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Study the association between rs1800629 polymorphism in tumor necrosis factor alpha gene and insulin-dependent diabetes mellitus

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Abstract

Background: Cytokines have vital roles in the autoimmune disease known as insulin-dependent diabetic mellitus (IDDM), which is genetically predisposed. Several studies elucidated that tumor necrosis factor-alpha gene (TNF-α) polymorphism was related to the evolution of diabetes mellitus. The current study was aimed to evaluate the cytokines TNF-α and interleukin-6 (IL-6) levels as well as to assess the gene polymorphism of TNF-α rs1800629 (-308G/A) variations among diabetic children. **Subjects and Methods:** The study was carried out on 161 IDDM cases and 120 non-diabetic subjects. The cytokine TNF-α and IL-6 levels were evaluated in serum by enzyme-linked immunosorbent assay (ELISA). The TNF-α -308G/A (rs1800629) gene polymorphism was examined with the real-time polymerase chain reaction (RT-PCR) method. **Results:** Serum levels of TNF-α and IL-6 were elevated among IDDM patients in comparison to controls, and a positive correlation was indicated between TNF-α and IL-6 (r=0.235, P<0.001). The association analysis showed a significant difference in the TNF-α gene between patients and controls. Specifically, the patients with the A/A genotype had a considerably greater risk of IDDM compared with the control. **Conclusion:** The findings indicated that the mutant TNF-α (308 A/A) (rs1800629) was a risk factor for IDDM vulnerability in children. Hence, TNF-α plays an essential role in the evolution and pathogenesis of IDDM, and it might be a useful indicator of the disease's progression.

Keywords: Insulin-dependent diabetes mellitus (IDDM); tumor necrosis factor-alpha (TNF-α); interleukin 6 (IL-6); diabetes mellitus (DM); gene polymorphism.

1. Introduction

One of the major global public health issues is diabetes mellitus (DM). It was estimated that 536.6 million individuals in the age range of 20–79 will develop diabetes in 2021, accounting for 90% of cases with noninsulin-dependent diabetic mellitus (NIDDM) [1]. Diabetes has evolved into greater health and financial burdens worldwide in recent years, particularly in low- and middle-income nations [2]. Diabetic mellitus that is insulin-dependent has received less attention than NIDDM in the majority of research evaluating diabetes burdens [3]. IDDM is a serious chronic autoimmune illness that often affects children and adolescents [4], and it has become more common globally in the last decade [5]. The incidence of IDDM in children under 14 has grown globally since 1989 by 3% each year [6], in addition to 8.4 million individuals expected to have the disease by 2021 [7]. The challenges include under diagnosis, misdiagnosis, a high likelihood of complications, and early mortality [8, 9]. As a result of an entire lack of insulin secretion, type 1 diabetes mellitus (T1DM) is regarded as a chronic condition that manifests clinically as hyperglycemia and a wide range of symptoms [10–12]. The autoimmune process that arises from a complicated interplay between cytokine inflammatory pathophysiology, genetic and environmental variables is responsible for this illness [13]. An imbalance between Th1 and Th2 inflammatory responses with cytokine secretion, immunological activation of T cells, and disruption of both peripheral and central tolerance are associated with the disease's development. These factors eventually result in the continuing deterioration of β-cells and the gradual loss of insulin secretion [14,15]. Evaluation of the role of inflammation in T1DM, as mediated by acute phase proteins and cytokines, showed that inflammation can either prevent or promote diabetes. The powerful pro-inflammatory cytokine and immunomodulator tumor necrosis factor alpha (TNF-α) is secreted by the natural killer cells, activated macrophages, monocytes, eosinophils, neutrophils, mast cells, CD4+ lymphocytes, and neurons. It plays a role in several metabolic diseases, including obesity, T1DM, and T2DM. Insulin resistance results from the suppression effects of insulin [16]. The promoter region of the human TNF-α gene has a large number of single-nucleotide polymorphisms (SNPs). The structural differences caused by these SNPs in the gene's regulatory regions may have an impact on TNF-α production or function [17]. TNF-α-308G and TNF-α-308A are the two alleles presented at this polymorphism location, which are related to the TNF-α308 guanine/adenine (G/A) (rs1800629). The most common genotype in normal people is TNF-α-308G homozygosity [18]. Regarding disease risk, one of the most significant TNF polymorphisms, for instance, IDDM vulnerability, is TNF-α-308 [19]. There are few contradicting

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reports regarding TNF-α's function in the involvement and development of IDDM. Thus, the purpose of the current research was to assess serum TNF-α level and the significance of the SNP (rs1800629) as a risk factor for IDDM susceptibility in Egyptian children, in addition to identify any potential association between this specific gene and the autoimmune disease's duration and risk factors.

