JÖKULL JOURNAL
JOKULL ISSN: 0449-0576
Vol 65, No. 2;Feb 2015

Genetic Variants Associated with the Risk of Diabetic Nephropathy

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Background: One of the most common complications of diabetes mellitus is diabetic nephropathy (DN). The objective of the study was to investigate the relationship between variants of transcription factor 7–like 2 (TCF7L2) and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PPARGC1A) gene polymorphisms individually or in combination with the progression of nephropathy in type 2 diabetes mellitus (T2DM) patients.

Methods: Eighty five T2DM patients (Forty five with nephropathy and forty without nephropathy), and forty five healthy control subjects were included in the study. The polymorphisms were evaluated by PCR/RFLP analysis.

Results: The frequency of TCF7L2 rs7903146 TT genotype and T allele were significantly associated with DN patients compared to normal controls (P= 0.016 and 0.008), respectively and compared to T2DM patients without nephropathy (P=0.023 and 0.018), respectively. A significant differences in AA genotype distribution for the Gly482Ser polymorphism of PPARGC1A gene was observed between T2DM with nephropathy cases compared to control subjects (P=0.002). Moreover, a significant association in A allelic frequencies was observed in DN cases compared to control subjects at P=0.019 and compared to T2DM patients without nephropathy at P= 0.035. No differences in the genotypic and allelic frequencies between cases and controls were found for the Thr394Thr polymorphism.

Conclusions: Our study suggests that candidate gene polymorphisms rs7903146 of TCF7L2 and Gly482Ser of PPARGC1A may serve as a susceptibility biomarker for nephropathy in type 2 diabetes mellitus patients.

Keywords.
Diabetic nephropathy,
Type 2 diabetes mellitus,
Transcription factor 7–like 2,
Peroxisome proliferator activated receptor gamma coactivator-1 alpha
Gene polymorphism

1. Introduction

Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus ***(Movva et al., 2007)***. Complications from diabetes can be categorized as microvascular or macrovascular. Microvascular complications include nervous system damage (neuropathy), renal system damage (nephropathy) and eye damage (retinopathy). Diabetes and its complications prove to be major cause of morbidity and mortality around the world and contribute largely to health care costs ***(Deshpande, 2008)***. In Egypt the prevalence of retinopathy was 41.5%, neuropathy ranged from 21.9% in hospital outpatient clinics to 60% in hospital inpatient clinics and nephropathy ranged from 6.7% in hospital outpatient clinics to 46.3% in hospital inpatients ***(Hamed et al., 2008)***. Several studies have revealed that optimal glycaemic control is the ideal primary preventive measure against the development of complications ***(Ho tang et al., 2007; Nam et al., 2011)***. Diabetic nephropathy is the major single cause of End Stage Renal Disease (ESRD) in developing countries and extrapolations suggest that this number will multiply in the future. Between 20 to 40 % of all diabetic patients are prone to developing kidney failure, and family-based studies suggest that a significant genetic component confers risk for DN ***(Krolewski, 1999; Iyengar et al., 2003)***.

The transcription factor 7–like 2 (TCF7L2) is a transcription factor that involved in the regulation of insulin secretion, incretin effects, and an enhanced rate of hepatic glucose production ***(Groves et al., 2006; Zhang et al., 2006)***. The TCF7L2 gene on chromosome 10q25, a part of the Wnt signalling pathway, was shown to have a strong association with the increased risk for developing type 2 diabetes mellitus ***(Florez et al. 2006)*** and may contribute to the etiology of nephropathy in type 2 diabetes in an interactive manner with other genes ***(Wu et al., 2009)***. Recently, Hussain and his colleges reported a significant association between the T allele and DN, but this association is not independent of T2DM ***(Hussian et al., 2014)***.

Peroxisome proliferator-activated receptor gamma coactivator 1 alpha, encoded by the PPARGC1A gene, a co-activator of nuclear receptors, was discovered as a molecular switch that turns on several key components of the adaptive thermogenic program in brown fat, including the stimulation of fuel intake, mitochondrial fatty acid oxidation, and heat production. PPARGC1A is strongly expressed in kidney, brown adipose tissue, heart, skeletal muscle, and brain, all of which are highly oxidative tissues ***(Liang and Ward, 2006)***. The PPARGC1A gene is located on chromosome 4p15.1, a genetic linkage analysis has indicated that this chromosomal region is linked with pre-diabetic phenotypes. Therefore, PPARGC1A gene is a strong biological and positional candidate for the susceptibility of T2DM ***(Pratley et al., 1998)*** and may play a role in susceptibility to diabetic nephropathy ***(Gayathri et al., 2010)***.

To our knowledge, no enough data are available concerning the association of TCF7L2 and PPARGC1A gene polymorphisms with nephropathy in type 2 diabetes mellitus Egyptian patients. Therefore the current study aimed, to analyze the

polymorphisms rs7903146 (C/T) within TCF7L2 gene and Thr394Thr (G/A) and Gly482Ser (G/A) within PPARGC1A gene individually or in combination, and to evaluate their contribution to susceptibility to nephropathy in T2DM patients.

