





TNF- α (-308) Gene Polymorphism and Type 2 Diabetes Mellitus in Ethiopian Diabetes Patients

This article was published in the following Dove Press journal:
Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy

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Background: Type 2 diabetes mellitus (T2DM) is a metabolic disorder resulting from insulin insufficiency or function. Predisposing factors for T2DM are mainly genetic and environmental. Genetic polymorphism of cytokines like tumor necrosis factor- α (TNF- α) is suggestive of interference with insulin-sensitive glucose uptake and induces insulin resistance that ultimately could lead to T2DM. In this study, we assessed the effect of TNF- α (-308) G/A gene polymorphism and its association with the development of T2DM in an Ethiopian population.

Methods: An institutional-based cross-sectional study was conducted on study subjects with T2DM and non-diabetic healthy controls. DNA was extracted and genotyping was carried out by using amplification refractory mutation system polymerase chain reaction. A genetic-polymorphism on TNF- α (-308) G/A with T2DM was evaluated by logistic regression and Student's *t*-test. A P-value <0.05 was considered as statistically significant.

Results: In the present study, we observed a significant association between T2DM and TNF- α (-308) gene polymorphism's GG genotype [χ^2 test P = 0.005, OR (95% CI) =2.667 (1.309–5.45d8)]. In contrast, no statistically significant differences were observed in the frequencies of genotypes AA and AG (χ^2 test P=0.132 and 0.067, respectively). Moreover, T2DM individuals had higher concentrations of lipid profiles for those carrying the TNF- α (-308) GG genotype as compared to the control group.

Conclusion: TNF- α (-308) genetic polymorphism may be implicated in the genetic susceptibility for, as well as the development of T2DM and lipid metabolism in the Ethiopian population. Therefore, a large-scale study and early screening of TNF- α (-308) genetic polymorphism may help in early management and control of diabetes and related outcomes.

Keywords: TNF- α , T2DM, North West Ethiopia

Background

Diabetes Mellitus (DM) is a chronic disease characterized by impaired metabolism of glucose due to defects in insulin secretion or action.^{1,2} Globally, the number of adults living with diabetes raised from 108 million in 1980 to 422 million.³ The global prevalence of diabetes is rapidly increasing, rising from 4.7% to 8.5% in the adult population. Accordingly, the magnitude of diabetes in Africa and Ethiopia including Gondar accounts for 7.1%, 3.8% and 2.6%, respectively.^{3–6} Recently, the burden of diabetes has risen faster in low- and middle-income countries and Type 2 Diabetes Mellitus (T2DM) accounts for more than 90% of the total DM patients.³

Several factors have been attributed to T2DM pathogenesis, of which genetic and environmental factors are the primary factors, and T2DM is also heavily linked to obesity and overweight.^{7–9} Obesity induces adiposity hypertrophy and changes in

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stromovascular cell composition to bust pro-inflammatory state which leads to interact adaptive cells with adipose tissue macrophages to modify their activation state.¹⁰ The inflammatory and immune-mediated T2DM development as a result of cytokines, including IL-6, TNF- α , IL-1, IL-10 and TGF- β has been also stated as one factor for the development of DM.¹¹ Furthermore, the influence of genetic polymorphisms in pro- and anti-inflammatory cytokine specific genes like TNF- α and IL 10 is the frequently observed risk factor for the development of diabetes.¹²

Tumor Necrosis Factor-alpha is a pro-inflammatory cytokine that is located in chromosome 6p21.33 within the HLA III region,^{13,14} and it acts in the regulation of cell proliferation, differentiation and apoptosis. Macrophages are a primary source of TNF- α . It can also be secreted by multiple cells, including T and B cells as well as osteoblasts, smooth muscle cells, endothelial cells, epithelial cells and tumor cells.^{15,16} Current studies have now demonstrated that TNF- α , which is an NF-kappaB regulated product as well as a potent activator of NF-kappaB,^{17,18} inhibits insulin receptor (IR) signaling by promoting insulin receptor substrate (IRS) serine phosphorylation that leads to an inhibition of insulin-stimulated tyrosine phosphorylation.^{19–21} Besides, experimental studies on an animal model have shown that neutralization of TNF increases insulin sensitivity.^{12,22–24} Despite this, the replacement of nucleotide G by A in the promoter region of the TNF- α gene (–308 G/A) can increase the transcription of the gene. Accordingly, a high gene transcription enhances the production of TNF- α cytokine that leads to the occurrence of T2DM. In addition, homozygous subjects with –308 A/A TNF- α indicate increased levels of fat and lower levels of HDL than homozygous subjects with G allele.^{23,25} From these, we can consider polymorphic genotype can be associated with an increased frequency of T2DM.^{12,26} From this, we can understand that there is a gap in the area of the study. Therefore, the aim of this study is to investigate the association between genetic polymorphisms TNF- α (–308 G→A) with T2DM Ethiopian patients and healthy population.

