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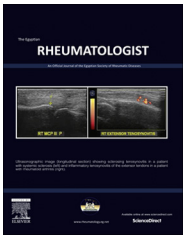

ORIGINAL ARTICLE

Association of interferon-γ and its (+874 T/A) gene polymorphism with type 2 diabetes mellitus in rheumatoid arthritis patients

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KEYWORDS

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Abstract *Background:* Rheumatoid arthritis (RA) patients are at an increased risk of developing type-2 diabetes mellitus (T2DM) compared to the general population. Interferon-γ (IFN-γ) was found to have a role in both RA and T2DM.

Aim of the work: To investigate the role of IFN-γ and its +874T/A gene polymorphism in the development of T2DM in RA patients.

Patients and methods: IFN-γ level and its +874T/A gene polymorphism were investigated in 70 RA patients with T2DM and in 80 without, in addition to 150 healthy controls.

Results: The level of IFN-γ was significantly higher in RA patients with (465 ± 64.4 pg/ml) and without (219 ± 50.3 pg/ml) T2DM compared to controls (110 ± 18 pg/ml) ($p < 0.0001$). IFN-γ +874T/A genotyping showed a significant increase in the frequency of AA genotype (42.9%) and a significant decrease in TT genotype (14.2%) in RA patients with T2DM compared to those without; similarly, the frequency of the T-allele was significantly lower ($p < 0.05$) and the A-allele increased ($p < 0.05$); however no significant differences in the genotypes distribution were found between non-diabetic RA patients and healthy controls. The TT-genotyped RA patients with (539.6 ± 4 pg/ml) and without (260 ± 59.6 pg/ml) diabetes had higher serum IFN-γ levels compared to other genotypes ($p < 0.001$), while in controls, no significant difference in IFN-γ levels according to genotype was observed.

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Conclusions: Serum IFN-γ and its gene polymorphism may play a role in the susceptibility of RA patients to T2DM. The homozygous genotypes AA and TT seem to be more commonly associated with diabetes in RA patients with special contribution of the A-allele.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by progressive inflammation and involvement of T-cells, B-cells and pro-inflammatory cytokines [1].

Many cytokines are key players in the pathogenesis of RA including tumor necrosis-α [2] and interleukin-6 (IL-6) [3] with involvement of their gene polymorphisms.

Patients with RA are at an increased risk of insulin resistance and type 2 diabetes mellitus (T2DM) due to the raised levels of systemic inflammation [4] and metabolic syndrome has been frequently reported in Egyptian RA patients [5].

Interferon-γ (IFN-γ), a T-helper 1 (Th1) cytokine has an important role in the pathogenesis of RA as it serves as a marker of activation of Th1 cells which promote and amplify autoimmune diseases, furthermore, it is involved in the pathogenesis of T2DM [3,4]. This cytokine has the potential to direct the inflammatory response by upregulating a variety of pro-inflammatory mediators including TNF-α and IL-6. Moreover, data suggest that IFN-γ may also be able to directly enhance activation of the pro-inflammatory nuclear transcription factor-κB (NF-κB) under certain conditions [6]. A cytosine–adenine (CA) repeat polymorphism was reported in the first intron of the IFN-γ gene and found to affect transcription. In addition, an adenine (7) to thymine (T) transition at position +874 (rs2430561) has been associated with increased IFN-γ expression [8]. Association of a polymorphic microsatellite located in the first intron of the IFN-γ gene, which is in complete linkage disequilibrium with the rs2430561 functional variant; with susceptibility to RA was reported [9]. Also, the association between IFN-γ gene +874 (A/T) polymorphisms and T2DM was reported [7]. However, other studies failed to confirm the presence of association between that gene polymorphism and RA [10,11].

The aim of the present study was to assess the serum IFN-γ levels and IFN-γ gene +874 (A/T) polymorphism in RA patients with and without T2DM in comparison to the level in control subjects.

