Clinical Journal of Diabetes Care and Control

Chembio Publishers
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Research Article *Volume 2; Issue 1*

Interlukin-4 (IL-4), Tumor Necrosis Factor-α (TNF-α), and its receptors Gene Polymorphism in Type 2 Diabetes Mellitus

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Received Date: February 12, 2019; **Published Date:** February 21, 2019

Abstract

Type 2 diabetes mellitus (T2DM) and its complications become a major cause of inactivity and death worldwide. The immune system plays a significant role in the development and complication of T2DM. Cytokines and its receptors were a major part of the immune system involved in T2DM complications. Recently, genetic factors become a focused area to evaluate the contribution of these cytokines with diabetes. Subjects and methods: 150 T2DM patients and 100 healthy males were recruited into Prince Mansour specialized hospital. For each individual, serum glucose, cholesterol, triglyceride, HDL-C, urea, creatinine, TNF-α, and IL-4 were measured for all individuals included in this work.

TNF-α (-308G/A) and (-238G/A), TNFRII (M196R), IL-4 (-590C/T), and IL-4Rα (I50V) gene polymorphism were detected. Results: TNF-α and IL-4 levels were elevated in T2DM patients ($P<0.01$). A positive correlation between serum TNF-α with glucose and HbA1c levels were detected. Serum IL-4 was positively correlated with HbA1c level ($P<0.01$). Genotypes of TNF-α (-308 GA and AA), TNFRII (196 MR) and IL-4 (-590 CT) were more susceptible to T2DM. Conclusion: both TNF-α and IL-4 gene polymorphisms may associate with T2DM. Both GA and GG genotypes of TNF-α (-308), and CT genotype of IL-4 may more susceptible to T2DM than other TNF-α and IL-4 genotypes. According to their receptors, only TNFRII RR genotype may at risk of T2DM.

Keywords: Type 2 diabetes mellitus; Polymorphisms; Cytokines; IL: Interleukin

Abbreviations: T2DM: Type 2 Diabetes Mellitus; TNF: Tumor Necrosis Factor; TNFR: Tumor Necrosis Factor Receptor; PBMC: Peripheral Blood Mononuclear Cells; ELISA: Enzyme-Linked Immune Sorbent Assay

Introduction

Type 2 diabetes mellitus (T2DM) is a multifactorial metabolic disorder characterized by elevated blood sugar resulting from a defect in insulin secretion or resistance

Citation: Alhazmi AS, et al. Interlukin-4 (IL-4), Tumor Necrosis Factor-α (TNF-α), and its receptors Gene Polymorphism in Type 2 Diabetes Mellitus. Clin J Dia Care Control 2019, 2(1): 180015.

to its action or both. T2DM becomes a significant health problem in the world [1]. Its prevalence in Saudi Arabia rapidly elevated and became the most health problem in the last 20 century [2]. Previously, a study demonstrated that a family history, genetic pictures, environmental factors, and chronic inflammatory responses play a significant role in the development of T2DM. Disturbance in inflammatory cytokines has a role in an insulin-producing cell (β-cell) destruction and insulin signaling impairment [3]. Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine produced by many cells including monocytes and macrophages with two varied and complex functions. On one hand it confers diseases resistance and, on the other hand, causes a pathological complication [4]. TNF-α is produced primarily as a membrane-bound protein from which the active soluble form released by the action of metalloproteinase called TNF-α converting enzyme (TACE). The gene of TNF-α located on the short arm of chromosome 6 and several polymorphisms were identified within the promoter region of this gene [5]. Both (-308) G/A and (-289) G/A are polymorphisms that may affect TNF-α expression [6].

These polymorphisms associated with several autoimmune and inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematous, and chronic bacterial infections. Previous studies reported that TNF-α is a possible cytokine involved in insulin resistance [7] and impairs its secretion by β-cells [8]. The metabolic activities of TNF-α mainly mediated through TNF receptor type II (TNFRII). Increased TNFRII expression levels have been observed in obesity, insulin resistance, and T2DM [9]. Interleukin-4 is an important anti-inflammatory cytokine produced by helper T cells type 2 (Th2), natural killer cells (NK), basophils, eosinophils, and mast cells [10].