2. Subjects and methods:

2.1. Study subjects

This study recruited 85 T2DM patients diagnosed at least 5 years before, the patients consecutively attended the diabetes clinic of Internal Medicine Department; Kasr El-Aini Hospital affiliated to Cairo University. Forty five of them (12 males and 33 females) with nephropathy, while the remaining 40 (9 males and 31 females) without nephropathy. The mean age of T2DM patients with nephropathy was 48.47±8.89 years compared to 54.43±8.45 years for the T2DM patients without nephropathy. The diagnosis of T2DM was based on the American Diabetes Association criteria, a fasting plasma glucose level >126 mg/dL and glycated hemoglobin > 6.5% and/or treated by oral hypoglycemic agents and/or insulin to achieve glycemic control. Diabetic nephropathy was defined by persistent albuminuria (albumin-creatinine ratio [ACR] > 30 mg/g creatinine) on at least two consecutive occasions over the previous six months. While patients with ACR < 30 mg/g creatinine were normoalbuminuric and had no nephropathy. To avoid misclassification diabetic patients as having; abnormal liver function, abnormal thyroid function, advanced renal diseases other than diabetes, cardiovascular disease were excluded.

Beside 45 healthy subjects (17 males and 28 females) with mean age 51.04±7.97 years and without any previous history of diabetes or renal disorders were enrolled as control group. The study protocol was approved by the Research Ethics Committee; Faculty of Pharmacy- Cairo University (REC-FOPCU), serial number of the protocol was BC (209). A written informed consent was obtained from each participant before testing.

2.2. Sampling

Venous blood was obtained from patients and controls after an overnight fast of 12 hours and was divided into two portions. One portion of blood (2 ml) was added to EDTA and stored at −20 °C for detection of TCF7L2 and PPARGC1A gene polymorphisms as well as glycated hemoglobin assay. A second portion of 1 ml was added to Fluoride + EDTA and centrifuged at 1000 ×g for 10 min; the plasma was then separated for determination of fasting plasma glucose level.

Morning urine samples were collected under aseptic conditions from patients and controls. Urine was centrifuged at 500 ×g to get rid of the cells and salts then the supernatant were used for determination of urinary albumin and creatinine concentrations.

Urinary albumin concentration was assessed using enzyme-linked immunosorbent assay (ELISA) kits provided DRG-USA according to the manufacturer's protocol. Fasting blood glucose, glycated hemoglobin and urinary creatinine were measured using commercially available kits following the manufacturer's instructions.

2.3. Isolation of genomic DNA

Genomic DNA was isolated from peripheral white blood cells using Qia-amplification extraction kit (Qiagen-USA) according to the manufacturer's instructions.

2.4. Genotyping method for rs7903146 (C/T) polymorphism of TCF7L2 gene

The genotyping of TCF7L2 rs7903146 (C/T) was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. A 266 bp fragment of the gene was amplified with primer sequences fwd: 5' CTGAACAATTAGAGAGCTAAGCACTTTTTAGGTA-3'; rev: 5' TTTCACTATGTATTGTTGCCAGT CAGCAAACAC-3' ***(Habalova et al., 2009)***. PCR amplification was performed with 5 pmol of each primer in combination with Taq PCR Master Mix (Qiagen, Valencia, CA, USA). The PCR cycling conditions were: initial denaturation at 94°C for 5 min, then the PCR was carried out for 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and final cycle of 72°C for 10 min (T-Gradient thermal cycler, Biometra, Germany). The PCR products were digested 2 hours at 37°C with 10 U of RsaI (New England Biolabs, Hitchin, UK). The digested products were resolved on 3% agarose gel and identified by Red-Safe staining. Allele T was detected as a 266-bp fragment, allele C as 233- and 33-bp fragments (Figure 1).

2.5. Genotyping method for Thr394Thr and Gly482Ser polymorphisms of PPARGC1A gene

The genotyping was carried out for two polymorphisms of PPARGC1A gene namely Thr394Thr and Gly482Ser polymorphisms. The two polymorphisms were genotyped by PCR-RFLP method. The sequences of the primers to detect Thr394Thr polymorphism were 5'-GCCAGTCAATTAATTCCAAACC-3' and 5'-TTGGAGCTGTTTTCTTGTGC-3' ***(Hara et al., 2002)*** while the sequences of the primers that used to detect Gly482Ser polymorphism were 5'-CAAGTCCTCAGTCCTCAC-3' and 5'-GGGGTCTTTGAGAAAATAAGG-3' ***(EK et al., 2001)***. The PCR cycling condition was: denaturation at 95°C for 5 min followed by 35 cycles a 95°C for 45 sec, 50°C for 45 sec, 72°C for 45 sec, and a final extension at 72°C for 10 min. Ten ul of the PCR products were analysed on 2% agarose gels stained with Red-Safe to detect the amplification of the 203 bp and 611 bp for Thr394Thr and Gly482Ser polymorphism respectively. The PCR products were digested 2 hours at 37°C with *Msp*I for Thr394Thr polymorphism and *Hpa*II for Gly482Ser polymorphism (New England Biolabs, Hitchin, UK). The digested products were then analysed by electrophoresis on a 3% agarose gel stained with Red-Safe and the genotypes were determined. There were three genotypes, homozygous AA (611 bp), GG (366 bp and 245 bp) and heterozygous GA (611 bp, 366 bp and 245 bp) for Gly482Ser polymorphism (Figure 2) while, homozygous AA (203 bp), GG (182 bp and 21 bp) and heterozygous GA (203 bp, 182 bp and 21 bp) for Thr394Thr polymorphism (Figure 3).

2.6. Statistical analysis

Data are expressed as means ± standard deviation for quantitative variables and frequency for qualitative variables. Quantitative variables were compared using independent Student t-test and One-way ANOVA LSD test was used for multiple post-hoc comparisons. On the other hand, qualitative variables were compared using chi square ($\chi 2$) test or Fischer's exact test. One-way ANOVA was used to compare the biochemical characteristics of patients divided according to genotypes. The Statistical Package for the Social Sciences software (SPSS 17.0, Chicago, IL, USA) was used. $P< 0.05$ was considered significant.