2. Patients and method

2.1. Study groups

The study included 150 RA patients, recruited from the Rheumatology Department, Sohag University Hospital, who satisfied the criteria of the American College of Rheumatology/European League Against Rheumatism Collaborative initiative for the classification of rheumatoid arthritis [12]. Seventy of these RA patients developed T2DM during the course of disease. The diagnosis of T2DM was considered according to the American Diabetes Association Criteria [13]. 150 healthy subjects, their age and sex were matched to the patients served as controls. Written informed consents were obtained from all enrollees and the study was carried out in accordance with the guidelines of the ethics committee of Faculty of Medicine, Sohag University and to the tenets of Helsinki declaration.

For all participants the following tests were done; C-reactive protein (CRP), fasting blood glucose (FBG), lipid profile parameters: total cholesterol (TC), high density lipoprotein (HDL-C), triglyceride and low density lipoprotein (LDL-C).

2.2. Assessment of serum IFN-γ and IFN-γ +874 T/A

Approximately 5 ml venous blood samples were collected from the participants after an overnight fasting and divided into two parts; one part (2 ml) was taken in EDTA tubes for DNA extraction and the other part (3 ml) was taken in plain tubes and the serum was separated after centrifuged at 3000 rpm for 15 min and used for routine laboratory tests and for the estimation of IFN-γ protein levels using ELIZA test kit supplied by R&D system. The puffy coats of EDTA samples were obtained after centrifugation at 3000 rpm for 15 min and used for DNA extraction using QIAamp kit supplied by Qiagen (USA).

2.3. Genotyping

Genotyping for the polymorphism of IFN-γ +874 T/A was carried out by the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) assay in two separate PCR reaction mixtures according to Sen et al. with modifications [14]. Two forward primes (one for A-allele; and the other for T-allele) and a common reverse primer were used for IFN-γ +874 T/A genotyping as following: forward A; 5-TTCTTA CAACACAAAATCAAATCA-3, forward T; 5-TCAACAAA GCTGATACTCCA-3; and common reverse primer; 5-TTCT TACAACACAAAATCAAATCT-3, in addition, an internal control primer pair to amplify human growth hormone (HGH) sequence to check for successful PCR amplification as following; forward; 5-CCTTC CAACCATTCCCTTA-3 and reverse; 5-TCACGGATTTCTGTTGTGTTTC-3. Amplification was carried out in a thermocycler (Biometra, Germany), using 25 μl reaction volume containing the following; 2.5 μl, ×10 PCR buffer (500 KCl, 100 Mm Tris–HCl, 1.0% Triton X-100), 0.75 μl 50 mM $MgCl_2$, 0.5 μl 10 mM dNTPs, 1.0 μl, of each primer (10 pmol/ml), 1.0 μl of template DNA and 0.125 μl Taq polymerase (5 U/μl). The PCRs were performed in the following conditions: 95 °C for 4 min, followed by 10 cycles of 95 °C for 15 s, 62° for 50 s and 72 °C for 40 s, and then 25 cycles of 95 °C for 20 s, 49 °C for 50 s and 72 °C for 50 s, and 72 °C for 10 min for the final extension. The amplified products were separated by electrophoresis on 2% agarose gel stained with 0.5 μg/ml ethidium bromide and

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Table 1 Demographic and laboratory data of the rheumatoid arthritis patients with and without type-2 diabetes mellitus and the control.

Parameter	RA patients (n = 150)		Control (n = 150)	p
	With T2DM	Without T2DM		
Female:male	50/20 (2.5:1)	50/30 (1.7:1)	105/45 (2.3:1)	>0.05
Age (years)	57.8 ± 4.6	57.4 ± 4.3	56 ± 5.2	>0.05
Dis. duration (years)	6.4 ± 2	7 ± 2.4	–	>0.05
Body mass index	26.2 ± 3.2	24.8 ± 2.5	25.6 ± 3	>0.05
FBG (mg/dl)	153.4 ± 16.7	95 ± 11.5	94 ± 10.5	<0.0001
TC (mg/dl)	260.9 ± 65.2	194 ± 30	186.9 ± 12.4	<0.0001
TG (mg/dl)	208.6 ± 60.8	136.6 ± 31	109 ± 29.4	<0.0001
HDL-C (mg/dl)	30 ± 7	33 ± 8	37.5 ± 4.5	<0.05
LDL-C (mg/dl)	168.4 ± 63.2	133 ± 18	101.8 ± 14.9	<0.001
CRP (mg/dl)	19.4 ± 3.3	9.5 ± 2.2	1.9 ± 0.1	<0.0001
INF-γ (pg/ml)	465 ± 64.4	219 ± 50.3	110 ± 18	<0.0001