2. Subjects and Methods

Study population

This was a cross-sectional observational study; the samples were conducted from the Centre for Diabetes and Endocrinology, Children Hospital in Giza, Egypt. The children with IDDM of both sexes were sequentially recruited as study participants. On the basis of the criteria provided by the American Diabetes Association [20], 161 cases with IDDM were included in the research. None of the diabetics were taking any kind of medicine, not even aspirin, antibiotics, anti-inflammatory medications, or immunosuppressive drugs. Exclusion criteria included steroid medication, macrovascular implications, autoimmune disorders (rheumatoid arthritis), any diabetic complications (retinopathy, neuropathy, and nephropathy), in addition to acute or chronic diseases. The controls comprised 120 healthy, non-diabetic subjects who were matched for age and sex and had fasting plasma glucose (FBG) less than 100 mg/dL, referred to as normal FBG. Their overall health was fine; they had no family history of IDDM, no clinical signs of the disease, no chronic illnesses, no visible anomalies, and they were not on any medications. The informed consent was obtained from the parents of the children to be enrolled in this study.

Anthropometric measurements

The equation for calculating body mass index (BMI) was weight divided by squared height (kg/m^2). Each subject's waist circumference (WC) was measured using a standard tape graduated in centimeters (cm) at the midpoint between the iliac crest and the lower rib margin. Hip was defined as the largest circumference of the abdomen at the greater trochanter level. By dividing the hip circumference by the waist, the waist/hip ratio was obtained.

Blood sample collection and storage

All participants had venous blood samples drawn, which were separated into 2 sections. After being incubated for 15 minutes at 37˚C, the first portion was put into a centrifuge tube and rotated at 3,000 rpm for 10 minutes. Following separation, the sera were kept at -80˚C until the ELISA method was applied to estimate the serum levels of TNF-α and IL-6. The remaining portion was placed in an EDTA-coated tube for whole blood DNA extraction and kept at -80˚C until the TNF-α (rs1800629) was analyzed using real-time polymerase chain reaction (RT-PCR).

Biochemical analysis

Fasting plasma glucose levels were measured with an enzymatic colorimetric method (Stanbio Laboratory, USA).

Assessment of serum insulin and cytokine levels of TNF-α and IL-6 (ELISA technique)

An enzyme-linked immunosorbent assay (ELISA) commercial kit (AviBion Human TNF-α ELISA Kit; IBL International GmbH, Germany and Orgenium Laboratories' human, Vantaa, Finland; Chemux BioScience, Inc., San Francisco; respectively) was used to measure the serum levels of TNF-α, IL-6, and human insulin in the study groups in accordance with the manufacturer's guidelines.

Analysis of (rs1800629 G/A) polymorphism for TNF-α -308 gene (Real-Time PCR)

The QIAamp Extraction Kit (QIAamp; Qiagen, Germany) was used to extract DNA from whole blood in accordance with the manufacturer's guidelines. Employing TaqMan TNF-α SNP genotyping tests, an allelic discrimination assay rs1800629 G/A acquired from Applied Biosystems, Foster City, CA, USA) [21], genotyping was performed by RT-PCR in accordance with the manufacturer's instructions. In summary, the analyses were carried out in a total reaction volume of 25µl, which included 1µl of genomic DNA, 1.25µl of the 20X working mix for the SNP genotyping assay, and 12.5µl of the 2X TaqMan® Universal Master Mix. The PCR system 2700 real-time PCR equipment (Applied Biosystems, USA) was used for performing the RT-PCR. The thermal cycling program steps comprised 10 minutes at 95˚C for denaturation, then 45 cycles at 92˚C for 15 s, and 60˚C for 60 s to complete the extension stages.

Statistical analysis

SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. The G*Power software was utilized to compute the statistical power and sample size version 3.1.9.2 (Franz Faul, Universität Kiel, Germany). For quantitative variables, data are presented as means ± standard deviation (SD), and for qualitative variables, as a number (%). Quantitative variables were compared using the t test for two variables and one-way ANOVA for more than two variables. To compare the observed and predicted frequencies of categorical variables, the Chi square test was employed. For statistical significance, a two-sided $p < 0.05$ was used. The additive, recessive, and dominant models served as the basis for these analyses, and the ORs

along with their 95% confidence intervals (CIs) were displayed. Logistic regression analysis was achieved to demonstrate the association of risk factors with IDDM. Person's correlation analysis was used to calculate the degrees of association between IL-6 and TNF-α. A receiver operator characteristic (ROC) curve was performed for insulin-dependent diabetic diagnosis depending on the accuracy of TNF-α and IL-6 levels.