3. Results:

3.1. Demographic and biochemical data of the studied groups:

Demographic and biochemical characteristics of type 2 diabetes mellitus (T2DM) patients with or without nephropathy and control subjects are shown in table (1). There was no significant difference among T2DM with and without nephropathy and control groups regarding sex distribution (M/F: 12/33; 9/31; 17/28, respectively). While regarding age, there was a significant difference between diabetic nephropathy (48.47±8.89) and diabetic without nephropathy groups (54.43±8.45) at P= 0.002. Furthermore, the diabetes duration was significantly higher in T2DM patients who developed nephropathy (11±4.8 years) compared to diabetic patients without nephropathy (8±4 years). In comparison with the healthy controls, the diabetic patients with nephropathy had significantly higher fasting plasma glucose level (86.3±7.99 and 289.3±98.35 mg/dl, respectively), and glycated hemoglobin percent (5.4±0.3 and 9.92±1.95, respectively) moreover, the diabetic with nephropathy patients had significantly higher fasting plasma glucose level reaching 117.5% of that of the diabetic without nephropathy patients, and significantly higher glycated hemoglobin percent reaching 112.7% of that of the diabetic without nephropathy patients. On the other hand, T2DM patients with nephropathy compared to those without nephropathy and control healthy subjects had significantly higher urinary total protein (449.8±130; 97.2±50; 34.6±9.3 mg/L, respectively), urinary albumin (90±66.4; 13.97±9.96; 6.9±1.9 mg/L, respectively), and albumin creatinine ratio (104.5±93.3; 12.95±9; 6.95±2.5 mg/g, respectively). There was statistical difference in the urine creatinine level between the two diabetic patients groups with (0.98±0.44 g/L) and without nephropathy (1.14±0.4 g/L) at P= 0.046. Moreover, compared to diabetic patients without nephropathy, the poor diabetic control was significantly higher in T2DM patients who developed nephropathy.

3.2. Genotype distributions and allele frequencies of rs7903146 (C/T) polymorphism in TCF7L2 gene

The TCF7L2 rs7903146 genotype distributions and its allele frequencies in the studied groups (Table 2) revealed that the mutant TT genotype was significantly higher in T2DM with nephropathy patients (20%) compared to T2DM without nephropathy patients (5%) and the normal controls (6.7%) at P= 0.023 and 0.016, respectively. In addition, the

frequency of the mutant T allele was significantly higher in T2DM with nephropathy patients (45.6%) compared to T2DM without nephropathy patients (28.8%) and controls (26.7%) at P= 0.018 and 0.008, respectively. When the pure mutant TT genotype was pooled with CT and CC, we found the TT genotype to be higher than CC+CT genotypes in the diabetic nephropathy group compared to the diabetic without nephropathy group at P= 0.04.

3.3. Genotype distributions and allele frequencies of Gly482Ser and Thr394Thr polymorphism in PPARGC1A gene

Regarding the Gly482Ser genotype distributions and allele frequencies in different studied groups (Table 2), the frequency of the mutant AA genotype was significantly higher in T2DM patients with nephropathy 17.8% compared to controls 0% (P = 0.002). Moreover, the mutant A allele showed a significantly higher frequency in T2DM with nephropathy group 43.3% compared to T2DM without nephropathy 28.8% and control 26.7% groups (P= 0.035 and 0.019, respectively). When the pure mutant AA genotype was pooled with GA and GG, we found the AA genotype to be higher in the diabetic nephropathy group than in the control group at P= 0.003.

On the other hand, the Thr394Thr (G/A) genotype distributions and allele frequencies in the studied groups are shown in table (2). There were no significant differences among different studied groups regarding the distribution of homozygous GG (51.1%; 62.5%; 53.3%), heterozygous GA (33.3%; 30%; 33.3%) and homozygous AA (15.6%; 7.5%; 13.4%) and. Also, no significant difference was detected in the mutant A allele among studied groups (32.2%; 22.5%; 30%).

3.4. Biochemical characteristics of T2DM patients with and without nephropathy with different rs7903146 genotypes in TCF7L2 gene

The biochemical characteristics of T2DM patients with and without nephropathy having different rs7903146 genotypes of TCF7L2 gene were showed in table (3). In T2DM with and without nephropathy patients, the biochemical characteristics showed no significant differences between different genotypes.

3.5. Biochemical Characteristics of T2DM patients with and without nephropathy with different Gly482Ser and Thr394Thr genotypes in PPARGC1A gene

Table (4) showed the biochemical characteristics of T2DM patients with and without nephropathy having different Gly482Ser and Thr394Thr genotypes of PPARGC1A gene. The Gly482Ser genotypes in T2DM with nephropathy patients, there was significant difference between the mutant GA and AA genotypes compared to the wild GG genotype regarding the fasting plasma glucose level (317.6±85.4 and 226.78±98.9, respectively) at P= 0.003 as well as urinary albumin concentration (141±78 and 78.5±38, respectively) at P= 0.014. However, in T2DM without nephropathy patients, the biochemical characteristics showed no significant differences between different genotypes.

Regarding to the Thr394Thr genotypes in T2DM with and without nephropathy patients, the biochemical characteristics showed no significant differences between different genotypes.

