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The Gene Polymorphisms (-308G/A) and the Tumor Necrosis Factor-alpha Levels in Type 2 Diabetic Patients with and Without Tuberculosis Infection

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Keywords: Gene polymorphism (-308G/A); TNF-α levels; Type 2 diabetic patients; Tuberculosis infection

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Abstract

BACKGROUND: The gene polymorphism (-308G/A) and tumor necrosis factor-alpha (TNF-α) levels influence development of disease in type 2 diabetic patients and tuberculosis patients.

AIM: In this study, we analyze the association between the TNF-α polymorphisms (-308G/A) and the levels of TNF-α in type 2 diabetic patients with and without tuberculosis infection.

METHODS: This study was an analytic observational with cross sectional approach consisting 40 type 2 diabetic patients with tuberculosis infection, 40 type 2 diabetic patients without tuberculosis infection and 40 healthy control (HC) subjects. The TNF-α gene polymorphism (-308G/A) was analyzed with polymerase chain reaction-restriction fragment lengths polymorphisms (PCR-RFLP) method. The TNF-α levels were measured using an enzyme-linked immunosorbent assay. The association between gene polymorphism (-308G/A) in study groups was analyzed by Fisher's exact test, tumor necrosis factor-alpha (TNF-α) levels in study groups was carried out using the Kruskal-Wallis test. Hardy-Weinberg Equilibrium also determined genotype deviation and allele frequencies.

RESULTS: The GG and GA+AA genotypes frequency in both of patient groups and HC subjects were not differ significantly (95% and 5% vs 95% and 5% vs 92.5% and 7.5%; $p > 0.05$). The TNF-α levels (pg/ml) of type 2 diabetic without tuberculosis infection were higher than those of type 2 diabetic with tuberculosis infection and HC subjects (7.42 ± 0.78 vs 2.23 ± 0.51 vs 2.57 ± 0.63; $p < 0.01$). The TNF-α levels in the GA+AA genotypes were higher than the GG wild-type genotype ($p > 0.05$). There was no significant deviation of genotype frequency and allele from Hardy-Weinberg Equilibrium.

CONCLUSION: The gene polymorphism (-308G/A) had no association with type 2 diabetic patients with and without tuberculosis infection and the gene polymorphism (-308G/A) was not influence the TNF-α levels but there was a significant differentiation of TNF-α levels between the groups.

Introduction

Diabetes mellitus (DM) is the disorder of metabolic pathway caused by the failure of the function and or production of insulin which increase blood glucose levels (BGLs) or hyperglycemia [1].According to the data of International Diabetes Federation (IDF) in 2015, Indonesia ranked as the seventh highest number of diabetic patients worldwide with 10 millions case [2]. In 2013, The Indonesia Ministry of Health stated there was approximately 205 thousand diabetic patients in North Sumatra. Hence, North Sumatra got 8th rank in terms of the number of diabetic patients in Indonesia [3].

Several evidences showed DM increases the risk of respiratory disease. World Health Organization (WHO) showed that DM will increase the risk of tuberculosis infection two to three times higher than healthy population [4]. The relationship of DM with tuberculosis infection was reported for the first time by Avicenna (Ibnu Sina) in XI century. Ibnu Sina reported that the tuberculosis infection is the main cause of death in diabetic patients. In postmortem autoption,

more than 50% diabetic patients had tuberculosis infection. *Mycobacterium tuberculosis* (Mtb) is the only cause of an infectious disease called tuberculosis. The bacteria were found by Robert Koch in 1882 [5]. The incident of TB were 10.4 million cases in 2016 and the TB mortality rate (per 100 000 population) fell by 37% between 2000 and 2016 year [6].

In DM, hyperglycemia leads a disorder of the immune system as the risk factor for activation of latent tuberculosis infection, or appearance of tuberculosis primer infection [7]. These would be resulting in disruption of proinflammatory cytokine production. Recently, experts suggest the metabolic disorders and infectious diseases were associated with interferon gamma, interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, etc [8], [9], [10].

TNF-α is a proinflammatory cytokine that synthesized by macrophages. The synthesis of TNF-αis regulated by specific TNF-α gene sequences. Single nucleotide polymorphism (SNPs) or substitution of one nucleotide base in the TNF-α gene influence the synthesis or levels of TNF-α [11]. Previous studies showedthere was an associationof TNF-α gene polymorphism (-308G/A) with diabetes incidence. Another studies showed allele A of the TNF-α gene at position (-308G/A) associated with risk for tuberculosis but those results were still conflicting [12], [13], [14], [15], [16], [17].