3. Results

Clinical and demographic characteristics of insulin-dependent diabetes mellitus patients and controls are summarized in Table 1. The results revealed that BMI, waist-hip ratio, and FBG were higher in IDDM cases than controls (P<0.01). Moreover, serum levels of TNF-α and IL-6 were higher among diabetic children. The data showed a significant positive correlation between TNF-α and IL-6 (r = 0.235**; P< 0001) (Figure 1) for diabetic children. The genetic models that were used to investigate the relationship between the TNF-α-308G/A polymorphism (rs1800629) and the possibility of IDDM were: 1) the genotypic model (GG, GA, and AA); 2) the dominant genetic model (AA+GA vs. GG) (A is the minor allele and G is the major allele); 3) the recessive genetic model (AA vs. GG+GA); 4) the co-dominant model (GA vs. GG+AA); and 5) the allelic model: A allele vs. G allele analysis (Table 2). The variations in TNF-α polymorphism among the various genotypes in diabetic and control groups were indicated in Table 3. There were statistically significant differences between diabetic patients and controls with a normal genotype and those with mutant genotypes for BMI, W/H ratio, and serum levels of IL-6 and TNF-α. The linear regression of the risk factors among IDDM subjects was indicated in Table 4. The receiver operating characteristic (ROC) analysis data obtained in Table 5 and Figure 2 were used to assess the accuracy of serum levels of IL-6 and TNF-α as markers for early diagnosis of IDDM in children.

Table 1: Demographic and clinical characteristics of insulin dependent diabetes mellitus (IDDM) patients and controls

Variables	Non-IDDM group (n=120)	IDDM group (n=161)	*P*-value
Age (years)	10.92±1.7	11.2± 1.14	0.051
Sex (F/M)	57/63	78/83	---
Duration (years)	---	3.68±2.3	---
Height (cm)	131.19±10.35	135.4±22.25	0.056
Weight (kg)	32.5±12.2	40.32±16.94	**0.000**
BMI (kg/m^2)	18.4±4.69	21.54 ±5.75	**0.000**
Waist (cm)	68.5±21.3	68.16 ±11.9	0.866
Hip (cm)	75.2±11.17	80.1±14.9	**0.003**
W/H	0.9±0.18	0.86±0.06	**0.003**
FBG (mg/dL)	148.2±2.98	86.35±1.97	**0.000**
Insulin (µIU/mL)	14.3±8.01	15.53 ±13.67	0.397
IL6 (pg/ml)	3.36 ± 0.3	7.33 ±2.58	**0.000**
TNF-alpha (pg/ml)	4.725±2.32	46.88 ±24.87	**0.000**

Variables are presented as mean ± SD or numbers
P values <0.05 are represented in bold font and considered as statistically significant

Table 2: Examination of the relationship between the G/A polymorphism in TNF-α-308 and the risk of insulin-dependent diabetic mellitus (IDDM)

rs1800629 SNP	IDDM (n=161)	Controls (n=120)	*P* values	OR	95%CI
Genotypic model					
GG	42 (26.1%)	10 (8.3%)	Ref.		
GA	96 (59.6%)	95 (79.2%)	**0.000**	0.241	0.114-0.507
AA	23 (14.3%)	15 (12.5%)	**0.035**	0.365	0.141-0.942
Dominant model					
GG	23 (14.3%)	15 (12.5%)	0.664	1.167	0.580-2.345
GA+AA	138 (85.7%)	138 (85.5%)			
Recessive model					
GG+GA	119 (73.9%)	110 (91.7%)	**0.000**	0.258	0.123-0.538
AA	42 (26.1%)	10 (8.3%)			
Co-dominant model					
GG+AA	65 (40.4%	25 (20.8%)	**0.000**	2.573	1.497-4.422
GA	96 (59.6%)	95 (79.2%)			
Allele model					
G	143 (69.8%)	105 (87.5%)			
A	62 (30.2%)	15 (12.5%)	**0.000**	3.035	1.636-5.629

Data are number (%), variables were compared using chi square (χ^2) test or Fischer's exact test
P values for comparison between diabetic and controls; OR: odd ratio; Cl: confidence interval
Bold values indicate significant difference *P* value < 0.05 was considered significant