3.6. The combined effect of TCF7L2 rs7903146, PPARGC1A Gly482Ser and PPARGC1A Thr394Thr genotype frequencies in T2DM patients with and without nephropathy

To evaluate the interaction between the genotypes, the combined effect of TCF7L2 rs7903146, Gly482Ser and Thr394Thr genotypes was examined (Table 5). The frequency of the TT/AA/AA combined mutant genotypes were only reported in diabetic patients who developed nephropathy (8.9%) compared to the diabetic without nephropathy group (0%) at P= 0.014. On the other hand, the wild genotypes CC/GG/GG had significantly higher combination in diabetic without nephropathy patients (35%) compared to diabetic without nephropathy patients (15.6%) where T2DM patients with wild genotypes CC/GG/GG and/or without mutant genotypes TT/AA/AA have a protective effect against nephropathy.

4. Discussion:

Diabetic nephropathy is a serious complication in diabetes. Determining the causes that trigger the functional and structural changes that result in the progression of nephropathy in T2DM patients is essential for diagnostic, prevention, and therapeutic purposes. There was available data suggest that multiple factors contribute to this complication. Poor glycemic control, metabolic abnormalities, hemodynamic alterations, hypertension, and genetic factors are the major contributing factors ***(Chowdhury et al., 1999)***.

An initial finding in this study is the significant elevation in both fasting plasma glucose level and glycated hemoglobin percent in diabetic nephropathy patients in comparison with diabetic without nephropathy and control groups. Moreover, the poor diabetic control was significant risk factor for progression of nephropathy in diabetic patients. In this regard, ***El-Wakf et al. (2011)*** showed that, fasting blood sugar and glycated hemoglobin were significantly increased in diabetic patients with nephropathy when compared to diabetic patients without nephropathy. Thus, indicating the importance of both glucose and glycated hemoglobin levels as predictors for developing nephropathy among diabetic patients. Our result could be explained on the basis that hyperglycemia is a crucial factor in the development of diabetic nephropathy because of its effects on glomerular and mesangial cells, but alone it is not causative. Mesangial cells are crucial for maintenance of glomerular capillary structure and for the modulation of glomerular filtration via smooth-muscle activity. Hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, as well as increased matrix production and basement membrane thickening ***(Harris et al., 1991; Heilig et al., 1995; Mishra et al., 2005; Lin et al., 2006)***. Furthermore, increased blood glucose level induces injury in the kidney through

various pathways, including increases in the formation of reactive oxygen species (ROS), as well as advanced glycation end products, (AGEs) which play a critical role in the development of diabetic nephropathy ***(Chen et al., 2001; Twigg et al., 2002)***. Among the most investigated AGEs is glycated hemoglobin (HbA1c) which is the product of a slow and largely irreversible reaction that occurs through non enzymatic glycation of hemoglobin ***(John, 1997)***. Chronic hyperglycemia, as measured by HbA1c, is an established risk factor for diabetes associated microvascular diseases and a reduction of 1% in HbA1c is associated with a 37% decrease in microvascular endpoints ***(Stratton et al., 2000)***.

The present study showed that there was a significant increase in urinary total protein, urinary albumin concentration, and albumin creatinine ratio of T2DM patients with nephropathy compared to those without nephropathy and control healthy subjects. Our finding is supported with ***El-Wakf et al. (2011)*** and this increase could be explained on the basis that albuminuria, elevated urinary albumin excretion, is presented as a powerful predictor of progress to nephropathy in patients with T2DM ***(Keane et al., 2003)***. Albuminuria may reflect underlying renal expression of vascular damage, hypertension, endothelial dysfunction ***(Stehouwer et al., 2004)***, and inflammation ***(Ritz, 2003)***. Therefore, it has become clear that albuminuria is not only indicator for diabetic renal disease, but also for progress to more advanced stages of the disease ***(Murussi et al., 2008)***. At first, small amounts of the blood protein albumin begin to leak into the urine in people who are developing kidney disease, a condition called microalbuminuria (albumin-creatinine ratio [ACR] < 30 mg/g). The kidney's filtration function usually remains normal during this period. As the disease progresses further, more albumin leaks into the urine, a stage known as macroalbuminuria (ACR > 30 mg/g) or proteinuria. As the amount of albumin in the urine increases, the kidney's filtering function usually begins to drop, resulting in the body's retention of various wastes and abnormalities of the glomerular endothelial barrier, causing excessive filtration as well as reduction of renal tubular cell albumin degradation and reabsorption ***(Stehouwer et al., 2004)***.

The present study demonstrated a significant increase in diabetes duration in diabetic albuminuric group compared to diabetic normoalbuminuric group. In agreement, ***Unnikrishnan et al. (2007)*** and ***El-Wakf et al. (2011)*** reported that duration of diabetes was significantly higher in patients with micro and macro- albuminuria as compared to normoalbuminuric group. Suggesting that progression of diabetic renal disease occurs in association with increase in duration of diabetes.

Despite multiple candidate gene association studies and genome wide scan investigations, major genes that play a key role in susceptibility to diabetic nephropathy have yet to be identified ***(Lindner et al., 2003; Tanaka and Babazono, 2005; Rich, 2006; Sale and Freedman, 2006; Savage et al., 2007)***. Identification of genes that contribute to the risk of DN will ultimately allow identifying patients who are at risk ***(Rich, 2006)***. Also,

it is important to address gene–gene interactions for describing a trait involving complex disease-related mechanisms, particularly when each involved factor only demonstrates a minor marginal effect ***(Carlborg and Haley, 2004; Bell et al., 2006)***. In an attempt to study rs7903146 (C/T) polymorphism in TCF7L2 gene and Thr394Thr (G/A) and Gly482Ser (G/A) polymorphisms in PPARGC1A gene, and find out their impact on susceptibility to nephropathy in T2DM patients, the genotype distribution and allele frequencies of the forementioned genes were evaluated in controls, as well as T2DM patients with and without nephropathy. In addition, the effect of different genotypes on the assessed biochemical characteristics of the patients was evaluated.