RA: rheumatoid arthritis, T2DM: type-2 diabetes mellitus, FBG: fasting blood glucose, TC: total cholesterol, TG: triglycerides, HDL-C: high density lipoprotein-cholesterol, LDL-C; low density lipoprotein-cholesterol, CRP:C-reactive protein, INF-γ: interferon-gamma.

visualized using gel documentation system. Two products were obtained; 261-bp for IFN-γ and 426-bp for HGH.

2.4. Statistical analysis

Data were expressed as mean ± SD or number and percent. Genotype distribution was tested for deviation from HWE (Hardy–Weinberg equilibrium) by x^2 analysis. Fisher Exact test was used to compare the genotypes frequencies in the patients and the controls. Continuous data were compared using Mann–Whitney and ANOVA tests. A two-tailed value of $p < 0.05$ was considered statistically significant. All statistical calculations were performed using the computer program SPSS (Statistical Package for the Social Science; SPSS, Chicago, IL, version 16 for Microsoft Windows, USA).

3. Results

The clinical and laboratory data of the participants are represented in Table 1. There were no significant differences between the participants regarding age, sex or disease duration ($p > 0.05$). Diabetic RA patients had significantly higher FBG, TC, LDL-C and lower HDL-C compared to non-diabetic RA and healthy controls ($p < 0.05$).

The IFN-γ serum levels were significantly higher in RA patients (diabetic or non-diabetic) compared to controls ($p < 0.0001$). Also there was a significant correlation between IFN-γ level and CRP level in both groups of RA patients (Fig. 1). In addition a significant correlation was found between FBG and IFN-γ levels in diabetic RA patients ($r = 0.36$, $p = 0.002$).

The IFN-γ +874 T/A gene polymorphism genotyping showed that there was no deviation from HWE for both the patients and the controls. Fig. 2 shows agarose gel electrophoresis of interferon-γ +874 T/A gene polymorphism. AA genotype showed a significant increase, TT genotype exhibited a significant decrease in diabetic RA patients compared to non-diabetic RA patients ($p < 0.05$). The frequency of the T-allele was significantly decreased ($p < 0.05$) and the A-allele significantly increased ($p < 0.05$) in diabetic RA patients compared to non-diabetic RA patients. No significant