Previous studies also showed there was relationship of TNF-α gene polymorphism with TNF-α levels in diabetic patients and relationship of TNF-α gene polymorphism with TNF-α levels in tuberculosis patients but there was no study assessed the relationship of TNF-α gene polymorphisms (-308G/A) and TNF-α levels in type 2 diabetic patients with tuberculosis infection and without tuberculosis infection [12], [13], [14], [15], [16], [17]. Therefore, we investigated the association of TNF-α polymorphisms (-308G/A) and TNF-α levels in Type 2 Diabetic Patients with TB infection and without TB infection in this study.

Methods

Ethics

The research was conducted after got an approval from the Medical Ethics Committee Universitas Sumatera Utara (No.227KOMET/FK USU/2018). The study was conducted from March until June 2018.

Subjects

This study was an analytic observational with cross sectional approach consisting 40 type 2 diabetic patients with tuberculosis infection, 40 type 2 diabetic patients without tuberculosis infection and 40 healthy control (HC) subjects. Type 2 diabetic patients with tuberculosis infection were recruited at the health care facilities for pulmonary disease (BP4) Medan city. Type 2 diabetic patients without tuberculosis were recruited at Padang Bulan primary health care in Medan city. The HC subjects were recruited at a gym place in Medan city. The match samples with inclusion and exclusion criterias were selected according to researcher's decision. Eligible patients had no taking drugs that affect the levels of TNF-α proinflammatory cytokines such as anti-inflammatory drugs and antibiotics at least 2 days before blood collection, willing to be a study participant and signed informed consent. Patient who consumed vitamin supplements, had immune deficiencies such as HIV, had organ transplant, kidney function disorders, liver function disorders, malignancies, pregnancy and lactation, extra pulmonary tuberculosis were excluded from this study.

The eligible HC subject had age and sex matched with patient groups, healthy, selected based on interviews using questionnaires, blood tests routine and blood glucose levels (BGLs), willing to be a research subject and signed informed consent. The HC subject who had family history of DM were excluded from this study.

Subjects and sample preparation

Demographic characteristics of subject were obtained by filling out questionnaire form. Measurements of BGLs, TNF-α levels and analysis of TNF-α gene polymorphism (-308G/A) were done at the Integrated Laboratory of the Faculty of Medicine, Universitas Sumatera Utara (USU). The study took place from March to July 2018. Five ml of blood samples were withdrawn from the mediana cubiti vein, put into ice box and delivered to the Integrated Laboratory of Faculty of Medicine, Universitas Sumatera Utara. All the blood samples were centrifuged in 10 minutes at 3000 rpm then serum was separated, and stored in refrigerator at -80°C for measurement of TNF-α levels. BGLs was measured within 2 hours after being taken. DNA was isolated from blood leukocytes by usinggenomic DNA kit commercial based on manual prosedur kit (Promega, USA). DNAisolate was then stored at temperature -80°C for determination of gene polymorphism.

Determination of gene polymorphism (-308G/A)

The amplification of isolated DNA was performed withthe polymerase chain reaction (PCR) technique [18]. The amplified products were observed on agarose gel at 107 bp. The PCR reaction mixture (10 µl) was digested using restriction fragment length polymorphism (RFLP) techniqueby the 5 U of

restriction NcoI enzyme and incubated at 37°C for 2 hours, [19]. The PCR-RFLP products were electrophoresed in a 4% agarose gel.

The measurement of biomarkers levels

BGLs (mg/dl) were measured using commercial glucose kit (Bt. Diagnostic) and read with a spectrophotometer at a wavelength of 500 nm. TNF-α levels were measured using commercial TNF-α kit (Biolegend kit) with enzyme linked immunosorbent assay (ELISA) method. The result could be read by the ELISA raider (pg/ml) at 450 nm within 30 minutes with a sensitivity of 3.5 pg/ml and specificity up to 50 ng/ml.

Statistical Analysis

Data were analyzed using statistical package for social sciences (SPSS v.22) software. Association between gene polymorphism (-308G/A) in study groups was analyzed by Fisher's exact test, whereas Hardy-Weinberg equilibrium (HWE) was analyzed by the Chi-Square test. Comparison mean of blood glucose levels (BGLs) and tumor necrosis factor-alpha (TNF-α) levels in study groups was carried outusing the Kruskal-Wallis test and followed by Mann-Whitney test for post hoc comparison test with the significance level (< 0.05). The differences of the TNF-α levels on genotype variants in this population study was checked by the Kruskal Wallis test.