It regulates proliferation, apoptosis, and differentiation of many hematopoietic cells. IL-4 also down-regulates the production of helper T cells type 1 (Th1) and switch the immunoglobulin class into IgG and IgE [11]. Also, IL-4 balances the inflammatory response by inhibiting the secretion of the pro-inflammatory cytokines such as TNF-α and IL-6 from macrophages [12]. Previously a study suggested that IL-4 protect islet cells from cytotoxic damage induced by pro-inflammatory cytokines and Th1 cytokines. Another study showed that, long-term exposure of rat islet cells to IL-4 leading to inhibition of islets functions [13]. IL-4 mediates its action by binding to a trans-membrane receptor called IL-4Rα [14]. Several studies suggest an association between some gene polymorphisms in IL-4 and IL-4Rα such as (-590) C/T and I50V respectively and the development of T2DM [3]. This study was aimed to investigate the association of gene polymorphisms of TNF-α, TNFRII, IL-4, and IL-4Rα with the development of T2DM in Saudi populations.

Subjects and Methods

Subjects

This study consisted of 150 Saudi male type 2 diabetic patients recruited from outpatient clinics of Prince Mansour specialist hospital in Taif. 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) [15]. One hundred healthy Saudi male used as a control group. They had no any signs of diabetes mellitus with fasting blood sugar <110 mg/dL.

Samples collection: Six mL of venous blood was drawn from each of the two groups (diabetic type 2 patients and healthy control) under complete aspect condition after an overnight fasting. Three mL of the blood was collected in EDTA-containing tube for separation of peripheral blood mononuclear cells (PBMCs) for determination of HbA1c percentage and TNF-α, TNFRII, IL-4 and IL-4Rα genotypes. The other three mL of blood were collected in anticoagulant free tubes used for detection of serum TNF-α, IL-4, glucose, urea, creatinine and total cholesterol, HDL-C, and triglyceride levels.

Methods

Biochemical analysis: After the samples collection, sera were separated immediately and stored at -20°C. Blood glucose, urea, creatinine, total cholesterol, HDL-C, triglyceride, and HbA1c were measured by using VITROS 250 Chemistry Auto analyzer. Levels of both TNF-α and IL-4 were measured by solid phase sandwich enzyme-linked immune sorbent assay (ELISA) [16].

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).

Amplification of (-308) G/A and (-238) G/A polymorphisms of the TNF-α gene

The TNF-α (-308) G/A promoter polymorphism was genotyped by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The PCR reaction mixture (2.5 μL) was made up of 100 ng of genomic DNA sample, 2.5 μL of *Taq* DNA polymerase buffer (10 X), 0.5 μL of magnesium chloride (1.5 mM), 0.5 μL of each dNTPs (10 mM), and 1 μL of each primer (25 ng/μL); forward 5`-TAAACTTGGGAGAACATGGT-3` and reverse 5`-TGGGGAAAGATAGAGTAATA-3`. The PCR

condition was started with denaturation at 95 ºC for 5 minutes, followed by 35 cycles of melting at 95 ºC for 50 seconds, annealing at 53 ºC for 50 seconds, and extension at 72 ºC for 45 seconds, with a final extension step of 5 minutes at 72ºC, using thermal cycler (C1000, Bio-Rad, Hercules, California, USA). After RFLP analysis, the product was digested with BsmFI at 65 °C for 3 hours.

On 2% agarose gel, three genotypes were detected: GG (137 + 103 + 88 bp), GA (191 + 137 + 103 + 88 bp), and AA (191 + 137 bp). While the genotyping of (-238) G/A was done by using the same previously method with forwarding primer 5'-AGAAGACCCCCCTCG-GAACC-3' and the reverse 5'-ATC-TGGAGGAAGCGGTAGTG-3'. The reaction mixture then incubated with MspI at 37 °C for 1 hour. After 2% agarose gel electrophoresis, three genotypes were detected by GG (133+19 bp), G/A (152+133+19 bp) and A/A (152 bp) [17]. Quality control measures include blinded analyses, replicates of 10% of samples, and positive controls (blood-derived DNA from all known genotypes), and negative controls for contamination (no DNA) were run routinely with the patient sample [18].