Table 3: Associations between TNF-α-308-G/A and clinical parameters in the studied groups

Variable	IDDM (n=161)			Controls (n=120)			*P*-value
TNF-α 308 G/A (rs1800629)	GG (n=42)	GA (n=96)	AA (n=23)	GG (n=10)	GA (n=95)	AA (n=15)	
BMI	20.05±4.654	21.62±6.19	23.92±4.92	23.49±3.5	17.46±4.6	21.0±1.71	**0.000**
W/H	0.862±0.018	0.86±0.078	0.83±0.04	1.27±0.46	0.87±0.05	0.87±.0404	**0.003**
Insulin	16.34±13.01	14.53±13.6	18.23±15.3	12.9±3.27	14.7±8.56	13.07±6.49	0.397
IL-6 pg/mL	8.043±2.539	7.313±2.81	6.157±0.21	3.4±0.105	3.34±0.32	3.5±0.293	**0.000**
TNF-alpha pg/mL	45.54±27.65	50.47±24.1	34.35±18.6	2.75±0.26	4.547±2.2	7.17±2.17	**0.000**

Numeric variables are presented as mean ± SD

Table 4: Regression analysis for the risk factors in insulin dependent diabetic patients

Independent Variables	B	S.E.	Wals x^2	Significant	Expected B	95% C.I. for EXP (B)	
						Lower	Upper
Age	-0.035	0.050	0.482	0.488	0.966	0.875	1.066
Duration	0.212	0.069	9.369	**0.002**	1.236	1.079	1.415
BMI	0.010	0.025	0.158	0.691	1.010	0.962	1.060
IL6	-0.137	0.06	5.17	**0.023**	.872	0.774	0.981
TNF	0.014	0.007	3.9	**0.048**	1.014	1.000	1.028

B: regression coefficient; BMI: body mass index; CI: confidence interval; SE: standard error

Table 5: ROC analysis for serum TNF-α and IL-6 levels in insulin dependent diabetic group

Variables	Area	Std. Error	Specificity	Sensitivity	Accuracy	*P*-value	95% Confidence Interval	
							Lower Bound	Upper Bound
TNF-alpha pg/mL	0.779	0.035	89%	71%	80%	**0.004**	0.710	0.847
IL-6 pg/mL	0.824	0.039	89%	68%	78.5%	**0.001**	0.747	0.901

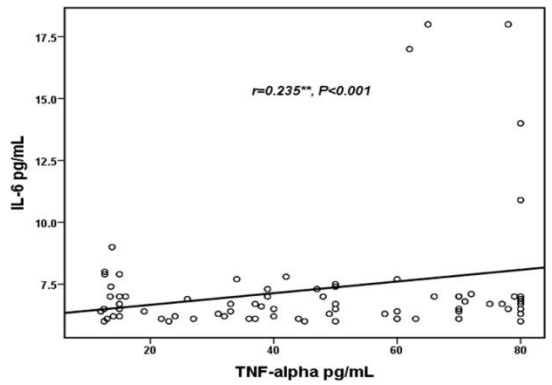

Figure 1: Correlation between TNF alpha and IL-6 levels in IDDM cases

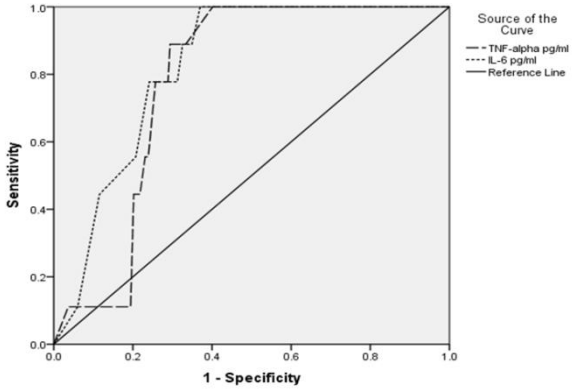

Figure 2: Receiver operating characteristic (ROC) curve analysis for serum TNF-α and IL-6 levels in insulin dependent diabetic group versus non-diabetic controls

