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RESEARCH ARTICLE

Association of TGF-β1 Polymorphism with Type 2 Diabetes Mellitus and Diabetic Nephropathy in Saudi Patients.

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Abstract

Diabetic nephropathy is the common process that is leading to ESRD. Several studies showed that TGF-β is a major anti-inflammatory cytokine involved in extracellular matrix deposition and thickening of basement membrane of glomeruli. This study is aimed to evaluating the association of TGF-β1 gene polymorphisms (869T/C) and (509C/T) with complicated and uncomplicated type 2 diabetes mellitus. Methods: 250 Saudi male classified into; 100 healthy males as control, 80 uncomplicated type 2 diabetes mellitus male patients and 70 type 2 diabetes mellitus male patients with nephropathy. Blood and urine samples were collected from all groups for measurement of plasma glucose, urea, cholesterol, triglyceride, HDL-C, urea, creatinine and TGF-β1. In addition a genotyping of TGF-β1 was done. Results: our results showed a statistical difference in TGF-β1 levels in all groups, also a positive correlation between hyperglycemia and HbA1c with TGF-β1 level was detected. According genotype, only 869T/C genotypes are involved in susceptibility to T2DM and diabetic nephropathy. Both TC and CC genotype are higher in T2DM patients compared with control (P<0.05), while only CC is higher in diabetic nephropathic patients compared with uncomplicated T2DM (P<0.05). Conclusion: an increase TGF-β level may be involved in processes of T2DM development and its nephropathic complication. In addition, the TC and CC genotypes of TGF-β1 869T/C are more susceptible to T2DM, while only CC genotype to diabetic nephropathy.

Introduction:-

Type 2 diabetes mellitus (T2DM) is still one of the most public health problems distributed all over the world. Sedentary lifestyle and obesity are the major risk factors triggering the development of this problem. Family history and genetic factor appear as additional risk factors besides obesity and lifestyle (Alhazmi et al., 2015). Multiple genes polymorphisms have been concluded by several studies to be a factor that influences the development of T2DM and its complications as nephropathy (Yih-Hsin et al., 2009). Previously, several studies reported that Chronic inflammation and immune system imbalance play a significant role in T2DM development. Pro-inflammatory and anti-inflammatory cytokines are expressed in a different manner during insulin resistance and T2DM stages (Navarro-Gonzalez and Mora-Fernandez, 2008). Transforming growth factor-β is an anti-inflammatory cytokine which inhibits the activation of macrophages. It is a family composed of 33 cytokines that exert their effects through a formation of type I and type II complex seriene/therionine kinase receptor (Heldin et al., 2009). This complex phosphorylates Smad family (mainly Smad 4), which translocate to the nucleus and regulate genes expression involved in growth arrest, angiogenesis, cell differentiation, and immune control (Qian et al., 2008). TGF-β1 is a member of TGF-β superfamily and is one of the most prominent fibrotic factor. Previous studies, found that elevated TGF-β1 in T2DM patients compared with control and a its level is positively correlated with fasting blood sugar. Moreover, *in vitro* studies showed that both high glucose and insulin concentrations stimulate the expression of TGF-β1 in cell culture. The T2DM reports as a primary cause of ESRD resulting from progressive nephropathy (Manabe, 2011; Goldberg, 2009). Diabetic nephropathy is a result of glomerular, tubular, interstitial,

and vascular lesions. Clinical evidence suggests that TGF-β plays a significant role in a final pathway that involves glumerolonecrosis and interstitial fibrosis which leads to end-stage renal disease (ESRD) (Qian et al., 2008). In vitro studies showed that TGF-β1 triggers hypertrophy and extracellular matrix deposition in tubular cells. The TGF-β1 gene present on chromosome 19, contains seven exons and large introns, and more than ten polymorphisms distributed in them (Bendicho et al., 2012). The previous study found that the 869 T/C polymorphism of TGF-β1 gene that involves the substitution of leucine for proline is associated with T2DM development among the Chinese population. They found that the genotype CC/CT was higher in T2DM compared with the control group (Wong et al., 2003). This polymorphism induces the production of TGF-β1, leading to a higher level of TGF-β1 compared with wild type. This higher level of TGF-β1 is associate with T2DM and the development of diabetic nephropathy (Herder et al., 2009). According to the genotype, the TGF-β1 869 T/C genotypes are associated with high risk of diabetic nephropathy and ESRD (Karina et al., 2014; Wang et al., 2005; Egger et al., 1997). This work was aimed to investigate the association of TGF-β1 genes polymorphism with T2DM and its nephropathic complication in Saudi males.