Results

The polymerase chain reaction (PCR) products of TNF-α gene had done visualized on 2% agarose gel and all samples detected had TNF-α gene at 107 bp. Digestion of PCR product of TNF-α gene by the Nco1 enzyme shown 3 bands at 107 bp, 87 bp dan 20 bp.

The distribution of TNF-α gene polymorphism (-308G/A) in groups of subjects can be seen in Table 1.

Table 1: Frequency of genotypes within the (-308G/A) region of TNF-α gene at each groups

Genotype	Type 2 diabetic patients with TB (N = 40)	Type 2 diabetic patients without TB (N = 40)	HC (N = 40)	p
GG	38 (95%)	38 (95%)	37 (92.5%)	0.864
GA+AA	2 (5%)	2 (5%)	3 (7.5%)	

TB = Tuberculosis infection; HC = Healthy control.

Frequency of GG and GA+AA genotypes in both of patient groups were not much different with healthy subjects (95% and 5% vs 95% and 5% vs 92.5% and 7.5%; $p > 0.05$). In this study, the comparison of mean BGLs and TNF-α levels between type 2 diabetic patients with and without tuberculosis infection and healthy control (HC) can be seen in Table 2.

Table 2: Comparison of BGLs and TNF-α levels in type 2 diabetic patients with and without tuberculosis infection and healthy subjects

Biochemical	T2 diabetic patients with TB (N = 40)	T2 diabetic patients without TB (N = 40)	HC (N = 40)	p
Glucose (mg/dl)	295.13^{a} (± 57.16)	246.15^{b} (± 73.30)	104.73 (± 19.29)	0.01
TNF-α (pg/ml)	2.23^{c} (± 0.51)	7.42^{d} (± 0.78)	2.57 (± 0.63)	0.01

TB = Tuberculosis infection; HC = Healthy control; Data shown as mean ± standard deviation; Mann-Whitney test for post-hoc comparison; ^{a} p = 0.01 vs HC; ^{b} p = 0.01 vs HC; ^{a,b} p = 0.01; ^{c} p = 0.46 vs HC; ^{d} p = 0.01 vs HC; ^{c,d} p = 0.01.

The mean of BGLs in type 2 diabetic patients with tuberculosis infection was higher (295.13^{a} (± 57.16) mg/dl) as compared to type 2 diabetic without tuberculosis infection and HC (246.15^{b} (± 73.30) and 104.73 (± 19.29) mg/dl) with $p < 0.05$. Post hoc comparison between each groups were statistically significant ($p < 0.05$). The mean of TNF-α levels in type 2 diabetic patients without tuberculosis infection was higher as compared to type 2 diabetic with tuberculosis infection and HC ($p < 0.05$). Mann-Whitney test for post hoc comparison was shown association between type 2 diabetic without tuberculosis infection and type 2 diabetic with tuberculosis infection ($p < 0.05$), also in type 2 diabetic without tuberculosis infection and HC ($p < 0.05$) but no association in type 2 diabetic with tuberculosis infection with HC ($p > 0.05$).

The association of genotypes of TNF-α gene polymorphism (-308G/A) with TNF-α levels shows in Table 3.

Table 3: The TNF-α levels in subjects with variant genotypes of TNF-α gene polymorphism (-308G/A)

Biomarker	Subjects (N = 120)		p
	GG (N = 113)	GA+AA (N = 7)	
TNF-α (pg/ml)	3.98 (± 0.36)	5.58 (± 0.33)	0.43

The TNF-α levels were higher in GA+AA mutant genotypes compared in GG wild-type genotype but the association between genotypes of TNF-α gene and TNF-α levels shows no significant difference performed by Kruskal Wallis test ($p > 0.05$).