Regarding the TCF7L2 rs7903146 C/T polymorphism, the mutant TT genotype and/or T allele were significantly associated with diabetic patients who developed nephropathy when compared to diabetic patients without nephropathy and control healthy subjects. Our finding was supported by ***Buraczynska et al. (2011)*** who showed that the mutant T allele of the rs7903146 polymorphism in the TCF7L2 gene is strongly associated with nephropathy, especially in early onset of diabetes. Recently, ***Buraczynska et al. (2014)*** and ***Hussain et al. (2014)*** reported that the T allele of the rs7903146 SNP in the TCF7L2 gene confers the risk of developing diabetic nephropathy. On the contrary, ***Wu et al. (2009)*** reported that the TCF7L2 gene was not associated with nephropathy in type 2 diabetic patients and the TCF7L2 gene may contribute to the etiology of nephropathy in T2DM patients in an interactive manner with other genes. Since the TCF7L2 plays an important role in the development of diabetic nephropathy this finding could be explained on the basis that AGEs induced TCF7L2 expression through transforming growth factor-beta (TGF-β) in mesangial cells then the TCF7L2 translocation from the cytoplasm to the nucleus increased and TCF7L2 bound to the activin receptor-linked kinase1 (ALK1) promoter, thereby resulting in increased ALK1 expression ***(Araoka et al., 2010)***. ALK1 is the upstream molecule for mothers against decapentaplegic homolog 1 (Smad1) in several cell types ***(Finnson et al., 2008)*** as in adult humans with DN, the expression of ALK1 and Smad1 are reemerged in the glomeruli ***(Abe et al., 2004; Matsubara et al., 2006)***; however, Araoka and his colleges reported that the role of ALK1 in the increase of Smad1 is uncertain ***(Araoka et al., 2010)***. Furthermore, increased ALK1 enhances the effect of TGF-β and further promotes the phosphorylation of Smad1. These interactions cause the phenotypic changes in mesangial cells, and these changes are characterized by the increased expression of α-SMA, thereby resulting in the development of glomerulosclerosis ***(Araoka et al., 2010)***. These findings suggest that the TCF7L2 plays a key role in the development of diabetic nephropathy.

Regarding Gly482Ser and Thr394Thr polymorphisms in the PPARGC1A gene, our results clearly showed a significant positive association between the mutant A allele of Gly482Ser polymorphism and T2DM patients who developed nephropathy compared to T2DM patients without nephropathy and controls. Furthermore, the mutant AA genotype

was significantly higher in DN patients than control healthy subjects. It has been demonstrated that PPARGC1A gene is a multifunctional coregulator of cellular energy metabolism ***(Puigserver and Spiegelman, 2003)*** and mediates the expression of genes involved in oxidative metabolism, adipogenesis and gluconeogenesis ***(Handschin and Spiegelman, 2006; Liang and Ward, 2006)*** so that PPARGC1A plays a significant role in many aspects of glucose and fat metabolism and energy balance ***(Weikard et al., 2005)***. Our results were in agreement with ***Gayathri et al. (2010)*** who suggested that the PPARGC1A Gly482Ser polymorphism is associated with diabetic nephropathy. Also several genetic association studies concerning this polymorphism was found to be associated with T2DM in different populations, including Danish ***(Ek et al., 2001)***, British ***(Andersen et al., 2002)***, Caucasians ***(Kunej et al., 2004)***, North Indian ***(Bhat et al., 2007)*** and Han Chinese ***(Zhu et al., 2009)***. However, the association of this polymorphism with T2DM was not detectable in French, Pima Indians and Austrians populations ***(Lacquemant et al., 2002; Muller et al., 2003; Oberkofler et al., 2004)***.

On the other hand, our results failed to show any detectable changes in genotype distributions and allele frequencies in Thr394Thr polymorphism within PPARGC1A gene among the studies groups. The results of the current study were supported by ***Gayathri et al. (2010)*** who failed to show any significant difference in genotype distributions and allele frequencies of Thr394Thr polymorphism between diabetic nephropathy patients and type 2 diabetic patients without nephropathy. However, in other studies, ***Yan et al. (2008)*** reported that the Thr394Thr polymorphism was significantly associated with T2DM in Chinese Han population and ***Vimaleswaran et al. (2005)*** demonstrated that PPARGC1A gene polymorphism (Thr394Thr) is associated with T2DM in Asian Indians.

According to the present study, there was significant difference between the Gly482Ser SNP in PPARGC1A gene and fasting plasma glucose level as well as urinary albumin concentration in diabetic nephropathy patients while, ***Vimaleswaran et al. (2005)*** reported that neither fasting plasma glucose nor urinary albumin concentration was significantly different among the different genotypes of Gly482Ser polymorphism. On the other hand, the results of our study revealed that Thr394Thr polymorphism in PPARGC1A gene did not significantly affect fasting plasma glucose, glycated hemoglobin, or urinary albumin concentration. Our results were in contrary to those noted by ***Vimaleswaran et al. (2005)*** who found that there was a significant difference in fasting plasma glucose among the different genotypes in the diabetic subjects. Moreover, current results showed lack of significant association between SNP rs7903146 of the TCF7L2 gene and fasting plasma glucose, glycated hemoglobin levels and urinary albumin concentration.