Subjects and Methods:-

Subjects:-

This study consisted of 220 Saudi males classified into two groups; the first group includes 120 diabetic type 2 patients recruited from the Diabetic Center of King Abdul-Aziz specialist hospital in Taif city. All patients diagnosed according to the World Health Organization criteria (fasting blood glucose >126 mg/dL or 2-hour postprandial blood glucose >200 mg/dL). The second group was used as a control including 100 healthy Saudi male without any signs of diabetes mellitus with fasting blood sugar <110 mg/dl. The diabetic type 2 patients group was further subclassified into; 1- diabetic type 2 patients complicated with nephropathy and 2- diabetic type 2 patients without nephropathy based on a questionnaire and laboratory results of blood albumin and creatinine and urine albumin (Howell and McAnulty, 2006).

Samples collection:-

Six mL of venous blood was drawn from each individual included in the study (diabetic type 2 patients and healthy control) under complete aspect condition after an overnight fasting. Three mL was collected in EDTA containing tube for separation of peripheral blood mononuclear cells (PBMCs) for determination of TGF-β1 genotypes. The other three mL of the blood was collected in anticoagulant-free tubes used for separation of serum to detect TGF-β1, blood sugar, urea, creatinine, triglyceride, cholesterol, C-HDL, and HbA1c concentrations. Moreover, the urine sample was collected from only diabetic patients for measurement of microalbuminuria.

Methods:-

Biochemical analysis:

After sample collection, sera were separated immediately and stored at -20°C. Blood glucose, urea, creatinine, cholesterol, triglyceride, C-HDL were measured by spectrophotometric method using chemistry autoanalyzer (Biomerieux, France). TGF-β1 blood level was measured by high sensitivity enzyme-linked immunosorbent assay (ELISA) (Halil et al., 2010). Microalbuminuria was detected by a quantitative nephelometric assay (Roche) through the measurement of microalbumin in 24 hour urine sample (American Diabetes Association, 2010).

DNA isolation:-

Genomic DNA was extracted from EDTA whole blood sample using a spin column method according to the protocol (QIAamp Blood Kit; Qiagen GmbH, Hilden, Germany) (Perrey et al., 1999).

Amplification of TGF-β1 (869 T/C) and (509 C/T) gene polymorphisms:-

Both 869 T/C and 509 C/T were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) including amplification by thermal cycler and digestion with a site-specific restriction enzyme. The forward and reverse primers for 869 C/T were 5′-TTCCCTCGAGGCCCTCCTA-3′ and 5′-GCCGCAGCTTGGACAGGATC-3′ respectively, while for 509 C/T the forward primers was 5′-GAGCAATTCTTACAGGTGTCTGC-3′ and the reverse was 5′-GAGGGTGTCAGTGGGAGGAG-3′. The PCR amplification protocol was composed of 35 cycles comprising three steps each: 75 s at 96 °C, 75 s at 62 °C and 75 s at 73 °C. The PCR products were digested with MspA1I for 869 C/T and with Eco81I for 509 C/T. All the products run on a 3% ethidium bromide-stained agarose gel (Pushplata et al., 2007).