Materials and Methods

Study Design, Period and Participants

A cross-sectional study from September to May 2018 was conducted at the University of Gondar Specialized Hospital Diabetic clinic. For this purpose, 150 participants (75 DM patients with clinically diagnosed and laboratory-confirmed T2DM and 75 healthy controls) were selected by convenient

sampling. Participants with confirmed diabetes mellitus or newly diagnosed diabetes using WHO guidelines,²⁷ fasting plasma venous glucose of ≥ 7 mmol/l (126mg/dl) or random plasma venous glucose of ≥ 11.1 mmol/l (200mg/dl), were included. Whereas both groups of participants less than 18 years old, with coronary artery disease, and/or other metabolic disorders, pregnant women and cancer were excluded.

Sample Collection and Processing

Following the acquisition of informed consent socio-demographic data, clinical data and 5 mL of whole blood were collected from each participant. The blood was poured into plane containers and centrifuged after clotting for lipid profile analysis. Serum was kept at –20 °C in sterile circumstances at immunology and molecular laboratory until the analysis was done. Triglyceride, LDL and Cholesterol were determined by means of enzymatic tests using the A25 Biosystem human (German). The normal value of each test was based on the reference of the enzymatic test of the A25 Biosystem human (German) kit. The standard operational procedure was addressed in the pre-analytic, analytic and post-analytic stages of laboratory services which constituted and impacted the overall quality of the laboratory analysis.

DNA Extraction and Genotyping

For this study genomic DNA was extracted from whole blood using salting out method.²⁸ The polymorphism (–308 G/A) in TNF- α gene was amplified and genotyped using thermo cyclic PCR in a 15 μ l reaction mixture containing 100ng of template DNA, buffer (100 mM Tris, pH 9.0; 500 mM KCl; 15 mM MgCl₂; 0.1% gelatin), 200 μ M dNTP, 10 pmol of each primer and 1.0 unit Taq DNA polymerase. Each reaction employed a generic antisense primer and one of the two allele-specific sense primers. The primers for SNPs TNF- α (–308) G/A were: forward primer G-5' ATAGGTTTTGAGGGGCATGG-3', primer A-5' AATAGGTTTTGAGGGGCATGA-3', reverse primer 5' TCTCGGTTTCTTCTCCATCG-3'. The amplified product was evaluated with 2% agarose gel stained with ethidium bromide and then a UV light gel documentation system was used to observe the band.^{28,29} The purity of extracted DNA was checked on a Nanodrop spectrophotometer. The DNA yield at 260/280nm between 1.8 and 2.0 was considered as pure.^{30,31}

Statistical Analysis

Data were entered into Epidata 3.1 and checked for completeness and clearance. Data were collected, summarized, tabulated and analyzed using SPSS version 20 software

(SPSS, Inc., Chicago, Illinois, USA). The differences in the distribution of genotypes and allele frequencies were analyzed using the Chi-square test. Odds ratios (ORs) and the corresponding 95% confidence interval (95% CIs) were calculated to assess the strength of the association between TNF- α gene polymorphism versus T2DM. A P-value <0.05 was considered as statistically significant. The logistic regression model and Student's t-test were also applied for comparison of parameters.

Results

To assess the frequency of TNF- α (-308 G/A) gene polymorphism and its association with T2DM, 150 participants (75 T2DM patients and 75 healthy controls) were included. Mean \pm standard deviation (SD) age of the T2DM cases and controls were 55.45 ± 9.998 and 34.41 ± 9.012 years, respectively. The sex distributions of males were found to be 47/75 (62.7%) and 41/75 (54.7%) in both study groups (cases and controls), respectively. The mean fast blood sugar (FBS) levels among T2DM cases and controls were also 192.89 mg/dl and 88.03 mg/dl, respectively. With regard to lipid profile, the mean triglyceride (TG), cholesterol and low-density lipoprotein (LDL) level of T2DM case were 192 mg/dl, 191mg/dl, 95 mg/dl, as well 163.59 mg/dl, 163 mg/dl and 60 mg/dl for control groups, respectively. There are statistically significant differences between two groups of serum TG and Cholesterol with P=0.012, 0.014, respectively (Table 1).

