genotype subgroups (all P >0.05). These results were similar to those obtained by Li et al., & suggested that adiponectin gene SNP45 may affect blood lipid metabolism in normal glucose subjects (16).

Table (3): Multivariate regression analysis of morbid risk factors of DM

Risk factors	P value	Odds Ratio	95% CI	
			Lower	Upper
BMI	0.197	1.033	0.455	6.889
TG & GG genotype	0.017	3.82	1.239	11.80
G allele	0.031	2.96	1.07	8.21
Adiponectin	0.012	- 4.006	2.254	9.623
HOMA - IR	0.010	3.227	1.770	8.021

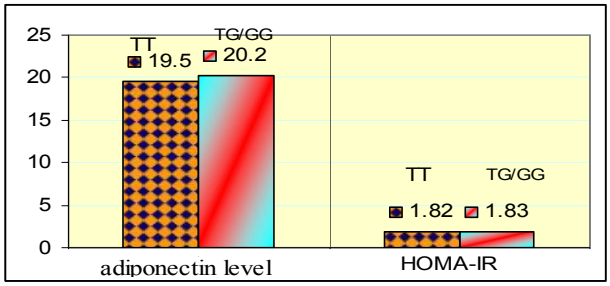

Figure (4): Comparison between TT&TG/GG genotypes NGT group regarding to adiponectin level and HOMA-IR

Using logistic regression analysis (table3) revealed that subjects with the TG/GG genotype were at increased risk for T2DM (OR 3.82, 95% CI 1.239 – 11.80) compared with those having the TT genotype. In a study done by Hara et al., stated that the SNP45 was significantly associated with risk of T2DM in Japanese (13). These results agreed with that obtained by Mohammad zadeh & Zarghami who concluded that subjects with the G/G + TG genotype of SNP 45 were at increased risk for T2DM (OR 2.574; 95% CI 1.051-6.302) compared with those having TT genotype (14). These findings are consistent with data showing that the G allele of SNP45 is significantly associated with T2DM. Consequently, it is logical to guess that the adiponectin SNP45 polymorphism may be interconnected with the incidence of T2DM where the G allele may be the vital risk factor for T2DM (16).

Meanwhile, in a study done in Qatari population by Rizk.,et al., concluded that inspite of significant differences in allele frequencies of SNPs 45 comparing controls with T2DM patients, but no significant association was found between this SNP and the risk for developing T2DM (p=0.760) (11). Also, In contrast to these results, a study done in Korea by Lee et al., found that there was no statistically significant differences in allele frequencies of SNPs 45 comparing controls with T2DM subjects (T frequency 68.3% vs. 71.6%, P = 0.13). The genotype distributions of these SNPs had no association with the risk of T2DM (18). Moreover, a study done in Polish Caucaian populations revealed no significant difference in G allele frequency comparing controls with T2DM patients (7% ,8% respectively p=0.48) (19). Furthermore, the study done in Italian population by Chiodini et al., revealed that there was no significant association between SNP 45 and the risk for T2DM (20).

In conclusion, the adiponectin SNP45 polymorphism may be associated with the T2DM & its risk is higher predictable with G allele carriers, which may be emphasized by variation in adiponectin concentrations in these G allele carriers. More prospective studies, including measuring the exact quantity of adiponectin expressed in white adipose tissues obtained from individuals with the SNP45 genotypes to explain the role of SNP45 in alteration of adiponectin expression. Also, to establish the means by which SNP45 influence insulin sensitivity and accordingly the risk of T2DM.

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