Results:-

This project was performed on 150 T2DM Saudi male patients enrolled into the Diabetic Centre in King Abdulaziz specialized hospital during 2015. A hundred Saudi healthy men were used as a control group. Table 1 showed a comparison between T2DM patients and the control group by using t-test in BMI, blood glucose, HbA1c, triglyceride, cholesterol, HDL-C, urea, creatinine, and TGF-β1 levels. It showed highly significant differences in BMI, glucose, HbA1c, and TGF-β1 in type 2 diabetic patients group (31.22±5.9, 8.45±2.56, 8.76±2.13 and 141.15±22.48 respectively) compared with the control group (23.30±2.3, 4.66±0.84, 5.33±4.20 and 21.36±6.5 respectively) ($P<0.01$). Also, significant differences were observed in blood cholesterol, triglyceride and HDL-C (6.60±3.43, 2.62±3.87 and 0.68±0.07 respectively in T2DM group) and (5.22±1.43, 1.17±1.04 and 1.43±0.09 respectively in control group) between the two groups ($P<0.05$). Table 2 represents a correlation by using Spearman`s test between BMI, glucose, HbA1c, triglyceride, cholesterol, HDL-C, urea, creatinine, and TGF-β1. The TGF-β1 showed positive correlations with glucose ($P<0.05$) and HbA1c ($P<0.01$). Table 3 showed statistical differences in creatinine, urea, albumin, microalbuminuria, and TGF-β1 between complicated diabetic patients (nephropathic) (129.56±36.53, 5.21±2.15, 2.85±0.45, 98.22±32.24 and 138.15± 20.87 respectively) compared with a noncomplicated one (79.05±10.11, 2.84±0.99, 4.20±0.39, 75.13±12.09 and 98.55±14.50 respectively) ($P>0.01$). The TGF-β1 genotypes statistics of both 689 T/C and 509 C/T in type 2 diabetic patient and control group are represented in table 4. The TC and CC genotypes of 869 T/C showed higher frequencies in T2DM patients compared with a control group ($P<0.05$). Also, the results showed a higher frequency of C allele in T2DM patients group compared with the control group ($P<0.05$). According to 509 C/T genotypes, there was no significant result observed. Table 5 represents the frequencies of the same TGF-β1 genotypes in T2DM with and without nephropathy. The CC genotypes of 869 T/C has a higher frequency in T2DM patients with nephropathy compared to T2DM patients without nephropathy ($P<0.05$). Moreover, the C alleles has a higher frequency in T2DM patients with nephropathy than without nephropathy ($P<0.05$). There is no significant differences between T2DM patients with and without nephropathy in 509 C/T genotypes.

Table 1: Comparison between biochemical parameters of control and type 2 diabetic patients

Parameters	Control(n=100)	Diabetic type 2 patients(n=150)	P value
BMI (Kg/m^2)	23.30 ± 2.3	31.22 ± 5.9	0.004**
Glucose (mmol/L)	4.66 ± 0.84	8.45 ± 2.56	0.003**
HbA1c	5.33 ± 4.20	8.76 ± 2.13	0.002**
Cholesterol (mmol/L)	5.22 ± 1.43	6.60 ± 3.43	0.040*
Triglyceride (mmol/L)	1.17 ± 1.04	2.62 ± 3.87	0.047*
HDL-C (mmol/L)	1.43 ± 0.09	0.68 ± 0.07	0.031*
Creatinine (μmol/L)	69.05 ± 22.11	76.27 ± 36.53	0.215
Urea (mmol/L)	2.70 ± 0.93	2.82 ± 2.15	0.091
TGF-β1 (Pg/mL)	21.36 ± 6.5	141.15 ± 22.48	0.007**

** $P < 0.01$ * $P < 0.05$

Table 2: correlation between biochemical parameters of diabetic type 2 patients

	BMI	Glucose	HbA1c	Cholesterol	Triglyceride	HDL-C	Creatinine	Urea	TGF-β1
BMI		0.008	0.005						
Glucose			0.001					0.04	0.03
HbA1c									0.008
Cholesterol					0.004		0.001		
Triglyceride							0.002		
HDL									
Creatinine								0.003	
Urea									
TGF-β1									

Table 3: Comparison between biochemical parameters of diabetic type 2 patients with and without nephropathy

Parameters	T2DM without nephropathy(n=80)	T2DM with nephropathy(n=70)	P value
BMI (Kg/m^2)	28.31±1.3	31.87 ± 2.9	0.041*
Glucose (mmol/L)	7.55 ± 1.84	7.48 ± 2.02	0.083
HbA1c	7.23 ± 3.45	7.76 ± 3.11	0.092
Cholesterol (mmol/L)	6.38 ± 1.88	6.60 ± 3.43	0.144
Triglyceride (mmol/L)	2.62 ± 1.37	2.72 ± 2.24	0.249
HDL-C (mmol/L)	0.72 ± 0.29	0.75 ± 0.17	0.189
Creatinine (µmol/L)	79.05 ± 10.11	129.56 ± 36.53	0.008**
Urea (mmol/L)	2.84 ± 0.99	5.21 ± 2.15	0.009**
Albumin (g/dL)	4.20 ± 0.39	2.85 ± 0.45	0.007**
Microalbuminuria mg/24 hours	75.13 ± 12.09	98.22 ± 32.24	0.007**
TGF-β1(Pg/mL)	98.55 ± 14.50	138.15 ± 20.87	0.008**