One of the aims of the present study was to look for combined genetic profiles that were associated with increased incidence of diabetic nephropathy. Our data suggested a synergistic effect between the TCF7L2 rs7903146, PPARGC1A Gly482Ser, and PPARGC1A Thr394Thr polymorphisms and diabetic nephropathy. The TT/AA/AA mutant

genotypes were only reported in diabetic patients who developed nephropathy suggesting a risk for nephropathy whereas the CC/GG/GG wild genotypes were more prevailed in diabetic without nephropathy patients than diabetic nephropathy patients suggesting a protective effect against nephropathy. Thus, we can hypothesize that TCF7L2 rs7903146 polymorphism may act synergistically with PPARGC1A Gly482Ser and PPARGC1A Thr394Thr polymorphisms to enhance nephropathy incidence within type 2 diabetic patients. As the number of studies related to TCF7L2-Gly482Ser-Thr394Thr interactions is limited, there is a scope to further explore the synergistic effects of these polymorphisms in diabetic nephropathy susceptibility.

Based on the present results of our study, TCF7L2 rs7903146, and PPARGC1A Gly482Ser polymorphisms individually or in combination were reported to be associated with susceptibility to nephropathy in type 2 diabetes mellitus patients. Thus we suggested that these results might shed more light on the functional significance of TCF7L2 rs7903146, and PPARGC1A Gly482Ser polymorphisms and its contribution as a risk factor for nephropathy in T2DM Egyptian patients. However, it is important to replicate the results in larger populations to decide whether to role TCF7L2 rs7903146, and PPARGC1A Gly482Ser polymorphisms measurement as a screening tool that might be useful for the prediction, prevention, and management of diabetic nephropathy. In conclusion, diabetics who are at high risk of developing nephropathy can be early identified in order to afford better clinical management.

Acknowledgments

We thank the patients who participated in this study.

Conflict of interest statement

I (we) verify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Table 1: Demographic and biochemical characteristics of T2DM patients with and without nephropathy and control groups.

	Control (n=45)	**T2DM without nephropathy** (n=40)	**T2DM with nephropathy** (n=45)	**P***	**P****
Sex					
Male	17 (37.8%)	9 (22.5%)	12 (26.7%)		
Female	28 (62.2%)	31 (77.5%)	33 (73.3%)	0.059	0.657
Age (years)	51.04±7.97	54.43±8.45	48.47±8.89[b]	0.15	**0.002**
Diabetes duration (years)	---	8±4	11±4.8[b]	---	
Treatment					
Insulin	---	16 (40%)	26 (57.8%)		
Non-Insulin	---	24 (60%)	19 (42.2%)	---	0.078
Pressure					
Normal	---	27 (67.5%)	23 (51.1%)		
High	---	13 (32.5%)	22 (48.9%)	---	0.095
Fasting plasma glucose (mg/dl)	86.3±7.99	246.3±76.1[a]	289.3±98.35[ab]	**<0.001**	**0.026**
Glycated haemoglobin (%)	5.4±0.3	8.786±1.45[a]	9.92±1.95[ab]	**<0.001**	**0.004**
Diabetic Control					
Good	---	26 (65%)	18 (40%)		
Fair	---	10 (25%)	5 (11.1%)		
Poor	---	4 (10%)	22 (48.9%)	---	**0.001**
Urinary total protein (mg/L)	34.6±9.3	97.2±50.3[a]	449.8±130.3[ab]	**<0.001**	**<0.001**
Urinary-albumin (mg/L)	6.9±1.9	13.97±9.96	90±66.4[ab]	**<0.001**	**<0.001**
Urinary creatinine (g/L)	1.05±0.27	1.14±0.379	0.98±0.437[b]	0.35	**0.046**
Albumin creatinine ratio (mg/g)	6.95±2.5	12.95±9.03	104.5±93.3[ab]	**<0.001**	**<0.001**

T2DM = Type 2 diabetes mellitus
Data are expressed as mean ± SD for quantitative variable or number (%) for qualitative one
[a] Significantly different from control,
[b] Significantly different from T2DM without nephropathy.
Bold values indicate significant difference P value < 0.05 was considered significant.

Table 2: Genotype distribution and allele frequencies of the TCF7L2 rs7903146, PPARGC1A Gly482Ser PPARGC1A and Thr394Thr gene polymorphisms in the studied groups