4. Discussion

A significant public health concern in the twenty-first century is diabetes. A chronic autoimmune disease with an increasing frequency is type 1 diabetes mellitus (T1DM), often referred to as insulin-dependent diabetes mellitus (IDDM) [22]. IDDM results from a sequence of physiological alterations, including genetic susceptibility, dysregulation of effector and regulatory T cells, loss of central and/or peripheral tolerance, and antagonistic or synergistic communications involving cytokines. These elements culminate in an inflammatory cascade that destroys the β cell and induces diabetes [15]. Children with diabetes have distinct developmental characteristics depending on the metabolic management, age at initially, and length of the disease [23]. Thus, 161 diabetic children who were age- and gender matching with healthy controls were included in our study. Nonetheless, the study's findings revealed a minor male predominance among children with diabetes. Our findings agreed

with the findings published by Hassan et al. [24] and Aljuhani et al. [25]. The findings of Gabri et al. [26], Zurita-Cruz et al. [27], and Lee et al. [28] showed that, in contrast to our results, 54% and 51.5% of their patients were female. Furthermore, the results demonstrated that children with diabetes had higher body mass indexes (BMI) and were taller and heavier than children without the disease. Similarly, Hassan et al. [24] observed that children with diabetes under control had higher BMIs and were taller and heavier than children with uncontrolled diabetes. Conversely, the findings of Khadilkar et al. [29] and Gaete et al. [30] showed that the T1DM group had lower BMI, weight, and height values than the control group. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that triggers the apoptotic and cytotoxic responses, increases adhesion molecule expression, and stimulates the production of IL-6 [31–33]. It is released by activated CD4+ T cells, causing necrosis and localized infiltration of pancreatic lymphocytes. This ultimately causes the β cell to die and prompts the pancreas to produce TNF-α. Consequentially, it immediately aids in the damage done to β cells and advances the pathophysiological process of T1DM [31, 34]. TNF-α has been observed to decrease the expression of insulin-regulated glucose transporter type 4, primarily found in skeletal muscles and adipocytes [35]. TNF-α is thought to be a potential mediator between insulin resistance and diabetes since it was thought to cause insulin resistance by preventing the phosphorylation of insulin receptor substrate 1 on the insulin signaling cascade [36]. According to our data, TNF-α and IL-6 levels were significantly higher in diabetes patients than in control subjects. Also, this relation has been established by the results of the positive correlation between IL-6 and TNF-α. Our findings are consistent with a prior study's finding that children with diabetes had greater TNF levels than controls [37]. Moreover, previous investigations conducted in Egypt revealed that patients having type 1 DM [38] and type 2 DM [39] had higher TNF levels. According to our data, individuals with insulin-dependent diabetes had more TNF-α gene polymorphism AA than people without diabetes, which may indicate more inflammatory activity. As well, Dos Santos Haber and colleagues reported similar findings [15]. Al-Terehi et al., on the other hand, came to the conclusion that TNF-α gene polymorphisms do not significantly differ between patients and controls [40]. These results may be explained by an in vitro investigation that displayed the evolution of diabetes was accelerated by targeted overexpression of TNF-α in transgenic mouse pancreatic cells [41]. In accordance with a different study conducted in adoptive transfer models, diabetes can be generated mostly by Th1 and Th2 cells [42]. In the recently-onset T1DM mouse model, anti-TNF therapy prevented the disease's advancement by re-establishing self-tolerance and glycemia homeostasis [43, 44]. On the contrary, the disease progressed more quickly when anti-TNF-α was administered at four weeks of age or later [45]. Nevertheless, this cytokine's systemic delivery provided protection against T1DM [46]. These investigations demonstrated that TNF-α may either trigger or suppress inflammatory pathways. The answer's direction is likely influenced by limited variables, such as genetic susceptibility and the disease's stage [34, 47].

5. Conclusions

Diabetes mellitus is considered a current pandemic worldwide and a major public health issue in Egypt. Diabetes is becoming more common to a disturbing extent. Children with diabetes have dysregulated levels of several inflammation-marker cytokines, which are known to promote disease. Our results demonstrated that the IDDM group had a considerably larger proportion of high-risk patients based on TNF-α value and that the TNF-α 308 G/A (rs1800629) polymorphism along with TNF-α level were significantly higher within IDDM participants in comparison to the controls. Moreover, a considerable positive correlation was observed between elevated TNF-α levels and circulating IL-6 levels in individuals with diabetes. As a result, TNF-α may act as an alternative indicator of diabetes and provide insight into the pathogenetic pathways and regulation of the disease process, which may help in the development of novel immunotherapeutic approaches. Our findings support the establishment of a national IDDM registry as well as the necessity of more multicenter epidemiological investigations encompassing the entire nation in order to determine the incidence of IDDM nationally and provide relevant health information for Egypt.

6. Conflicts of interest

No conflict of interest.

7. Authors' contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved the submission.

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