** $P < 0.01$ * $P < 0.05$

Table 4: genotypic and allelic frequencies of TGF-β1gene polymorphism in T2DM patients and control subjects.

Genotype (869T/C)	Control n (%)	T2DM n (%)	X^2	P value
TT	70 (70)	63 (42.00)	0.103	0.390
TC	28 (28)	71 (47.33)	4.891	0.041*
CC	2 (2)	16 (10.66)	4.099	0.049*
Allele				
T	168 (84.00)	197 (65.66)	0.027	0.654
C	32 (16.00)	103 (34.33)	4.113	0.047*
Genotype (509C/T)	Control n (%)	T2DM n (%)	X^2	P value
CC	68 (68%)	96 (64%)	0.098	0.960
CT	29 (29%)	48 (32%)	0.0910	0.980
TT	3 (3%)	6 (4%)	0.086	0.890
Allele				
C	165 (82.25%)	240 (80%)	0.012	0.761
T	35 (17.5%)	60 (20%)	0.023	0.702

** $P < 0.01$ * $P < 0.05$

Table 5: genotypic and allelic frequencies of TGF-β1gene polymorphism in T2DM patients with and withou nephropathy.

Genotype (869T/C)	T2DM without N n (%)	T2DM with N n (%)	X^2	P value
TT	40 (50%)	23 (32.86%)	0.202	0.390
TC	37 (46%)	34 (48.57%)	0.652	0.128
CC	3 (4%)	13 (18.57%)	4.011	0.036*
Allele				
T	117 (73.13%)	80 (57.14%)	0.301	0.654
C	43 (26.69%)	60 (42.86%)	3.165	0.049*
Genotype (509C/T)	T2DM without N n (%) n (%)	T2DM with N n (%)	X^2	P value
CC	51 (63.75%)	45 (64.28%)	0.088	0.933
CT	25 (31.25%)	23 (32.86%)	0.084	0.980
TT	4 (5%)	2 (2.86%)	0.081	0.890
Allele				
C	127 (79.38%)	113 (80.71%)	0.022	0.807
T	33 (20.63%)	27 (19.29%)	0.029	0.722