Amplification of TNFRII polymorphism

The subjects were genotyped for TNFRII (M196R) by PCR–RFLP as described previously. The region surrounding the polymorphism was amplified with forwarding primer 5'-ACTCTCCTATC-CTGCCTGCT-3' and reverse primer 5'-TTCTGGAGTTGGCTGCGTGT-3'. After RFLP analysis, the product was digested with NlaIII at 65 °C for 3 hours. On 2% agarose gel, the 242-bp PCR product was uncleaved in the 196R allele and cleaved into two fragments of 133- and 109-bp in the 196 M allele [9].

Amplification of IL-4 (-590 C/T) polymorphism

The subjects were genotyped for IL-4 (-590 C/T) polymorphisms by using PCR-RFLP. The PCR product of IL-4 (-590 C/T) was a 195-bp fragment and was digested with AvaII into 175-bp and 20-bp fragments. The digested products were run on a 2% agarose gel.

Amplification of IL-4Rα (I50V) polymorphism:

The genotypes for IL-4Rα (I50V) were determined by PCR-RFLP as described previously. The IL-4Rα (I50V) region was amplified by using; forward primer 5'-GGCAGGTGTGAGGA-GCATCC-3' and reverse primer 5'-GCCTCCGTTGTTCTCAGGGA-3'. The amplified product was digested with RsaI yielded 273 bp for I allele and 254-bp fragment when the V allele was present [19].

Statistical analysis

SPSS software version 16 (SPSS Inc., Chicago, IL, USA) was used in the performance of statistical analysis. The correlations were tested using Spearman`s test. The t-test was used in comparisons performance. The Chi-square test was used to compare frequency of each genotype and allele. Both comparisons and correlations were considered statistically significant when $P<0.05$.

Results

The results of this project were represented by numbers of tables. Serum level of glucose, HbA1c, cholesterol, HDL-C, triglyceride, creatinine, urea, TNF-α, and IL-4 levels were represented as (Mean ± SD) of both control and T2DM groups in Table 1. Blood glucose level showed a significant statistical difference between T2DM (8.45 ± 2.56) group compared with control group (4.66 ± 0.84) (P=0.003). In addition, HbA1c showed a significant statistical difference in T2DM (8.76 ± 2.13) compared with control group (5.33 ± 4.20) (P=0.002).

Lipid profiles including cholesterol and triglyceride showed significant statistical differences in T2DM group (6.60 ± 3.43 and 2.62 ± 3.87) compared with control group (5.22 ± 1.43 and 1.17 ± 1.04) (P=0.040) and (P=0.047) respectively. HDL significantly lower in T2DM (0.68 ± 0.07) compared with control group (1.43 ± 0.09) (P=0.031). Both TNF-α and IL-4 showed significant statistical differences in T2DM (18.25±7.09 and 24.03 ± 9.24) compared with control group (9.80 ± 3.05 and 12.44 ± 5.02) (P=0.007) and (P=0.005) respectively. Figure 1 represents the serum biochemical parameters levels in both groups. Table 2 represents a correlation between all parameters measured by using Spearman`s test. It was showed a positive correlation of glucose level with HbA1c, urea, TNF-α (P=0.001, 0.04 and 0.003 respectively). Also, HbA1c was showed a positive correlation with TNF-α and IL-4 levels (P=0.004 and 0.008 respectively).

Parameters	Control (n=100)	Diabetic type 2 patients (n=150)	P value
BMI (Kg/m2)	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 (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
TNF-α (ng/ml)	9.80 ± 3.05	18.25±7.09	0.007**
IL-4 (pg/ml)	12.44 ± 5.02	24.03 ± 9.24	0.005**

Table 1: Biochemical parameters of both control and type 2 diabetic patients (ANOVA).
* *P*< 0.05 is considered significant ** *P*< 0.05 is considered significant.