TNF- α -308 (G/A) Genotype and Allelic Distributions in T2DM Patients and Healthy Controls, at the University of Gondar Hospital, 2018

Calculated frequencies of genotype and allelic expression TNF- α gene polymorphism using Hardy Weinberg equilibriums have been shown in Table 2. Among T2DM patients, for TNF- α -308 G/A genetic polymorphism, 45/75 (60%), 24/75 (32%) and 6/75 (8%) were GG, AG and AA genotypes, respectively. Similarly, from 75 healthy controls 36/75 (48%), 27/75 (36%) and 12/75 (16%) were GG, AG and AA genotypes, respectively. Likewise, the allelic frequency of -308*G and -308*A alleles in T2DM patients were 114 (76%) and 36 (24%) respectively; meanwhile allelic frequency of -308*G and -308*A were found to be 90 (60%) and 60 (40%) in healthy controls, respectively [$\chi^2 = 0.004$, Fisher exact

Table 1 Socio-Demographic Risk Factors and Biochemical Profiles of the Study Participants, North West Ethiopia, 2018

Parameter		T2DM (n=75), N (%)	Control (n=75)	P-Value
Sex	Male	47 (62.7%)	41 (54.7%)	0.320
	Female	28 (38.3%)	34 (45.3%)	
	Total	75 (100%)	75 (100%)	
Age in years (Mean \pm SD)		55.45 \pm 9.998	34.41 \pm 9.012	0.000
FBS (Mean \pm SD)		192.89 \pm 56.23	88.03 \pm 11.599	0.000
Cholesterol (Mean \pm SD)		191 \pm 32	163.29 \pm 23.820	0.012
TG (Mean \pm SD)		192 \pm 35	163.59 \pm 24.476	0.014
LDL (Mean \pm SD)		95 \pm 42	60.01 \pm 23.753	0.145

Abbreviation: FBS, fast blood sugar; TG, triglyceride; LDL, low-density lipoprotein; SD, standard deviation.

test=0.002, OR (95% CI) =0.0474]. Besides, with regard to TNF- α (-308) genotype frequencies among case and controls, it was noted that T2DM patients in our study showed a significantly higher frequency of homozygote genotype GG [χ^2 P=0.005, fisher's exact test P=0.005, OR (95% CI) =2.667 (1.309–5.458)]. On the other hand, the frequencies of genotypes AA and AG [χ^2 p=0.132, fisher's exact test P=0.208, OR (95% CI) =0.457 and $\chi^2=0.067$, fisher's exact test P=0.066, OR (95% CI) =0.51 (0.248–1.043)], respectively, were not statistically different.

Association of TNF- α (-308G/A) Genotype with Biochemical Parameters of T2DM and Healthy Controls

We have summarized the association between TNF- α (-308 G/A) genotype and lipid profile (TG, Cholesterol and LDL) within each of T2DM patients and control groups (Table 3). Moreover, TG, cholesterol and LDL levels in TNF- α (-308) GG genotype (206 mg/dl, 207mg/dl, 92 mg/dl, respectively) were significantly higher than genotype AG (201mg/dl, 188 mg/dl, 96 mg/dl, respectively) and AA (172 mg/dl, 166 mg/dl, 73.5mg/dl, respectively) genotypes in T2DM patients group. Generally, there was a high level of TG, cholesterol and LDL in T2DM than the comparison groups (P= 0.003, P=0.012, P=0.024).