** $P < 0.01$ * $P < 0.05$ N: nephropathy

Discussion:-

Type 2 diabetes mellitus is a complex, chronic and rapidly growing disorder occurring due to either defect in insulin secretion, action or both. It accounts for about 95% of diabetes worldwide (Stumvoll et al., 2005). The development of T2DM is affected by multiple genetic and environmental factors (Ahlqvist et al., 2011). Several studies in the last years focused on pro-inflammatory cytokines, and they found that these cytokines implicated in the development of T2DM and its complication (Yih-Hsin et al., 2009). TGF-β is an anti-inflammatory cytokine which induces immune tolerance and preventing autoimmune reactions (Li MO et al., 2006). Moreover, it diverse proliferation, differentiation, survival of T cells and inhibits macrophages activation (24). TGF-β1 is a member of TGF-β superfamily and several studies showed that hyperglycemia and hyperinsulinemia stimulate and increase TGF-β1 expression *in vitro* as well as *in vivo* (Sarafidis and Ruilope, 2006). Moreover, *in vitro* studies found that TGF-β1 is responsible for development of mouse β-cells, and this may be explain the increase of TGF-β1 in T2DM which result from increase insulin demand. Previously, several studies showed high TGF-β1 levels in T2DM (Yener et al., 2008). Present study is similar to previous studies which shows higher TGF-β1 level in T2DM. Also, the TGF-β1 level is positively correlated with fasting blood glucose level and HbA1c, so this result may give us an idea about the effect of hyperglycemia on the TGF-β1 level. In the past, many authors concluded that there is a correlation between elevated TGF-β1 and diabetic nephropathy (Singh and Ramji, 2006). Nephropathy characterized by deposition of extracellular matrix (ECM) and hypertrophy of glomerular cells followed by glomerulosclerosis. These changes are followed by consequences of microalbuminuria, albuminuria, uremia, and finally ESRD (Goldberg, 2009). The TGF-β1 has been considered to be involved in the development of diabetic nephropathy. It is highly expressed in mesangial cells of diabetic glomeruli and its inhibition prevent glomerular fibrosis. Previous studies conclude that increase TGF-β1 expression in mesangial, epithelial and tubular cells induced by hyperglycemia and hyperinsulinemia (Anderson et al., 1997). **In 1999, Rivarola** and her colleague found a positive correlation between albuminuria and urinary TGF-β1 level in diabetic patients complicated with nephropathy. Our results are similar and showed elevation in serum TGF-β1 levels of diabetic nephropathic patients compared with uncomplicated T2DM patients. In addition a positive correlation was present between albuminuria and serum TGF-β1 levels in T2DM patients with nephropathy. The human *TGF-β1* gene located on chromosome 19q13.1-13.3 (Fujii et al., 1996), and there are several polymorphisms distributed along this gene (Watanabe et al., 2002). The TGF-β1 869 T/C polymorphism that resulted in a substitution of proline for leucine at tenth amino acid usually associated with increases *TGF-β1* gene expression *in vitro* (Awad et al., 1998). **In 2013, Sherif`s** study found that there is an association between TGF-β1 869T/C polymorphism and T2DM development. Moreover, another study done on Chinese population showed that the TGF-β1 869 T/C is associated with increased TGF-β1 level and the development of T2DM (Teresa et al., 2003). Our result showed that TC and CC have a high frequency in T2DM compared with the control group. Also, the study showed that the C allele is more frequent in T2DM. TGF-β1 509 T/C genotypes did not show any statistical differences in T2DM patients compared with healthy group. According to diabetic nephropathy, a recent study done by **Raina et al., 2015** found that the CC genotype of TGF-β1 869 T/C increased the risk of nephropathy by 3.1–4.5-fold in Indian populations. Also, **Sherif et. al., 2013** found an association between TGF-β1 869 T/C polymorphism and T2DM development and severity of its nephropathy. They found that the TT and TC genotypes were more susceptible to the development of T2DM. In association with the risk of diabetic nephropathy, only TC is highly associated with diabetic nephropathy (Sherif et al., 2013). **Teresa study** done upon Chinese population showed that TC and CC genotypes of TGF-β1 869 T/C have a higher frequency in type 2 diabetic nephropathy compared with T2DM patients without nephropathy. Our result is in accordance with **Buraczynska et al.,** study done among Poland population which showed that only the CC genotype of TGF-β1 869 T/C is more susceptible to T2DM nephropathy. Another studies done among Mexicans and Cucasians didn't confirmed this association, and this may be related to ethical and race differences. According to TGF-β1 509 C/T, **Ng et al., 2003** study concluded that there is no association between 509 C/T polymorphism and susceptibility to diabetic nephropathy. **Wang et al., 2013** meta-analysis suggests that the TGF-β1 gene 509 C/T polymorphism would not be a risk factor for IgA nephropathy in Asians but might play a role in Europeans. Recently, **Raina et al., 2015** study done on Indian population found that the TT genotype provided 5.5-fold risk towards ESRD cases from Jammu and Kashmir and no risk for the cases from Punjab. Our results upon TGF-β1 509 C/T is in agreement with Wang and Ng studies, which found no association between its genotypes and susceptibility to diabetic nephropathy.

Recommendation and conclusion:-

Our study was done on a small sample size, so a large sample size in a different populations is recommended to give a broad picture of involvement of these polymorphisms in T2DM and its nephropathic complication. Also, the study

of other cytokines and its genes polymorphisms is recommended to give a broad picture of involvement of inflammatory reaction in risk of T2DM and its complications. From our results, we can conclude that the increase TGF-β level may involve in processes of T2DM development and its nephropathic complication. Moreover, the TC and CC genotypes of TGF-β1 869T/C is more susceptible to T2DM, while only CC genotype to diabetic nephropathy.

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