Discussion

In this present study, we analyzed the TNF-α gene from all samples in type 2 diabetic patients with and without tuberculosis infection groups and healthy control group. The TNF-α gene was detected at 107 bp and polymorphism (-308G/A) showed three type of genotypes, namely GG, GA and AA. Hardy-Weinberg Equilibrium was done analysis in all groups and the results were consistent with the HWE ($p > 0.05$), it

means the relative proportions of genotype in the three groups were constant from one generation to the next. The genotype in this population still in equilibrium is due to the possibility of random marriage, and no migration to the population [20]. Several mutations and polymorphisms have been described for TNF-α gene. The TNF-α gene is located on chromosome 6 (6p21.3) between HLA-B and DR, in the class III region at the Major Histocompatibility Complex (MHC) [11]. The gene polymorphism (-308G/A) is located in the promoter region of the gene and involves in the substitution of a guanine (G) by an adenine (A) of the TNF-α gene sequence. Polymorphism is a change of nucleotide sequences in genes that result in variations in protein function. Polymorphism can determine susceptibility to disease. The impact of polymorphism is a change in the vulnerability of a population to illness [21]. Our result showed the TNF-α gene polymorphism (-308G/A) had no significant difference between type 2 diabetic patients with and without tuberculosis infection and HC subjects. Previous study showed there was an association of gene polymorphism (-308G/A) with DM [14], [22]. Another study showed that A allele of TNF-α at position (-308) was associated with tuberculosis infection [12]. Wu et al., (2017) notice that TNF-α gene polymorphism (-308G/A) was associated with severity of tuberculosis infection in a Chinese Han Population [23]. In this context, previous study showed that the GG wild type genotype significantly decreased in diabetic patients and also in tuberculosis patients compared to healthy subjects, different with our study that found the GG genotype frequency was highest in all groups. Study by Jamil et al., reported that *TNF-α* −308G/A polymorphism was a potent risk factor for diabetes in higher age (> 45) groups [24].

We also analyzed association between TNF-α levels in type 2 diabetic patients with and without tuberculosis infection and healthy control group. We found that TNF-α levels in type 2 diabetic patients without tuberculosis infection was higher compared to type 2 diabetic patients with tuberculosis infection and healthy control subjects. Previous study byde Souza Bastos et al., showed the increasing levels of TNF-α in uncontrolled and controlled of diabetic patients compared to the healthy subjects [25]. Another previous study also showed that there was an association of TNF-α levels, blood glucose levels and HbA1c in type 2 diabetic patients [26]. Study by de Andrade Junior et al., showed that the increasing levels of TNF-α related to the severity and complications in tuberculosis infection [27]. In tuberculosis infection, disruption of immune system of the body carried out by Mtb. The body's defences against Mtb is the secretion of TNF-α. The cytokine will enhance the ability of phagocytosis of macrophages and induce macrophage apoptosis infected with these Mtb [9].

TNF-α is a type of proinflammatory cytokine which also has role as mediator in inflammatory reaction. The action of TNF-α as an inflammatory cytokine via induction of other cytokines such as IL-1 and IL-6 [11], [21]. In DM, the inflammation process was induced by increasing of glucose oxidation in hyperglycemia condition [28]. Hyperglycemia correlated with increase reactive oxygen species (ROS) and lipid peroxides such as malondialdehyde (MDA) [24], [29]. The process continues to decrease antioxidants levels. Previous study showed that there was a decrease of glutathione peroxidase and glutathione in DM patients compared to healthy subjects [30]. The other study showed that decrease of SOD level occurred in tuberculosis patients with DM compared to healthy subjects [31]. Decline of antioxidants levels stimulate oxidative stress in the cell and trigger insulin resistance. Several studies showed that insulin resistance was associated with the production of TNF-α [32], [33].

Our study did not find association between gene polymorphism (-308G/A) and TNF-α levels in study groups. Previous studies also showed that there was no an asscoiation between TNF-α gene polymorphism (-308G/A) and TNF-α levels in type 2 diabetic patients [7]. Our result was contradictive with study by Joshi et al., which showed an associationbetween genotypes TNF-α gene polymorphism (-308G/A) and TNF-α levels in tuberculosis patients [9]. The TNF-α levels can be affected by many factors. The unmodifiable or irreversible factor in nature was genetic profiles. Gene polymorphism as genetic profile referred to the individual characteristics was affected by ethnicity. The modifiable or reversible factor (environmental) were nutrition intake, lifestyle, etc [20], [34]. The limitation of this study was nutrition intake, lifestyle of patients group and healthy subjects did not assessed. The TNF-α levels in this population could be affected by an environmental factor due there was no association between TNF-α gene polymorphism (-308G/A) and TNF-α level in patients group and healthy control subject.

In conclusion, there was no association of gene polymorphism (-308G/A) with type 2 diabetic patients with and without tuberculosis infection but the TNF-α levels showed the different result. The genotypes distribution of gene polymorphism (-308G/A) were not affect the TNF-α levels in this population study. Further study should be conducted to analyse the environmental factors on the research subjects.

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