Discussion

In the present study, we determined the extent of G to A substitution at position -308 TNF- α gene and its association with the development of T2DM and biochemical

Table 2 Frequencies of TNF- α -308 (G/A) Allelic and Genotype Polymorphisms Among Study Participants, North West Ethiopia, 2018 (N= 150)

TNF- α -308 (G/A)		T2DM (N=75) N (%)	Control (N=75) (%)	χ^2 p-value	Fisher's Exact Test p-Value	OR (95% CI)
Alleles	A	36 (24)	60 (40)	0.004*	0.002*	0.474 (0.279–0.802)
	G	114 (76)	90 (60)			
Genotypes	AA	6 (8)	12 (16)	0.132	0.208	0.457 (0.142–1.413)
	AG	24 (32)	36 (48)	0.067	0.066	0.51 (0.248–1.043)
	GG	45 (60)	27 (36)	0.005*	0.005*	2.667 (1.309–5.458)

Notes: *P < 0.05, p-value for HWE test in controls shell.

Abbreviations: TNF- α , tumor necrosis factor-alpha; OR, odds ratio; CI, confidence interval.

Table 3 Association Between Lipid Profiles and TNF- α (-308 G/A) Genotypes in T2DM Patients and Controls

Biochemical Parameter	T2DM Patient (N=75)				Control (N=75)			
	AA (n=6)	AG (n=24)	GG (n=45)	P-Value	AA (n=12)	AG (n=36)	GG (n=27)	P-Value
FBS (mean)	162	191	198	0.002*	89	88	87.8	0.344
TG (mean)	172	201	206	0.003*	159	151	168	0.309
Cholesterol (mean)	166	188	207	0.012*	167	158	164	0.653
LDL (mean)	73.5	96	92	0.024*	55	54	61	0.675

Note: *P < 0.05 was considered significant.

Abbreviations: FBS, fast blood sugar; TG, triglyceride; LDL, low-density lipoprotein.

parameters in an Ethiopian population. Cytokine gene polymorphisms may influence their transcription, levels of production, and could be implicated in inducing susceptibility or resistance to several diseases.³² Findings from several studies have demonstrated that cytokine gene polymorphisms are associated with T2DM and related complications in a context of TNF- α mediator of obesity-linked insulin resistance and resultant of T2DM.^{33–35} It suggested that a single-nucleotide polymorphism (SNP) in cytokine gene has a specific role in determining diabetes susceptibility.³⁶ TNF- α G/A polymorphism, located on the upstream of the gene at -308, and is known to influence TNF- α plasma level.³⁷ In the present study, TNF- α -308 GG gene polymorphisms showed significant association with lipid metabolism (TG, Cholesterol and LDL) in each group (T2DM patients and control) and within the group. Moreover, TNF- α (-308) GG genotype in TG, cholesterol and LDL were significantly higher than genotype AG and AA in diabetic patients. There was a high level of TG, cholesterol and LDL in T2DM than the comparison groups (P= 0.003, P=0.012, P=0.024). For this reason, we hypothesized that TNF- α GG genotype will increase the expression and production of TNF- α . However, contrary to the authors' hypothesis, as indicated by Um JY et al, the -308 A allele has been shown to be associated with increased TNF- α

expression after in vitro stimulation.^{38–41} It appears that whether the A allele or the G allele of TNF- α -308 G/A polymorphism is associated with an increased TNF- α production remains to be determined in this Ethiopian population. It is notable that TNF- α production could be influenced by multiple genetic polymorphisms located in the TNF- α gene and/or other immune-related genes. Its production increases plasma lipid metabolism through the inhibition of adipose lipoprotein lipase activity and/or a stimulation of hepatic lipogenesis, induces lipolysis and suppresses free fatty acid (FFA) uptake there by an increasing FFA production will induce hepatic gluconeogenesis opening the door for hyperglycemia.⁴² An article by Mirhafez et al⁴³ did not show an association of the possession of the GG genotype of the TNF- α gene -308 G/A polymorphism with metabolic syndrome in their study population (P<0.05). Further, in their study, the AA genotype of TNF- α -308 G/A polymorphism was related to an increased level of triglyceride in metabolic syndrome patients, compared to the control group.⁴³ Our study indicated that TNF- α gene promoter -308 G/A polymorphism's GG genotype was associated with a significantly increased risk of T2DM in an Ethiopian population, but this association remains to be confirmed in larger, independent studies in populations of Ethiopian ancestry, given that the current study had small sample sizes (i.e., only 75