	BMI	Glucose	HbA1c	Cholesterol	Triglyceride	HDL	Creatinine	Urea	TNF-α	IL-4
BMI		0.008**	0.005**						0.006**	
Glucose			0.001**					0.04*	0.003**	
HbA1c									0.004**	0.008**
Cholesterol					0.004**		0.001**			
Triglyceride							0.002**			
HDL										
Creatinine								0.003**		
Urea										
TNF-α										
IL-4										

Table 2: Correlation between biochemical parameters of diabetic type 2 patients (Spearman`s test).
* *P*< 0.05 is considered significant ** *P*< 0.05 is considered significant.

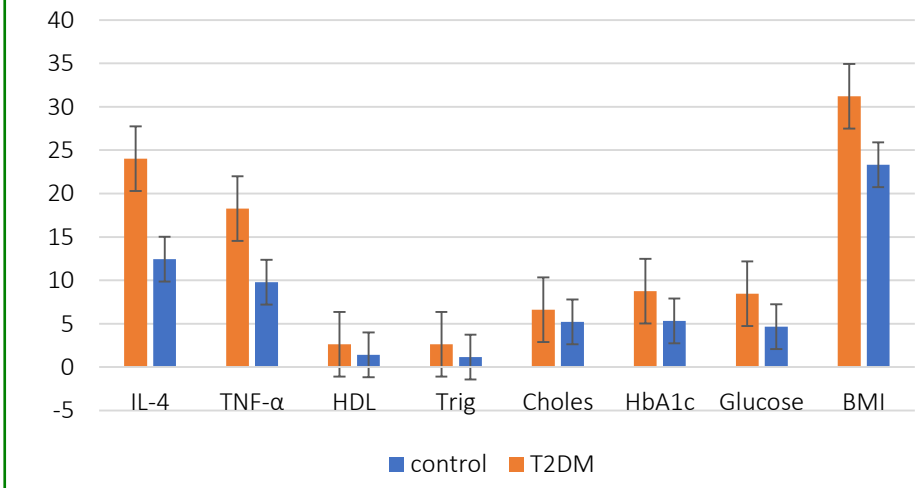

Figure 1: Biochemical parameters in control and T2DM groups.

Table 3 represents the genotype and allele frequency of TNF-α and its receptor in T2DM patients compared with the control group. Both GA and AA genotypes of TNF-α (-308) were showed a high frequency in T2DM patients compared with the control group (P=0.023 and 0.012 respectively). The A allele had a higher frequency in T2DM patients compared with the control group (P= 0.011). The RR genotype of TNFRII (196MR) was showed a higher frequency in T2DM patient compared with the control group (P= 0.042). In Table 4, represent the genotype and allele frequency of IL-4 and its receptor. Only CT genotype of IL-4 (-590) was showed a high frequency in the T2DM patient compared with the control group (P=0.049). Moreover, the T allele was showed a higher frequency in the T2DM patients (Figure 2-4).

Genotype TNF-α (-308)	Control n (%)	T2DM n (%)	X2	P value
GG	82 (82)	83 (55.33)	12.4	0.0004**
GA	16 (16)	52 (34.67)	5.38	0.023*
AA	2 (2)	15 (10.00)	6.05	0.012*
Allele				
G	180 (90.0)	218 (72.67)	2.72	0.193
A	20 (10.0)	82 (27.33)	5.42	0.011*
Genotype TNF-α (-238)				
GG	80 (80.0)	124 (82.66)	0.086	0.768
GA	18 (18.0)	22 (14.66)	0.021	0.883
AA	2 (2.0)	4 (2.66)	0.602	0.822
Allele				
G	178 (89.00)	270 (90.0)	0.032	0.856
A	22 (11.00)	30 (10.0)	0.117	0.731
Genotype TNFRII (196 MR)				
MM	54 (54)	62 (41.33)	1.56	0.441
MR	38 (38)	57 (38)	6.77	0.010
RR	8 (8)	31 (20.66)	1.101	0.042*
Allele				
M	146 (73.0)	181 (60.33)	2.67	0.322
R	54 (27.0)	119 (39.66)	4.871	0.042*

Table 3: Genotypic and allelic frequencies of TNF-a and TNFRII gene polymorphisms in T2DM patients and control subjects.
* $P< 0.05$ is considered significant ** $P< 0.05$ is considered significant.