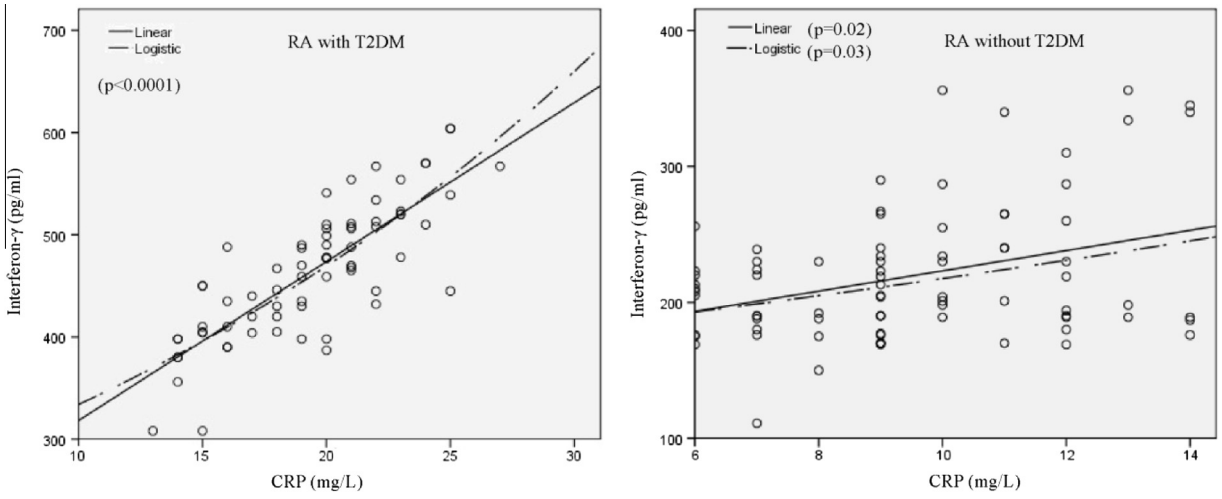

Figure 1 Correlation between the interferon-γ serum level and the CRP level in rheumatoid arthritis patients with type-2 diabetes mellitus (T2DM) (left) and those without T-2DM (right).

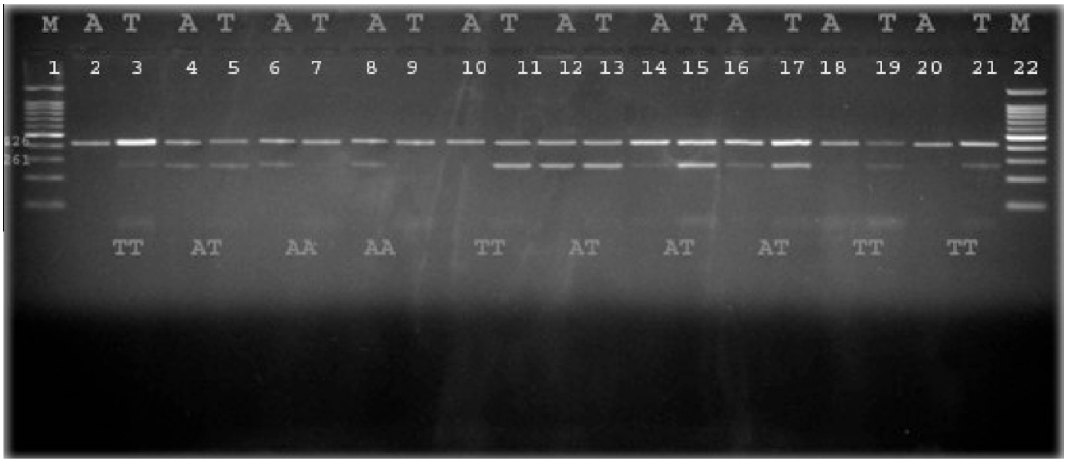

Figure 2 Agarose gel electrophoresis showing interferon-γ +874 T/A gene polymorphism. Each genotype is represented by 2 lanes; lanes 1 and 22 represent 100 bp ladder, lanes 2 and 3, 10 and 11, 18 and 19, 20 and 21 represent TT genotypes; Lanes 4 and 5, 12 and 13, 14 and 15, 16 and 17 represent AT genotype; lanes 6 and 7, 8 and 9 represent AA genotype.

Table 2 IFN-γ +874T/A genotypes of the rheumatoid arthritis patients with and without type-2 diabetes mellitus and the control.

INF-γ +874T/A genotype *n* (%)	RA patients (*n* = 150)		*p*	Controls (*n* = 150)	*p*
	With T2DM (*n* = 70)	Without (*n* = 80)			
TT	10 (14.2)	25 (31.25)	<0.05	40 (26.7)	>0.05
TA	30 (42.9)	42 (52.5)	>0.05	80 (53.3)	>0.05
AA	30 (42.9)	13 (16.25)	<0.05	30 (20)	>0.05
T-allele	50 (35.7)	92 (57.5)	<0.05	160 (53.3)	>0.05
A-allele	90 (64.3)	68 (42.5)	<0.05	140 (46.7)	>0.05

INF-γ: interferon-gamma, RA: rheumatoid arthritis, T2DM: type-2 diabetes mellitus.
N.B: the number of alleles equals double the number of the participants.