participants in each of T2DM patients and control groups). In previous studies, a study of Kolla et al, had similar findings, such that the GG genotype of TNF- α promoter -308 G/A polymorphism was significantly associated with an increased risk of diabetic neuropathy compared to healthy controls,⁴⁴ while there are some studies with dissimilar outcomes.^{23,45,46} The dissimilarity may be due to differences in genetic makeups among different study populations and even variabilities in their environmental risk factors. The logistic regression analysis showed that individuals who had GG genotype at the promoter region of TNF- α at (-308) position have 2.66 times higher risk of developing T2DM when compared to AA and AG genotypes. In addition, we found a significant association between G allele and T2DM (P=0.002). These demonstrate that carriage of the G could be associated with a great risk of T2DM. In the current study, we did not quantify TNF- α serum levels in T2DM patients and control groups, and thus we could not clearly show which TNF gene -308 G/A genotype is associated with an increased TNF- α serum level in each of the two study groups. However, in the study population, the G allele is postulated to increase the expression of plasma TNF- α level, which is an important mediator of insulin resistance that might result in diabetes through its ability to inhibit the tyrosine kinase activity of the insulin receptor, interfering with insulin action.^{37,47} Based on our findings, for G-to-A substitution at position -308 in the TNF- α gene, the GG genotype is more frequently observed in T2DM patients than in healthy controls in the Ethiopian population. However, our study sample sizes are relatively small, and further larger studies are needed to confirm our results. TNF- α is one of the most important pro-inflammatory mediators that could contribute to the development of insulin resistance, and possibly the pathogenesis of T2DM. Consequently, a better understanding of the genetic factors allows clinicians to have an early prediction, screening and diagnosis of T2DM among the Ethiopian population, which eventually could be important in accurate management and reduce DM-related complications. Furthermore, the lack of control of potential cofounders in the logistic regression model was considered as the limitation of the study. In this study, only studied a single SNP, i.e., -308 G/A polymorphism, in the promoter gene of the human TNF gene, and only performed single-SNP analysis on the genetic association of this SNP with T2DM risk was also a limitation. However, there are multiple SNPs located in the TNF gene promoter region besides

the -308 G/A polymorphism, which could play important regulatory roles of TNF- α gene transcription, e.g., -238 G/A, -857 C/T, -863 C/A and -1031 T/C promoter polymorphisms.⁴⁸ Therefore, in addition to the -308 G/A polymorphism, these other promoter polymorphisms of TNF- α could be studied, and haplotype inference based on the multiple linked SNPs residing in the TNF- α gene could be performed, which would provide more valuable genetic information for multiple linked genetic variants than single SNPs,⁴⁹ and the inferred haplotypes could be applied to conduct haplotype-based association analysis, which could be more informative than single-SNP-based association analysis⁵⁰ to reveal the causal genetic variant.

Conclusion

In this study, we found that Ethiopian T2DM patients had significantly higher frequencies of GG genotype of TNF- α -308 G/A polymorphism. It is possibly that individuals with GG genotype could develop diabetes faster than their counterparts who carry the AA/AG. Consequently, patients with the GG genotype would develop diabetes faster than their counterparts who have the AA genotype. Our finding also supports the hypothesis that TNF- α -308 G/A polymorphism might play a role in impairing lipid profiles in T2DM patients. Early identification of this genetic marker will help health professionals to provide proper advice and counseling so that the patients could have a regular follow up and modification in lifestyle risk factors and daily diet intakes to protect them from having an early manifestation of T2DM.

Abbreviations

HDL, High-Density Lipoprotein; LDL, low-density lipoprotein; MHC, major histocompatible complex; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphisms; TG, triglyceride; TNF- α , tumor necrotic factor-alpha; T2DM, Type 2 Diabetes Mellitus.

Consent

The authors confirm that all patients provided informed consent forms.

Ethical Approval and Considerations

This project was reviewed and approved by the University of Gondar ethical review committee. Informed written consent was obtained from each study participant before they were asked to provide socio-demographic data and clinical specimens. In the beginning, the objective, risks and benefits

associated with this study were briefly explained to each participant using their local language, including the right not to incorporate into the study. Finally, those who provided a written consent were included.

Data Availability

Data supporting these findings are contained within the manuscript and further data can be shared upon reasonable request to the corresponding author.

Author Contributions

Conception of the research idea: BA and NB; All the authors contributed toward data analysis, critically revising the paper, gave final approval of the version to be published, and agreed to be accountable for all aspect of the work.

Funding

This study was not funded by any governmental or non-governmental funders.

Disclosure

The authors declare that they have no competing interests in this work.

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