Genotype (-590)	Control n (%)	T2DM n (%)	X2	P value
CC	70 (70)	98 (65.33)	0.094	0.316
CT	18 (18)	48 (32)	4.433	0.049*
TT	12 (12)	4 (2.76)	1.099	0.411
Allele				
C	158 (79.00)	244 (81.33)	0.097	0.356
T	42 (21.00)	56 (18.67)	4.029	0.498
Genotype IL-4R (I50V)				
II	44 (44.0)	69 (46.0)	0.000	0.988
IV	40 (40.0)	67 (44.67)	0.072	0.787
V	16 (16.0)	14 (9.33)	0.0000	0.998
Allele				
I	128 (64.0)	205 (68.33)	0.484	0.486
V	72 (36.0)	95 (31.67)	2.001	0.157

Table 4: Genotypic and allelic frequencies of IL-4 gene polymorphism in T2DM patients and control subjects.
* $P< 0.05$ is considered significant ** $P< 0.05$ is considered significant.

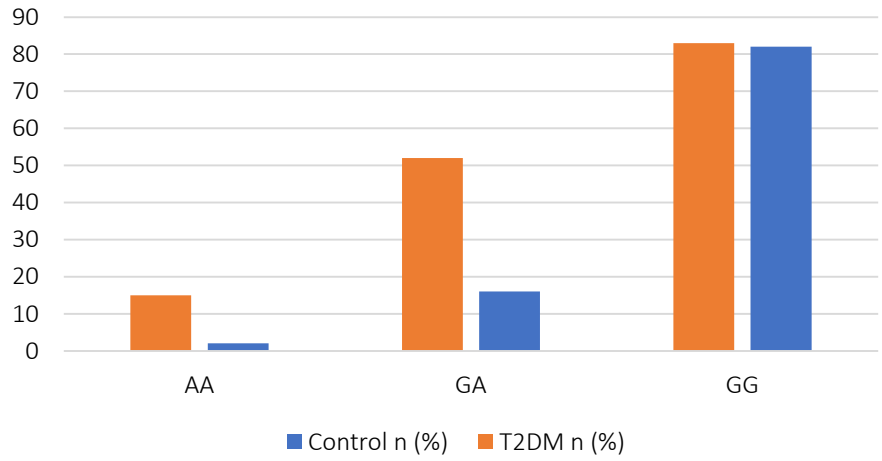

Figure 2: frequency of TNF-α (-308) genotype in both control and T2DM groups.

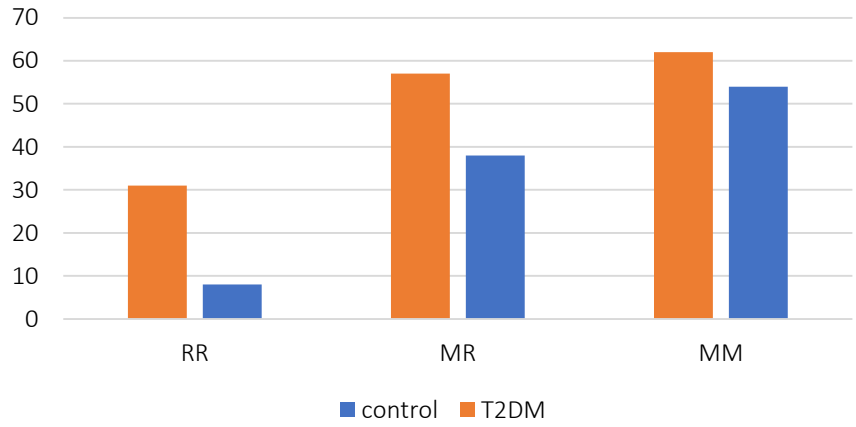

Figure 3: frequency of TNFRII (196 MR) genotype in both control and T2DM groups.