Genotype	**Control** (n=45)	**T2DM without nephropathy** (n=40)	**T2DM with nephropathy** (n=45)	**P***	**P****
TCF7L2 Genotype					
CC[a]	24 (53.3%)	18 (45%)	**13 (28.9%)**		
CT	18 (40%)	20 (50%)	23 (51.1%)	0.064	0.327
TT[b]	3 (6.7%)	2 (5%)	**9 (20%)**	**0.016**	**0.023**
TT+CT[a]	21 (46.7%)	22 (55%)	32 (71.1%)	**0.018**	0.123
CC+CT[b]	42 (93.3%)	38 (95%)	36 (80%)	0.063	**0.04**
Allele					
C	66 (73.3%)	57 (71.3%)	49 (54.4%)		
T	24 (26.7%)	23 (28.7%)	**41 (45.6%)**	**0.008**	**0.018**
Gly482Ser Genotype					
GG[c]	21 (46.7%)	20 (50%)	14 (31.1%)		
GA	24 (53.3%)	17 (42.5%)	23 (51.1%)	0.421	0.162
AA[d]	0 (0%)	3 (7.5%)	**8 (17.8%)**	**0.002**	0.069
AA+GA[c]	24 (53.3%)	20 (50%)	31 (68.9%)	0.13	0.076
GG+GA[d]	45 (100%)	37 (92.5%)	37(82.2%)	**0.003**	0.159
Allele					
G	66 (73.3%)	57 (71.3%)	51 (56.7%)		
A	24 (26.7%)	23 (28.7%)	39 (43.3%)	**0.019**	**0.035**
Thr394Thr Genotype					
GG[c]	24 (53.3%)	25 (62.5%)	23 (51.1%)		
GA	15 (33.3%)	12 (30%)	15 (33.3%)	0.927	0.525
AA[d]	6 (13.4%)	3 (7.5%)	7 (15.6%)	0.754	0.178
AA+GA[c]	21 (46.7%)	15 (37.5%)	22 (48.9%)	0.833	0.29
GG+GA[d]	39 (86.6%)	37 (92.5%)	38 (84.4%)	0.764	0.25
Allele					
G	63 (70%)	62 (77.5%)	61 (67.8%)		
A	27 (30%)	18 (22.5%)	29 (32.2%)	0.747	0.157

Data are number (%), variables were compared using chi square (χ^2) test or Fischer's exact test.
[a] CC vs CT+TT
[b] TT vs CC+CT
[c] GG vs GA+AA
[d] AA vs GG+GA
*P values for comparison between T2DM with nephropathy & controls,
**P values for comparison between T2DM with nephropathy & T2DM without nephropathy.
Bold values indicate significant difference P value < 0.05 was considered significant.

Table 3: Biochemical characteristics of T2DM patients with and without nephropathy with different rs7903146 genotypes in TCF7L2 gene

	TCF7L2 (without nephropathy)			**TCF7L2 (with nephropathy)**		
	CC (n= 18)	**CT+TT** (n=22)	**P* value**	**CC** (n= 13)	**CT+TT** (n=32)	**P** value**
Fasting plasma glucose (mg/dl)	237.44±72	253.5±80	0.513	320±110.66	276.88±91.84	0.186
Glycated haemoglobin (%)	8.76±0.97	8.8±1.78	0.93	9.84±2.34	9.95±1.8	0.866
Urinary albumin (mg/L)	14.76±10.86	13.3±9.37	0.656	77.35±28.78	94.6±76.46	0.436

Data are expressed as mean ± SD
One-way ANOVA test was used to compare the biochemical characteristics of patients divided according to genotypes.
*P values for comparison within T2DM without nephropathy group,
**P values for comparison within T2DM with nephropathy group.

Table 4: Biochemical characteristics of T2DM patients with and without nephropathy with different Gly482Ser and Thr394Thr genotypes in PPARGC1A gene

	PPARGC1A Gly482Ser (without nephropathy)			**PPARGC1A Gly482Ser (with nephropathy)**		
	GG (n= 20)	**GA+AA** (n=20)	**P* value**	**GG** (n= 14)	**GA+AA** (n=31)	**P** value**
Fasting plasma glucose (mg/dl)	240±73.5	252.5±8	0.61	226.78±99	317.6±85	**0.003**
Glycated haemoglobin (%)	8.9±1.34	8.7±1.6	0.67	9.8±2.6	9.98±1.6	0.754
Urinary albumin (mg/L)	15.7±10	12.3±9.5	0.28	78.5±38	141±78	**0.014**
	PPARGC1A Thr394Thr (without nephropathy)			**PPARGC1A Thr394Thr (with nephropathy)**		
	GG (n= 25)	**GA+AA** (n=15)	**P* value**	**GG** (n= 23)	**GA+AA** (n=22)	**P** value**
Fasting plasma glucose (mg/dl)	228.7±71	275.6±77	0.058	289±104	289.7±94	0.982
Glycated haemoglobin (%)	8.77±1.2	8.8±1.9	0.921	10.14±2.2	9.68±1.6	0.43
Urinary albumin (mg/L)	14.1±1	13.8±10	0.941	76.6±40	103.2±85	0.182

Data are expressed as mean ± SD
One-way ANOVA test was used to compare the biochemical characteristics of patients divided according to genotypes.
*P values for comparison within T2DM without nephropathy group,
**P values for comparison within T2DM with nephropathy group.
Bold values indicate significant difference from GG wild genotype group at P<0.05.

Table 5: The combined effect of TCF7L2 rs7903146, PPARGC1A Gly482Ser, and PPARGC1A Thr394Thr genotype frequencies in T2DM patients with and without nephropathy