Table 3 Serum INF-γ levels according to genotype in rheumatoid arthritis patients with and without type-2 diabetes mellitus and the control.

INF-γ (pg/ml)	Genotype			*p*
	TT	TA	AA	
RA patients				
With T2DM	539.6 ± 4	504.8 ± 34.7	400.4 ± 38.6	<0.001
Without	260 ± 59.6	212 ± 28.6	176 ± 21.8	<0.001
Controls	113.8 ± 19.2	109.4 ± 18	106.9 ± 16.4	>0.05

INF-γ: interferon-gamma, RA: rheumatoid arthritis, T2DM: type-2 diabetes mellitus.

differences in the genotypes distribution were found between non-diabetic RA patients and the controls (Table 2). RA patients (diabetic or non-diabetic) with TT-genotype had highest IFN-γ levels and those with AA-genotype had the lowest and patients with TA-genotype had intermediate levels. However, IFN-γ levels didn't differ according to the genotype in the controls (Table 3).

4. Discussion

In the present study, IFN-γ serum levels were found elevated in RA patients either diabetic or non-diabetic, and significantly correlated to CRP levels. The elevated levels of pro-inflammatory cytokines, including IFN-γ in RA and their correlation to disease activity have been reported in previous studies [15–19]. Interferon-γ has also been implicated as a biomarker for radiographic damage [20] and anemia [21] in RA patients. Diabetic RA patients had significantly elevated CRP and IFN-γ levels compared to non-diabetic RA patients, indicating more disease activation and immune stimulation in those patients. RA patients are at an increased risk of insulin resistance and T2DM due to the associated systemic inflammation [22].

The findings of IFN-γ +874 T/A gene polymorphism genotyping were similar to those previously reported [7,10,11], however this is the first study to investigate the role of IFN-γ +874 T/A gene polymorphism in the association of RA patients to T2DM. IFN-γ was found to be involved in the pathogenesis of diabetes mellitus and the frequency of the low IFN-γ production allele (A-allele) was found significantly higher in type-2 diabetics compared to controls [7]. IFN-γ +874 T/A gene polymorphism has been reported to affect its gene

expression. The AA genotype has been linked to lower IFN-γ levels, compared with the TT genotype, while the heterozygous (TA) genotype has been characterized by intermediate levels [8]. IFN-γ +874 T/A gene polymorphism overlap with the middle of an assumed NF-κB binding site that may be of functional concerns for the transcription of IFN-γ gene [23]. On the other hand, in a cohort of Brazilian RA patients there was no significant difference between the patients and controls regarding the IFN-γ +874 T/A polymorphism [24].

Rheumatoid arthritis is characterized by immune dysfunctions with predominance of the pro-inflammatory responses exhibited by Th1 and Th17. Recent data suggested that IFN-γ had a protective function in RA and involved in the down-regulation of disease-related chronic inflammation [25]. IFN-γ has been suggested to have a key role in the regulation of visceral adipose tissue inflammatory response and endothelial dysfunction in type 2 diabetes [25]. IFN-γ +874 T/A gene polymorphism was found associated with many diseases, including infectious diseases as hepatitis B, Helicobacter pylori gastritis and tuberculosis [26–28] and autoimmune diseases like SLE and scleroderma [29,30].

A longitudinal study taking into consideration the effect of medications received, disease activity, radiographic severity and other components of the metabolic syndrome is recommended to further demonstrate the important role of INF-γ and its gene polymorphism in rheumatoid arthritis and its associated comorbidities.

In conclusion, IFN-γ and its gene polymorphism may play a role in the susceptibility of RA patients to T2DM. The homozygous genotypes AA and TT seem to be more commonly associated with diabetes in RA patients with special contribution of the A-allele.

Conflict of interest

None.

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