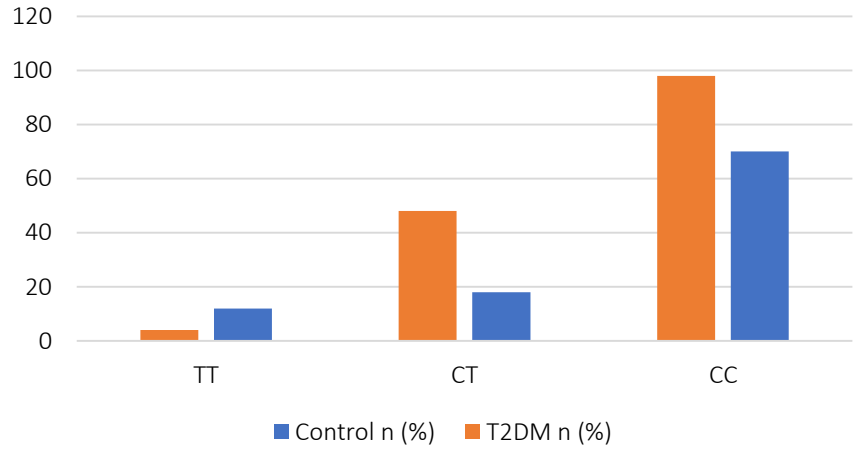

Figure 4: frequency of IL-4 (-590) genotype in both control and T2DM groups.

Discussion

T2DM is a metabolic syndrome associated with the disturbance in carbohydrate, lipid and protein metabolism. It is occurring due to a defect in insulin secretion or resistance to its action or both. Obesity is one of a significant factor that plays a significant role in the development of T2DM. Previously, a study concludes that genetic factor and the disturbance in the immune system are predisposing factors of T2DM development [20]. Cytokines such as IL-6, IL-1 and TNF-α act on hepatocytes and induce VLDL production, obesity, and insulin resistance [21]. TNF-α is a polypeptide composed of 157 amino acid residues with a molecular weight of about 17 kDa. Beside monocyte and macrophage, TNF-α also produced by adipocyte.

Obesity is usually associated with the elevated TNF-α level and may induce insulin resistance either by stimulating lipolysis or inactivation of insulin receptor substrate that affects the translocation of glucose transporter [22]. Recently, several studies showed increased TNF-α expression in insulin resistance and T2DM patients [17]. Our result showed a high serum TNF-α level in T2DM patients compared with healthy subjects. Jatla et al. [23] found a positive correlation between BMI and TNF-α, our result is in accordance with this outcome. We can conclude that the increase in fat deposition will induce TNF-α expression in adipocytes. Yih-Hsin et al. [3] found a positive correlation of TNF-α with triglyceride and VLDL levels while a negative correlation with HDL-C. In our result, we did not find these correlations, but we found a positive correlation between TNF-α level and levels of both glucose and HbA1c. Diana et al. [17] found a positive correlation between TNF-α level and serum creatinine and urea levels, but our results did not show this correlation.

Some cytokines gene polymorphisms were associated with increased expression of these cytokines and may correlate with insulin resistance and susceptibility to T2DM. Previously, a number of studies demonstrate an association between TNF-α gene polymorphisms and susceptibility to insulin resistance and T2DM. Their results are contradictory. Ying et al. [24] found no association between TNF-α -308 G/A polymorphism and susceptibility to T2DM, also Ishii et al. [25], who performed their study upon Japanese population found no association between TNF-α -308 G/A polymorphism and development of insulin resistance and T2DM. Also, Diana et al. [17] did not confirm the association between both TNF-α -308 G/A and 238 G/A polymorphisms and the development of T2DM.