TCF7L2+Gly482Ser+Thr394Thr genotypes			**T2DM without nephropathy (n=40)**	**T2DM with nephropathy (n=45)**	X^2	**P value**
TCF7L2 Thr394Thr	**Gly482Ser**					
CC	**GG**	**GG**	14 (35%)	7 (15.6%)		
CC	GG	GA	2 (5%)	0 (0%)	0.958	0.328
CC	GA	GG	0 (0%)	2 (4.45%)	3.407	0.065
CC	GA	GA	2 (5%)	3 (6.7%)	1.213	0.27
CC	AA	GG	0 (0%)	1 (2.2%)	1.833	0.176
CT	GG	GG	1 (2.5%)	3 (6.7%)	2.431	0.119
CT	GG	GA	3 (7.5%)	2 (4.45%)	0.079	0.778
CT	GG	AA	0 (0%)	1 (2.2%)	1.833	0.176
CT	GA	GG	8 (20%)	7 (15.6%)	0.655	0.418
CT	GA	GA	4 (10%)	8 (17.8%)	3.422	0.064
CT	GA	AA	2 (5%)	0 (0%)	0.958	0.328
CT	AA	GG	1 (2.5%)	1 (2.2%)	0.224	0.636
CT	AA	GA	1 (2.5%)	0 (0%)	0.489	0.484
CT	AA	AA	0 (0%)	1 (2.2%)	1.833	0.176
TT	GG	GG	0 (0%)	1 (2.2%)	1.833	0.176
TT	GA	GG	0 (0%)	1 (2.2%)	1.833	0.176
TT	GA	GA	0 (0%)	1 (2.2%)	1.833	0.176
TT	GA	AA	1 (2.5%)	1 (2.2%)	0.224	0.636
TT	AA	GG	1 (2.5%)	0 (0%)	0.489	0.484
TT	AA	GA	0 (0%)	1 (2.2%)	1.833	0.176
TT	**AA**	**AA**	0 (0%)	4 (8.9%)	6.061	**0.014**

Data are expressed as number (percentage).
Bold value indicate significant different from T2DM without nephropathy at P<0.05.
The missed genotypes combination are not found in the studied groups.

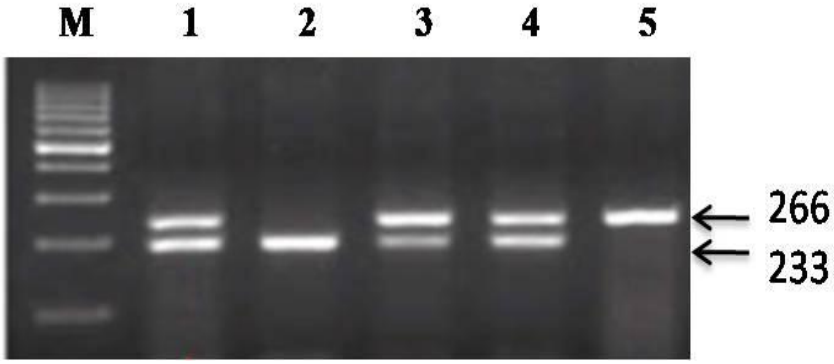

Figure 1: Agarose gel electrophoresis of TCF7L2 rs7903146 products after digestion with RsaI restriction enzyme.
M lane: DNA Ladder (250 bp).
Lanes 2: homozygous CC genotype yielded 1 band of 233 bp.
Lanes1, 3 and 4: heterozygous CT genotype yielded 2 bands of 266 bp and 233 bp.
Lanes 5: homozygous TT genotype yielded 1 band of 266 bp.

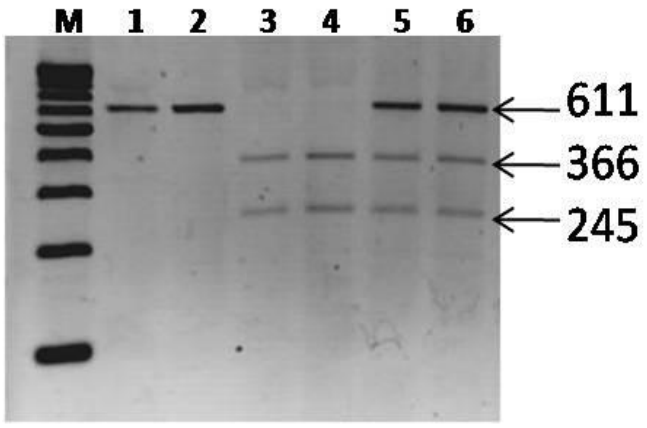

Figure 2: Agarose gel electrophoresis of PPARGC1A Gly482Ser products after digestion with HpaII restriction enzyme.
M lane: DNA Ladder (100 – 1000 bp).
Lanes 3 and 4: homozygous GG genotype yielded 2 bands of 366 bp and 245 bp.
Lanes 5 and 6: heterozygous GA genotype yielded 3 bands of 611 bp, 366 bp and 245 bp.
Lanes 1 and 2: homozygous AA genotype yielded 1 band of 611 bp.

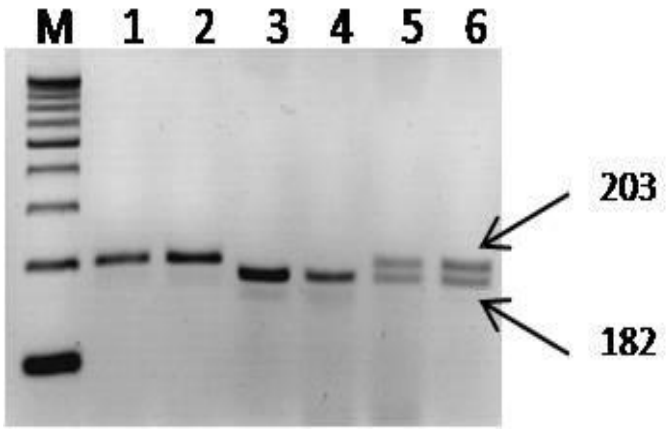

Figure 3: Agarose gel electrophoresis of PPARGC1A Thr394Thr products after digestion with MspI restriction enzyme.
M lane: DNA Ladder (100 – 1000 bp).
Lanes 3 and 4: homozygous GG genotype yielded 1 band of 182 bp.
Lanes 5 and 6: heterozygous GA genotype yielded 2 bands of 203 bp and 182 bp.
Lanes 1 and 2: homozygous AA genotype yielded 1 band of 203 bp.