Positive results between TNF-α -308 G/A and -238 G/A polymorphisms and susceptibility to T2DM were observed in Chang et al. [22] results which showed that the GA genotype of both TNF-α -308 and -238 was more susceptible for T2DM. Our results showed an association between TNF-α -308 G/A, but not -238 G/A with the development of T2DM. Our study showed that GA and AA genotypes of TNF-α -308 G/A more susceptible to T2DM than GG genotype. Moreover, A allele showed a higher frequency in T2DM than G allele. In 2006, Hollegaard [6] and his colleagues found that TNF-α gene polymorphisms at -308 and -238 were associated with increased expression of TNF-α. Our results also confirmed this information.

The TNF-α mediates its action through interaction with surface TNFRII, and TNFRII genotypes mainly M196R polymorphism associated with many metabolic and autoimmune disorders [26]. The TNFRII M196R, which results in the substitution of methionine for arginine at position 196, is postulated to affect the proteolytic cleavage of surface TNFRII to form a soluble type of these receptors. It can be speculated that this may alter the spectrum of the receptor activity, and hence might lead to pathological conditions. Tabassum et al. [27] investigated the association of TNFRII M196R genotypes with risk of T2DM in the Indian population and they conclude there is no association between this polymorphism and susceptibility of T2DM.

Also, Rubina et al. [9] found no association between this polymorphism and the development of T2DM. Our results are in agreement with both previous studies that showed no association between TNFRII M196R polymorphism and susceptibility with T2DM. The IL-4 is an anti-inflammatory polypeptide consisting of 129 amino acid residues. It is secreted by many cell lines such as Th2, mast cells, basophils, and eosinophils. Our results showed that the IL-4 level is higher in T2DM patients compared with the control group. This result is in accordance with Bid et al. [28], Yung-Luen Shih et al. [29].

The level of IL-4 in T2DM patients may increase to induce glucose tolerance and insulin sensitivity in these patients by inhibiting pro-inflammatory cytokines production such as TNF-α which induce insulin resistance by inhibiting insulin signal transduction. The IL-4 level is positively correlated with only HbA1c, and this may occur to tolerates glucose tolerance and induces insulin sensitivity. This result is supported by Yung-Luen et al. [29] study which found that transgenic mice with overexpression of IL-4 show better glucose tolerance by boosting insulin signaling. IL-4 expression and activities are affected by different IL-4 gene polymorphism at a various site of IL-4 gene. One of the important IL-4 gene polymorphism that

associated with various autoimmune disorders and inflammatory responses is IL-4 -590 C/T.

The previous study was done by Al-said et al. [30] found that IL-4 -590 CT genotype is associated with risk of T2DM in Egyptian patients, while wild-type CC is at low risk. Another study among Taiwanese T2DM patients also showed that IL-4 -590 CT genotype is more susceptible to T2DM [28]. On the other side, a study done by Kazemi on the Iranian population did not confirm this association [31,32]. Our result agrees with Al-said et al. [30]; we found that IL-4 -590 CT genotype is more susceptible for T2DM. IL-4 binds to surface receptor IL-4Rα, which a crucial component in IL-4 signal transduction. Polymorphism in IL-4Rα alters the binding affinity to IL-4 and affects its activity and associated with several diseases [33]. Our results showed no association between IL-4Rα I50V and susceptibility to T2DM.

Conclusion

From our result, we can conclude that both TNF-α and IL-4 gene polymorphisms may associate with T2DM. Both GA and GG genotypes of TNF-α (-308), and CT genotype of IL-4 may more susceptible to T2DM than other TNF-α and IL-4 genotypes. According to their receptors, only TNFRII RR genotype may at risk of T2DM.

Recommendation

Another large size study was recommended to improve the precise result because our study included a small size sample. Also, tissue culture study is recommended to take a broad picture of these cytokines effect during insulin resistance and development of T2DM.

Acknowledgment

We are grateful to all staff of medical laboratory department of Prince Mansour specialized hospital in Taif for their great help throughout this project.

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